DIVERSE FUNCTIONS OF MUCOSAL RESIDENT MEMORY T CELLS

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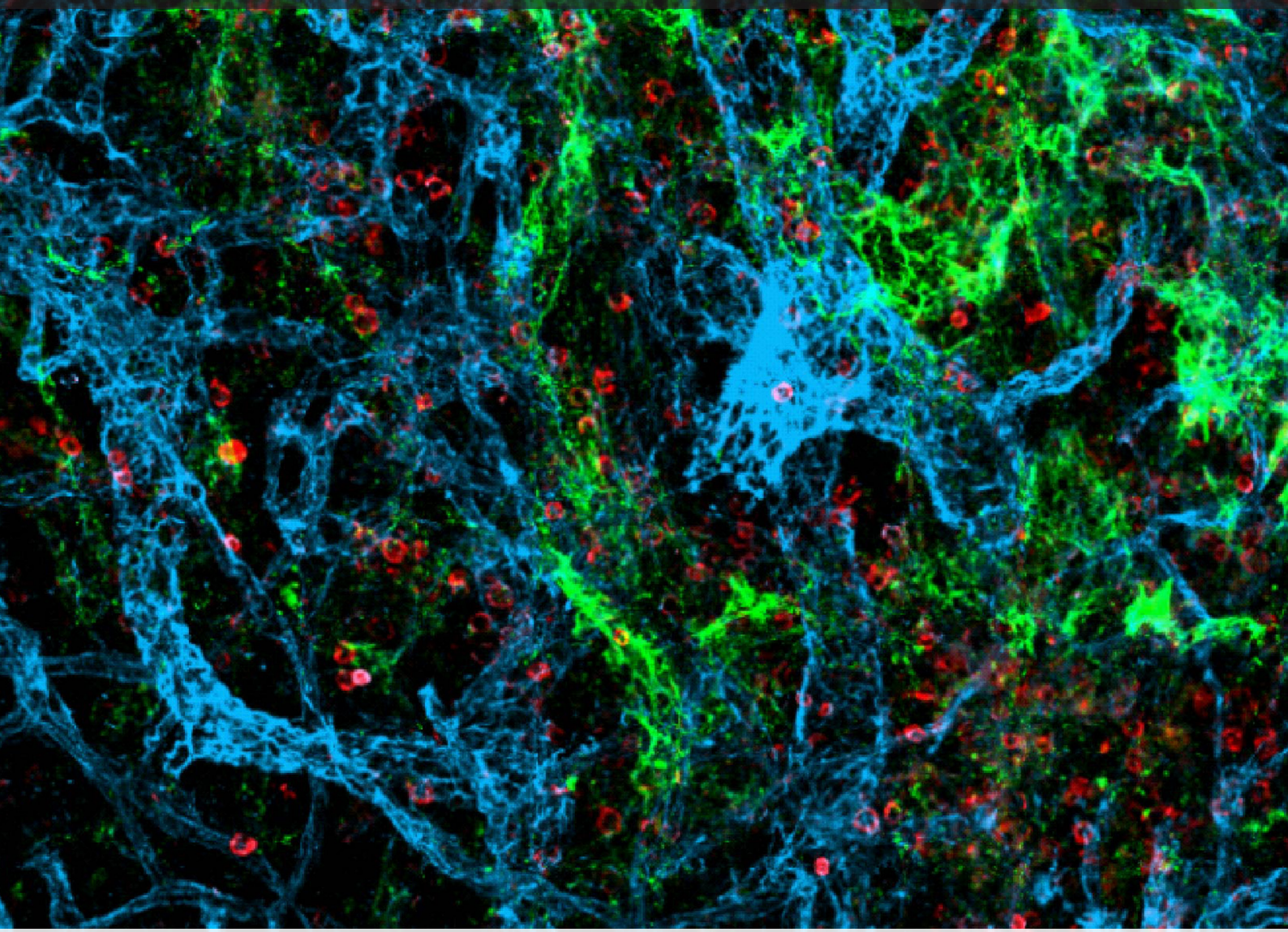

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DIVERSE FUNCTIONS OF MUCOSAL RESIDENT MEMORY T CELLS

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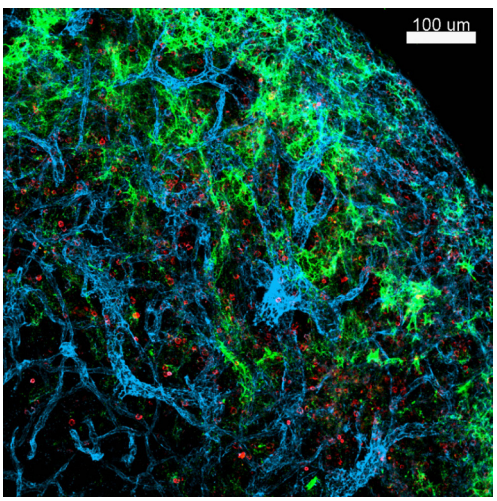

Whole mount 3D reconstruction of the draining lymph node 5 days after skin infection illustrating the complexity of the lymph node architecture. Whole draining lymph node from mice infected with vesicular stomatitis virus (VSV) was stained for the lymphatic vessels (Lyve-1; green), and the blood vessels (CD31; ligh blue) and VSV-N specific CD8+ T cells (MHC class-I tetrmaer; red). The image shows CD8 T cells egressing from the LN using the lymphatic sinuses. The image represents a 3D reconstruction of a 65 um merged z-stack. Image provided by Dr. Kamal Khanna.

Early studies recognized the unique phenotype and attributes of T cells found in mucosal tissues, such as the intestines, skin, lung and female reproductive tract. This special topic issue will cover many aspects of mucosal-resident T cell biology during infection and disease and is dedicated to Leo Lefrancois, a pioneer in this field who recently passed away. A major proportion of these mucosal T cells are memory T cells, now recognized as a major constituent of memory T cells referred to as tissue-resident memory T cells. Unlike central and effector memory T cell subsets, tissue-resident memory T cells exhibit tissue specificity with minimal systemic migration. Nonetheless, tissue-resident memory T cells share a similar origin and display some overlapping phenotypes with their other memory T cell counterparts. Articles in this issue will describe the different types of memory T cells residing in mucosal tissues, their origins and functions as well as how they vary among discrete mucosal sites. Manuscripts will consider the unique physiological environments and cellular constituents which facilitate tissue residency while preserving tissue function. Additionally, there will be descriptions of the various mechanisms responsible for the migration and segregation of tissue resident memory CD8 T cells from

the peripheral T cell pool. Although the mechanisms facilitating the sequestration of tissue-resident memory T cells within a respective tissue has not well characterized, various theories will also be discussed. Lastly, how these T cells contribute to immunity to pathogens, cancer, and autoimmunity and could be modified through vaccination or therapeutic intervention will be described. As mucosal tissues are the major portals of pathogen entry and frequent transformation, the activities and persistence of tissue resident memory T cells is crucial for mediating protection at these sites.

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EDITORIAL
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Protecting the borders: tissue-resident memory T cells on the front line

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Keywords: tissue-resident memory cells, T cells, mucosal tissues, migration, T cell differentiation

This research topic of Frontiers in Immunology focuses on T cells residing in mucosal tissues and is dedicated to a pioneer in the field, Leo Lefrancois. As mucosal tissues are the major portals of pathogen entry, the generation and functions of tissue-resident T cells are crucial for mediating protection and immune homeostasis at these sites. Unlike previously described T cell subsets, these tissue-resident T cells exhibit regional specificity with minimal systemic migration, most being previously activated or memory T cells. While tissue-resident memory T cells (Trm) display some overlapping phenotypes with other circulating T cell subsets, their origins and developmental pathways remain elusive.

The most extensively described tissue-resident T cells are those present in intestines, lung, and skin. While CD8 Trm derived from these mucosal tissues express core genetic profiles (1), unique patterns also exist, suggesting some tissue-specific programing *in situ*. Reviews in this issue by Shane et al. (2) and Mueller et al. (3) highlight the role of the respiratory and cutaneous microenvironment, respectively, on full commitment to the Trm lineage. Both reviews describe the cellular interactions and other regional cues that may support Trm differentiation and sublocalization (2, 3). Interestingly, dependency on cytokines and lifespan differs between these sites suggesting that not all Trm are equal. These and many other studies reviewed in this issue analyze Trm cells after tissues dissociation. Unfortunately, this approach isolates only a fraction of immune cells present *in situ* and, importantly, fails to reveal spatiotemporal cell–cell interactions. Undeniably, studies using microscopy allow one to gain a better perspective of the dynamics of T cells motility and migration patterns within the unique architecture of specific tissues. Benechet et al. (4) reviews the literature that has utilized various imaging technologies to decipher the migration activities of T cells. Future analysis of unperturbed Trm within their privileged niche will likely reveal unappreciated interactions and behaviors that improve our understanding of Trm biology.

Most studies investigating Trm focus on CD8 T cells; however, pathogen-specific CD4 Trm in mucosal tissues have also been discovered. In this issue, three reviews cover CD4 T cells starting with a global description of mucosal resident CD4 T cells presented by Turner and Farber (5). This is followed by a more focused description of the CD4 T cell responses resulting from encounters with intracellular bacteria, such as *Salmonella* and Chlamydia, which invade the intestines and female reproductive tract, respectively (6). Lastly, Gratz and Campbell (7) provide a new paradigm that includes T regulatory cells (Treg) among subsets of Trm. Indeed, Treg are enriched in the mucose to maintain a tolerogenic environment.

To understand the normal forces exerted on mucosal resident T cells, knowledge of the unique microenvironment inhabited by these T cells is essential. Despite the inherent tolerogenic nature of the mucosa, pathogen exposure and associated inflammation overrides the naturally suppressive environment, promoting Trm development. Inflammatory cytokines regulate the expression of chemokine receptors and other homing molecules to promote the migration of effector T cells to distinct locations where they will commit to the Trm lineage. Kim and Harty (8) highlight the effects of inflammatory cytokines present during different stages of the immune response on resultant CD8 T cell differentiation. Specifically, they describe an unappreciated role of IL-15, which promotes trafficking to inflamed tissues as well as the contrasting roles of TGF-β in the formation and retention of Trm. In addition to infection, commensal bacteria also likely influence Trm cells. The review by Spasova and Surh (9) describes how the gut microbiota is sensed by pattern recognition receptors such as TLR and NOD-like receptors. Subsequently, how unique immune cells populations highly represented in the intestines (ILC, specific subsets of DCs, Th17, Tregs, and IEL) interpret these signals and influence the gut microenvironment, including the persistence and functions of Trm, is also discussed. Together, these reviews draw attention to the complexity of specific microenvironments and their impact on Trm development and function.

While improving our knowledge of Trm will surely help to develop better clinical strategies to promote mucosal immunity, Sowell and Marzo (10) suggest that we should proceed with caution. In their opinion piece, they suggest that Trm may be refractory or inhibited by certain strategies, which modify conventional memory T cell responses, and vice versa. This is exemplified by inhibition of mTOR signaling that enhances memory T cell generation at the expense of effector T cell accumulation in the mucosa. As such, understanding how to specifically enhance development of Trm during vaccination will be of great value in the future.

In summary, this special issue highlights our evolving understanding of tissue-resident T cells. But many questions remain unanswered. For example, while tissue-resident T cells were originally described as a population unique to mucosal tissues, more recent studies have identified T cells with similar attributes in

non-mucosal tissues, such as the brain and lymph nodes (11, 12). Does this indicate that all tissues can harbor a permanent T cell population or only those with little access to circulating immune cells? Furthermore, are specific Trm pools within a given site undergoing continued attrition and replacement with repeated infections? Filling in these knowledge gaps will be essential to expanding our understanding of Trm during pathogen infection or other mucosal perturbations. Subsequently, strategies that exploit the functional responses of tissue-resident T cells can be developed.

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Every breath you take: the impact of environment on resident memory CD8 T cells in the lung

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Resident memory T cells (T_{RM}) are broadly defined as a population of T cells, which persist in non-lymphoid sites long-term, do not re-enter the circulation, and are distinct from central memory T cells (T_{CM}) and circulating effector memory T cells (T_{EM}). Recent studies have described populations of T_{RM} cells in the skin, gut, lungs, and nervous tissue. However, it is becoming increasingly clear that the specific environment in which the T_{RM} reside can further refine their phenotypical and functional properties. Here, we focus on the T_{RM} cells that develop following respiratory infection and reside in the lungs and the lung airways. Specifically, we will review recent studies that have described some of the requirements for establishment of T_{RM} cells in these tissues, and the defining characteristics of T_{RM} in the lungs and lung airways. With continual bombardment of the respiratory tract by both pathogenic and environmental antigens, dynamic fluctuations in the local milieu including homeostatic resources and niche restrictions can impact T_{RM} longevity. Beyond a comprehensive characterization of lung T_{RM} cells, special attention will be placed on studies, which have defined how the microenvironment of the lung influences memory T cell survival at this site. As memory T cell populations in the lung airways are requisite for protection yet wane numerically over time, developing a comprehensive picture of factors which may influence T_{RM} development and persistence at these sites is important for improving T cell-based vaccine design.

Keywords: CD8+ T cells, memory T cells, tissue-resident memory cells, influenza A virus, lung

INTRODUCTION

The adaptive immune system is defined by its ability to mount an antigen-specific immune response and generate long-lived memory cells. $CD8^+$ memory T cells (T_{mem}) respond rapidly upon secondary encounter with the same antigen and can provide protection against the development of severe disease or chronic infection in the absence of neutralizing antibodies (1). This attribute of T_{mem} is particularly attractive in the context of vaccine design for viral infections such as HIV or influenza, which rapidly modify antibody targets as a result of high mutagenic rates and immune pressure.

The efficiency of T_{mem}-mediated protection is in part a direct result of activated T cells initiating divergent developmental and migratory programs, which provide the host with a multifaceted immune response following challenge. This T_{mem} diversity is acquired as a result of different levels of co-stimulation, inflammation, or T cell help, which not only vary throughout the course of a single infection but are also impacted by infection route. Initially, memory T cells were broadly categorized into two populations based on homing preferences, circulating between secondary lymphoid organs as central memory T cells (T_{CM}) or less discretely throughout the periphery, including non-lymphoid tissues, defined as effector memory T cells (T_{EM}) (2). These memory pools are distinguished from one another by their differential expression of the lymph node homing molecules L-selectin (CD62L) and CCR7, with T_{CM} expressing high levels of these molecules for lymph node entry and retention (3) and T_{EM} cells expressing low levels. While this simplified T_{CM}/T_{EM} paradigm predominated T_{mem} classification for several years, subsequent studies using parabiotic mice (4) and adoptive transfer systems (5) demonstrated that at least one additional T_{mem} pool exists with tissue-specific residency and little migratory potential. Additional studies confirmed the existence of these tissue-locked T_{mem} at portals of pathogen entry and led to the T resident memory cells (T_{RM}) nomenclature.

As relative newcomers to the T cell memory scene, T_{RM} cells have not been characterized to the same extent as T_{CM} and T_{EM} cells, and our definition of this memory population, as well our understanding of its origin is still evolving. Nonetheless, specific $CD8^+$ T_{RM} populations have been identified in many peripheral sites including the gut (6), skin (7), brain (8), female reproductive mucosa (9, 10), and the lung (11). Despite some similarities with T_{EM}, lack of equilibration of T_{mem} between specific tissues of parabiotic mice as well as general "hallmarks" of T_{RM} have been identified as defining characteristics. These distinguishing features include the expression of CD103 (α_E integrin) and CD69, molecules traditionally associated with adhesion within epithelial layers and recent activation, respectively (12, 13). A recent paper by Mackay et al. defined a common transcriptional signature shared by $CD103^+$ T_{RM} cells isolated from the skin, gut, and lung consisting of 37 genes differentially expressed compared to T_{EM} or T_{CM} cells, demonstrating that T_{RM} cells are a distinct T_{mem} lineage (14). Additionally, this study determined that T_{RM} cells from distinct anatomical sites also possessed unique gene transcription

patterns, with 127 being unique to the gut, 86 unique to the skin, and 25 unique to the lung, indicating additional diversification within the T_{RM} pool, likely environmentally driven.

Despite the relative juvenescence of the T_{RM} field, the importance of this cell population has been alluded to for some time. T_{RM} cells are positioned at the site of pathogen encounter as a front line of defense, and several studies have highlighted their role in defense against pathogenic challenges (7, 15–17). Indeed, in the case of influenza virus infection, the number of antigen-specific $CD8^+$ T cells located within the respiratory tract correlates with the highest degree of heterosubtypic immunity (18, 19), and recently it has been shown that T_{RM} specifically are responsible for this protection (20). Defining the characteristics that lead to T_{RM} development, and determining how they persist at sites of infection may lead to novel ways to enhance vaccine efficacy. This review will focus on the development, characteristics, and maintenance of $CD8^+$ T_{RM} cells in the respiratory tract, which develop after acute respiratory infection, primarily with influenza and Sendai viruses. How the lung environment affects the developmental transition and tissue residency of $CD8^+$ T_{RM} cells will be discussed, from the primary activation of the antigen-specific cell through the return to homeostasis and during resting conditions.

PART I: FACTORS INFLUENCING T_{RM} DEVELOPMENT

There is great interest in deciphering the T_{RM} developmental pathway, as understanding this mechanism could lead to modulation of the responses in ways, which could enhance the establishment of this T_{mem} pool. The development of T_{RM} cells will have two main requirements: (1) the ability to survive through contraction (become a T_{mem}) and (2) the ability to differentiate into the appropriate memory lineage (become a T_{RM} cell as opposed to a T_{EM} or T_{CM} cell). In this section, we will discuss factors that may influence T_{RM} development in the early priming environment of the lymph node, and subsequently in the inflamed lung. Recent evidence demonstrates that T cell differentiation into distinct T_{EM} and T_{CM} subsets occurs soon after T cell priming (21), which begs the question: does a population of cells that is destined to become T_{RM} cells also develop during or soon after initial activation in the lymph node? Or, do T_{RM} arise only after tissue-specific entry based on specific cues within the microenvironment of tissues, like the lung? These scenarios are not necessarily mutually exclusive and full commitment to the T_{RM} lineage is likely due to a combination of these two possibilities as will be further discussed and as described in **Figure 1**.

EARLY DIFFERENTIATION SIGNALS IN THE LYMPH NODE: DEVELOPING T_{RM} POTENTIAL

Activation of $CD8^+$ T cell requires three signals: detection of cognate peptide/MHCI complex, co-stimulation, and a cytokine signal (22). The combination of these three signals, which may vary in intensity and type, results not only in clonal expansion and acquisition of effector function, but also influences long-term cellular fate (21). In many cases, the overall T_{mem} potential of the antigen-specific $CD8^+$ T cell is driven by lineage-associated transcription factors and acquired epigenetic changes (23), which can be experimentally monitored. These programing signals are influenced by the type of (priming) APC, antigen availability, and inflammatory properties of the pathogen, which can vary based on the individual pathogen and the route, which infection is acquired. While an early T_{RM} lineage-specific transcriptional program has not been identified, specific migratory signals facilitating peripheral tissue entry, with subsequent acquisition of T_{RM} characteristics implies that at least some early signals help polarize cells toward a T_{RM} fate. Here, we will discuss the possible early signals encountered in the lung draining mediastinal lymph nodes (MdLN), which may promote the development of respiratory T_{RM} cells.

During influenza infection, activated, antigen-laden respiratory DCs migrate to the MdLN to interact with naïve $CD8^+$ T cells. The majority of these migratory DCs fall into two subsets,

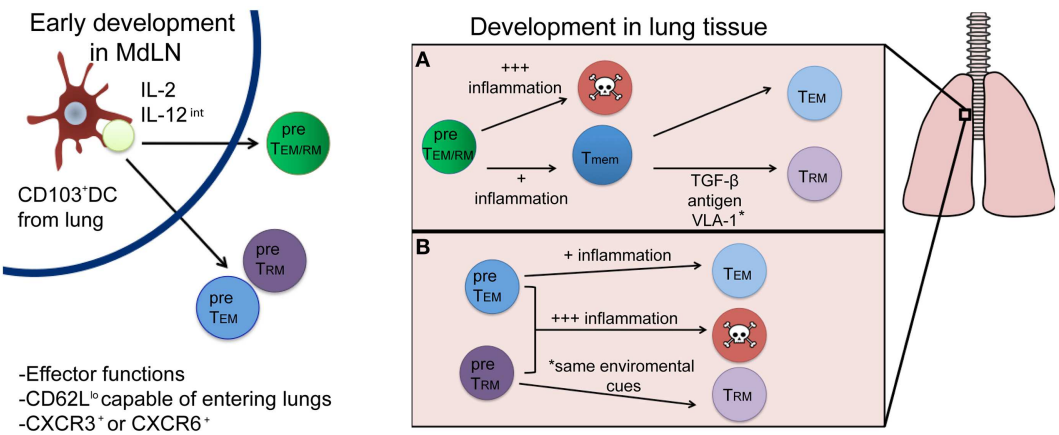

FIGURE 1 | Proposed developmental pathways for T_{RM} cells following respiratory infection. Priming by a $CD103^+$ DC and appropriate cytokine signals (left) results in the generation of either a common $T_{EM/RM}$ precursor cell (green) or individual pre-T_{EM} or T_{RM} cells (blue and purple, respectively). Once in the lung (right), environmental factors will drive subsequent cell fate decisions, resulting in either terminal differentiation (and death) or the generation of memory cells. While most current evidence supports the differentiation route depicted in **(A)**, where a common precursor differentiates first into memory, followed by environmentally driven lineage differentiation into T_{RM} or T_{EM} cells, it is plausible that differentiation into a T_{RM} fate occurs immediately following priming in the lymph node **(B)** and is distinguished by yet to be discovered phenotypic or genetic markers.

airway localized CD103$^+$ DCs and lung parenchyma CDllb^{hi} DCs (24). In addition to their localization in the lung during a resting state, these DC populations differ in their induction of CD8$^+$ T cell effector functions, with CD103$^+$ DCs requisite for complete effector differentiation, defined by expression of standard effector markers and their potential to enter inflamed tissues (CD25^{hi}, T-bet^{hi}, and Blimp-1^{hi} and CD62L^{lo} CCR5^{hi}). In contrast, CD11b^{hi} DCs are more likely to prime CD8$^+$ T cells, which largely remained in the lymph nodes, expressing molecules associated with the development of T_{CM} (CD62L^{hi}, T-bet^{lo}, Blimp-1^{lo} CD25^{lo}, and CD127^{hi} (25). Thus, as entry into peripheral tissues is a defining characteristic of T_{RM} cells, it is likely that T_{RM} precursors are activated in the draining MdLN by activated respiratory CD103$^+$ DCs, where they not only acquire effector function, but more importantly, the ability to accumulate in lung tissue, which is requisite for T_{RM} development. Priming by CD103$^+$ DCs may also be one of the reasons that T_{RM} have a propensity to develop following induction of the responses in mucosal tissues, as similar CD103$^+$ epidermal associated DCs are found predominately in these sites (26) and may be a common method promoting CD8$^+$ T cell migration into peripheral tissues. In support of this, intranasal vaccination gives rise to populations of long lasting T_{mem} in the female reproductive tract, a phenomenon that is not observed following systemic infection (27–29). This indicates that a common priming requirement (possibly CD103$^+$ DCs) can induce CD8$^+$ T cell migration into more restrictive sites, and vaccination at certain mucosal surfaces may broadly confer protection at expanded peripheral sites (30). However, it should be noted that certain systemic infections, such as lymphocytic choriomeningitis virus (LCMV), can produce populations of T_{RM} cells in a wide variety of tissues, including the intestinal tract, brain, and female reproductive tract, as well as organs such as the kidney, heart, and pancreas, although in this study respiratory T_{RM} were not assessed (31). LCMV, a true systemic pathogen, can replicate in multiple cell types and locations, suggesting that pathogen promiscuity could result in activation of CD103$^+$ DC and induction of T_{RM} independent of mucosal infection. For the lung, it seems that priming via the respiratory route [intranasally (i.n.)] is necessary for T_{RM} formation, as priming with influenza virus intraperitoneally (i.p.) fails to generate T_{RM} cells (20). The difference here is that influenza will not produce a productive infection when given by the i.p. route, limiting presentation to CD103$^-$ DCs (32). It will be important for future studies to distinguish whether the lung is truly a restrictive site, limiting T_{RM} generation only after infection via the i.n. route, or if a systemic or mucosal challenge at a divergent site can induce lung T_{RM} populations under the right conditions.

Another important factor which can be highly variable during infection is the presence of particular cytokines, which influence both memory cell potential (33, 34), and the specific pool of T_{mem} that develops (35). The potential for an effector T cell (T_{eff}) to become a T_{mem} cell has been defined based on the expression of CD127 and KLRG1 (36). T_{effs} largely fall into one of three categories: terminally differentiated short-lived effector cells (SLECs, KLRG1^{hi}/CD127^{lo}), early effector cells (EECs, KLRG1^{lo}/CD127^{lo}) or memory precursor effector cells (MPECs, KLRG1^{lo}, CD127^{hi}). It is the latter population, which develops into long-lived, bonafide T_{mem} of various phenotypes, including T_{CM}, T_{EM}, and T_{RM}. Therefore, the formation of MPECs is a necessary step in T_{RM} development, although the timing in which a cell begins to express these markers may differentially impact its memory phenotype. MPECs can form early in the lymph node, or once at the site of infection they can arise from EECs, which have the potential to differentiate into both SLECs and MPECs (37). The inflammatory cytokine IL-12 is detectable at 48 h following influenza infection, and is important for the development of IFN-γ producing cells early in the immune response (38). In regard to memory development, IL-12 promotes the development of terminally differentiated SLECs in a dose dependent manner via induction of the transcription factor T-bet (33). Interestingly, graded induction of IL-12 is observed after systemic infection with two different pathogens: *L. monocytogene*s (LM) induces a high concentration of IL-12, whereas vesicular stomatitis virus (VSV) induces much lower IL-12 levels. High concentrations of IL-12 during LM infection promote a skewed development favoring SLECs while VSV infection (lower IL-12) favored EECs (37). Since T_{RM} cells arise from KLRG1^{lo} precursors (14), high levels of IL-12 would likely negatively impact T_{RM} development. Nonetheless, a minimum threshold of IL-12 (and T-bet) expression is required to not only promote the requisite development of T_{eff} but promote migration into peripheral sites. In support of this, it has been shown that CD103$^+$ DCs isolated from the small intestine are capable of producing IL-12 following TLR stimulation (39). However, high levels of IL-12 signaling had a direct effect on CD8$^+$ T cells, leading to the down-regulation of CXCR3, a molecule necessary for the accumulation of antigen-specific CD8$^+$ T cells in the airways following influenza infection (40). These data would suggest that CD8$^+$ T cells at the site of priming need just the right amount of IL-12 to reach their full T_{RM} potential. In terms of cytokines important for parsing T_{mem} into defined subsets, the common gamma chain cytokines IL-2 and IL-15 have been shown to play a role in CD8$^+$ T cell differentiation into T_{CM} and T_{EM} cells. T_{CM} cells can be identified as a distinct population arising from MPECs as early as 5 dpi, and are formed through IL-15 signaling (when IL-2 is limited), whereas IL-2 signaling leads to T_{EM} phenotypes (41). As previously mentioned T_{CM} cells develop early after infection from the MPEC population in the lymph node, and these cells may never enter peripheral tissues. Thus, T_{RM} cells may arise from T_{effs}, which do not receive early T_{CM} biasing signals in the lymph node, and retain the ability to enter peripheral sites.

While the evidence we have presented thus far suggests that specific cellular interactions and cytokines present in the lymph node at the time of priming could form a population of cells with the potential to become T_{RM} cells, an early development pathway completely unique to T_{RM} remains unlikely. Traditional cell surface markers and functional characteristics associated with T_{eff} cells or T_{EM}, such as low levels of CD62L expression, are indistinguishable from T_{RM} early after infection. Moreover, the prototypical T_{RM} cell surface marker, CD103, does not appear until after a certain period of tissue residency in the epidermis (14). Interestingly, T_{RM} populations in the skin require the expression of CXCR3 for entry into the epithelium and subsequent T_{RM} differentiation as cells lacking CXCR3 remained largely outside of the epidermis and T_{RM} recovered from the skin were numerically reduced. Conversely, mice lacking CCR7 expression have CD8$^+$

T_{effs}, which fail to leave the skin via the lymphatics and harbor larger numbers of T_{RM} cells, suggesting environmental factors are required for complete T_{RM} development (14). Signals encountered in the MdLN after respiratory infection likely generate a population of T_{eff} cells with the potential to enter the lung and fully develop into true T_{RM} cells. However, $CD8^+$ T cells isolated from the respiratory tract phenotypically resemble T_{EM} cells, T_{RM} cells, and terminally differentiated SLECs (our unpublished observations), suggesting not all T_{eff} that enter the lung become T_{RM}. More likely, cells immigrating to the respiratory tract enter as a common $T_{EM/RM}$ precursor (**Figure 1A**). This common $T_{EM/RM}$ precursor population is likely primed by $CD103^+$ DCs, expresses high levels of CD25, and encounters intermediate levels of IL-12, akin to the development of T_{EM} cells. Therefore, T_{RM} and T_{EM} cells may share similar early developmental pathways, with later signals in the lung further differentiating and diverting true T_{RM} from a common $T_{EM/RM}$ precursor. Indeed, evidence supports the development of a common T_{EM}/T_{RM} precursor. As previously noted, the development of T_{EM} cells is dependent on IL-2 (and not IL-15) (41) and IL-15 is also dispensable for $CD8^+$ T_{mem} that develops following a respiratory infection (42), which generates substantial T_{RM} compared to systemic infection (20). In contrast, systemic infections produce large amounts of T_{CM} cells, and T_{mem} in these infections require IL-15 for maintenance over time (43). Although the evidence suggests a common developmental pathway for T_{EM} and T_{RM} cells after initial activation in the lymph node, the possibility remains that they are distinct lineages by the time of lymph node egress (**Figure 1B**), identifiable by phenotypic markers or gene expression patterns yet to be discovered. Nonetheless, the full commitment to the T_{RM} lineage will continue in the specific peripheral tissue, where these cells will be retained.

ENVIRONMENTAL SIGNALS COMMIT T_{EM}/T_{RM} PRECURSOR CELLS TO A T_{RM} LINEAGE

If T cell priming in the MdLN results in the migration of a common $T_{EM/RM}$ precursor population of cells to the lung, what factors in the lung facilitate the development of "full-fledged" T_{RM} cells? At the site of infection multiple factors will continue to influence emigrating $T_{EM/RM}$ precursors. Evidence in cutaneous infection models suggests that commitment to the T_{RM} lineage is a two-step process characterized by the sequential up-regulation of Bcl-2 and CD69, followed by CD103 (14). This suggests that T cells first acquire a memory phenotype, or an increased chance of survival, prior to differentiating into T_{RM} cells based on the current T_{RM} phenotypic markers. This section will discuss the respiratory factors that influence the transition to a memory phenotype and specific environmental components present in the lung that polarize these anti-viral $CD8^+$ T cells toward a T_{RM} lineage.

The inflammatory environment of the lung

The pioneer T_{eff} cells immigrating to the lung arrive ~5–6 days after initial respiratory infection. Prior to their arrival, innate immune cells have accumulated, keeping viral titers low, and as a result, some local tissue damage has occurred via cytolysis of infected epithelial cells, affecting barrier function. The inflammatory effects of this local immune response in the lung are still very present at the time of T cell entry, and can influence the development of T_{RM} cells. However, since anti-influenza T_{eff} migrate to the lung asynchronously over several days (peaking at ~10 days post viral infection), all T cells do not encounter equivalent levels of inflammation which will likely affect the fate of individual T_{eff} clones.

The first $CD8^+$ T_{effs} to arrive at the site of infection will encounter the greatest level of inflammation, as infectious virus is still present (at least until ~d8 post influenza infection) and innate effectors such as NK cells are producing local IFN-γ (38). Inflammatory monocyte-derived DCs arrive in the inflamed lung at the same time as T_{eff} and function as lung APCs, amplifying the inflammatory milieu and locally expanding the emigrating T_{eff} (44). Additionally, $CD8^+$ T cell proliferation continues in the lung, a process requisite for viral control after influenza infection (45). This additional expansion, however, is not without a cost. Increased levels of cellular division is not only associated with increased levels of apoptosis within the highly dividing populations (46), the aforementioned cytokines also promote terminal differentiation of the T cells and the formation of $KLRG1^+$ SLECs (47, 48). Therefore, this early inflammatory environment skews cells away from becoming memory cells, yet may paradoxically pave the way for resolution from infection and inflammation so that later immigrants may develop into T_{mem}.

$CD8^+$ T_{eff} themselves produce cytokines in the lung, including IL-2, IFN-γ and TNF-α which enhance the overall inflammatory response (49). Interestingly, while $CD8^+$ T cells activated in lymph nodes rapidly gain the ability to produce the inflammatory cytokine IFN-γ, entry into the lung tissue imparts IL-10 production (50, 51) in a manner seemingly dependent on the inflammatory lung environment (52), indicating that an enhanced activation status resulting from high levels of inflammation induces the $CD8^+$ T cells to produce regulatory cytokines. IL-10 is also produced at high levels by regulatory T cells (T_{regs}) activated in the lung following influenza infection (53). The production of regulatory cytokines by T_{regs} and $CD8^+$ T cells is important to initiate "dampening" the immune responses in the lung to prevent excessive damage and loss of function of this essential organ. Importantly, the production of IL-10 can directly impact the development of memory cells by inducing MPEC populations in a STAT3 dependent manner (54), however, it is unclear whether IL-10 has any direct consequences on the development of T_{RM}.

A variety of other cytokines produced after influenza infection is known to modulate anti-viral $CD8^+$ T cell responses. Thymic stromal lymphopoietin (TSLP), an epithelial derived cytokine that can be produced in the infected lung (55, 56), promotes expansion of the $CD8^+$ T cells at the site of infection directly (56) and indirectly via $CD11b^+$ inflammatory DCs (57). Additionally, transpresentation of IL-15 by pulmonary DCs has been shown to increase the survival of T_{effs} (58) and is an important component of T_{RM} development in the skin (14). However, IL-15 does not seem necessary for the overall development of memory in the lungs or the airways following influenza infection (42), although this study as well as the TSLP studies did not address T_{RM} populations specifically.

Localization as an important step in development of T_{RM} cells

As residence at the peripheral site is a requisite for T_{RM} development, cells destined to become T_{RM} cells must first gain access into peripheral tissues, often into physically restricted areas such as within an epithelial layer or closely associated with the underlying basil lamina (within the parenchyma). The route of migration used by T cells trafficking to the lung, however, is not well understood. Cells can enter the lung via two circulatory systems: the bronchial system, which provides oxygenated blood to the lung tissue, and the pulmonary circulation, which includes vessels that bring deoxygenated blood to alveoli and subsequently drain oxygenated blood back to the heart (59). The lung epithelium surrounding the airway spaces share a fused basal lamina with the adjacent capillary endothelium to allow gas exchange and could facilitate direct blood to airway traffic. Because pulmonary vessels are small in diameter and thin walled, blood pressure in these vessels is relatively low, thus allowing lymphocytes to traverse the endothelium independent of the multistep paradigm described for lymphocyte migration through larger vessels, which are dependent on selectins, integrins, and chemokines (60). However, histological sections of lung tissues depict memory cells localized close to the airways, but within the lung parenchyma, evoking a blood → lung → airway route (19, 61).

Broadly speaking, activated $CD8^+$ T_{effs} cells can gain access to peripheral sites by virtue of their expression of CD11a and CD44 with concomitant loss of CD62L expression on their cell surface (60, 62).While access into distinct anatomical sites within other mucosal tissues such as the skin and gut is highly correlated with expression of tissue-specific homing receptors (63–65), analogous molecules have not yet been identified for lung homing $CD8^+$ T cells. Nonetheless, some chemotactic signals are associated with T_{eff} migration into inflamed lung tissues including CXCR3 (66) and CXCR6 (67). CXCR6 is specifically up-regulated on $CD8^+$ T cells isolated from the lung and lung airways following intranasal immunization and mice lacking CXCR6 have reduced protection against tuberculosis challenge (67), indicating that CXCR6 expression may be important for the establishment of $CD8^+$ T cells at sites of protection. The expression of CXCR3 is important to establish migration of $CD8^+$ T cells specifically to the airways (68). While T_{RM} populations were not assessed in this study, $CXCR3^-$ antigen-specific $CD8^+$ T cells isolated from the lung expressed lower levels of CD69 than WT cells occupying the airways where antigen is present. CD69 expression is upregulated on T_{RM} populations, and contact with antigen has been suggested to be necessary for T_{RM} formation (8). Therefore, expression of CXCR3 may be a requirement for the development of T_{RM} cells in the lungs, akin to the requirement for CXCR3 in the skin (14).

As influenza virus replicates primarily in epithelial tissue, the localization of $CD8^+$ T cells adjacent to antigen may expose them to unique cytokines available in and near the epithelium such as TGF-β. TGF-β plays a role in both the contraction of effector T cells (69) and the establishment of T_{RM} cells by inducing the expression of CD103 (70). The role of TGF-β in the development of T_{RM} cells has been well described in the intestinal mucosa and the skin, and has also been implicated in the development of T_{RM} in the lung (71). Although TGF-β can be transiently activated by influenza virus (72, 73), it likely has lower constitutive production in the lung than other barrier sites as over-expression of TGF-β can promote pulmonary fibrosis and lung disease (74). Due to the localization of TGF-β production, CD103 expression may be specific to only those cells, which are found within epithelial layers and not necessary for T_{RM} in the lung parenchyma, a concept discussed later in more detail. Interestingly, following influenza infection a large majority of antigen-specific $CD8^+$ T cells begin to express the α1β1 integrin VLA-1 (61). T_{effs} localized cells to the collagen-rich areas near the airways and basement membranes that are VLA-1^+ have a survival advantage over those that do not express VLA-1 at the peak of the $CD8^+$ T cell response (61). The localization and retention of cells within the lung parenchyma, as well as the survival advantage may make VLA-1 expression a unique marker for cells destined to become lung T_{RM} cells. However, this possibility has yet to be explored.

PART II: CHARACTERISTICS AND MAINTENANCE OF COMMITTED $CD8^+$ T_{RM} IN THE LUNG

Following the resolution from infection, antigen-specific $CD8^+$ T cells will persist at the site of infection (19). As previously noted, these T_{mem} cells exist in the lung in two basic compartments, the airways and the lung parenchyma. Airway $CD8^+$ T cells exist outside of the body, within the lumen of the respiratory tract, or they can exist much like they do in the intestinal epithelium as intraepithelial cells. Cells within the airways, and very likely some intraepithelial cells, can be isolated by performing a bronchoalveolar lavage (BAL), while the remaining parenchyma cells can be isolated through a process involving the enzymatic digestion of collagen. Additionally the localization and characterization of these cell populations can be defined by microscopic analysis of lung tissue sections, although phenotyping cells by this method is limited. It is important to distinguish between these two populations of cells in the discussion of T_{RM}, as airway cells are likely comprised of both true T_{RM} cells and circulating T_{mem}, which migrate to the airways following the resolution of infection.

Cells in the airways are subject to the external environment of the lung, where mucous and pulmonary surfactants decrease the potential for their long-term persistence. Therefore, it is thought that memory $CD8^+$ T cells in the lung airways, at least for some period of time, are partially maintained by the continual recruitment to the airways. In support of this, Slutter et al. showed that CXCR3 is required for the continual recruitment of cells into the airways, and that loss of CXCR3 expression results in the accelerated loss of antigen-specific $CD8^+$ T cells specifically from the airways (40). Tracking the entry of T_{mem} from the circulation is also possible by monitoring CD11a expression, which is lost ~40 h after $CD8^+$ T cell emigration into the airways (75). Indeed, when T_{mem} are extracted from the airways (up until at least 13 months post infection), portions of the antigen-specific $CD8^+$ T cells express high levels of CD11a. Together, these data confirm that at least a proportion of airway $CD8^+$ cells may not be bona fide T_{RM} based on presence within this site alone. In support of this argument, CD103 expression is reduced on antigen-specific T_{mem} isolated from the airway when compared to T_{mem} isolated from the lung parenchyma both in terms of frequency (11) and on a per cell basis (76). Finally, while evidence suggests that a circulating population of cells is actively recruited into the lung airways

during steady state conditions (40, 75) it is clear that these recruits are not sufficient (either in number or function) to provide protection against heterosubtypic influenza challenge, as protection wanes while recruitment continues. Perhaps the limited migration and supplementation of competent T_{mem} cells from within the lung parenchyma may augment this pool and maintain heterosubtypic immunity, at least temporarily. However, cell tracking studies have not confirmed this possibility.

T_{mem} also exist in the respiratory tract within the lung tissue or parenchyma. As the lung is a highly vascularized organ, it can be difficult to discern at time of tissue harvest, which antigen-specific cells are trafficking through the vasculature of the lung (trapped within small capillaries) and which are truly within the parenchyma. Experiments using intravascular staining whereby antibodies are injected directly into the blood stream immediately before the lungs are examined to "tag" circulating cells demonstrated a large number of cells isolated from the lung tissue are circulating cells (naïve or T_{EM}) despite perfusion. This method has been useful in characterizing both $CD4^+$ (77, 78) and $CD8^+$ (11) T_{RM} cells in direct contrast to the circulating pool. Using this method to distinguish circulating vs. resident cells has, and will, continue to provide a clearer picture of what T_{RM} cells look like in the resting lung.

Microscopic analysis of lung tissue sections has also been useful in determining the precise localization of T_{RM} in the respiratory tract to gain better insight regarding the cellular associations and tissue microarchitecture, which may be important for supporting T_{RM} development and/or survival. Turner et al. showed that $CD4^+$ T_{RM} cells established following influenza infection were clustered together in the lungs, in regions both close to the airways and to the pulmonary blood vessels (78). This would position the cells in an ideal place to encounter antigen entering the body. The clustering of cells in this location is not a new observation, nor is it exclusive for the $CD4^+$ T cell population. In 2004, Ray et al. showed that influenza specific $CD8^+$ T cells persisted in the highly collagenized area between the airways and the blood vessels, and that this retention was dependent on the expression of VLA-1 (61). VLA-1 binds to type IV and type I collagen (79, 80), which are important structural components of the lung interstitium, specifically between the bronchi and the vasculature, and the basement membranes of both the pulmonary vasculature and the epithelium of the airway, respectively (81, 82). The co-localization of T_{RM} and collagen below the epidermal cell layer of the airways shows that T_{RM} cells also exist within the lung parenchyma. The collagen-rich environment of the lung may provide a framework or scaffold in which T_{RM} cells can persist close to the site of antigen acquisition, yet not actually within the epithelial layer of the lung where they may be subject to the harsh environment of the airways. Additionally, it is quite possible that this collagen matrix could also trap or capture soluble growth factors important for T_{RM} maintenance.

THE PERSISTENCE OF T_{RM} CELLS IN THE RESPIRATORY TRACT: ROLE OF THE LUNG ENVIRONMENT

Like other mucosal barrier sites, the resting lung is engaged in a constant balancing act regarding immunity and tolerance. It is estimated that we breathe in 10,000 l of air per day, with each breath containing a plethora of allergens, environmental pollutants, and pathogens. Inappropriate response to non-harmful antigens could lead to persistent inflammation and pulmonary disease. To prevent this, multiple layers of innate protection exist in the lung to preclude any inappropriate initiation of an immune response. The most basic of these is the mucosal barrier itself. The lining of the upper respiratory tract is composed of ciliated epithelial cells and mucus-secreting goblet cells, which together function as a "mucociliary escalator" facilitating expulsion of these innocuous agents, as well as some commensal organisms, out of the respiratory tract without activation of the adaptive immune response. However, the mucus would also prevent T_{RM} cells from persisting in the airways of the upper respiratory tract, leading to the accumulation of T_{RM} either within the epithelium, the parenchyma, or in the airways of the lower respiratory tract. While the lower respiratory tract does not contain mucous, it is characterized by numerous "pockets" where gas exchange occurs termed alveoli. The cells lining the alveoli are specialized epithelial cells known as type I and type II alveolar epithelial cells, which form the structural architecture of the alveoli and secrete immunosuppressive pulmonary surfactants, respectively (83). The role that these lung derived factors may play on $CD8^+$ T cells at the site is further complicated by conditions of an inflamed lung, such as asthma and allergy. Allergens can induce the upregulation of pulmonary surfactants, which in turn can protect against allergic disease via local IL-13 inhibition (84). Due to the proximity of surfactants and T_{RM} cells in the lower respiratory tract, and the essential role for surfactants in regulating respiratory inflammation, it is possible that T_{RM} persistence could be dynamically regulated by perturbation in surfactant (and mucus) activity. However, this has not been analyzed.

T_{RM} persist long-term in many non-lymphoid tissues, albeit with different kinetics. For example, VSV-specific T_{RM} cells exist as long as 120 dpi in the brain (8) while cutaneous herpes simplex virus T_{RM} cells persist for the lifetime of a mouse (85). This is shown to occur independently of increased proliferation (8, 15) and maintained populations are not dependent on replenishment from lymphoid organs (6, 78). Perhaps somewhat unique to the respiratory tract is that T_{mem} cells within this site appear to have a limited life-span, steadily decreasing over time (19). The lack of long-term survival of T_{mem} cells lung airways, and perhaps certain populations in the lung itself, has functional consequences since heterosubtypic immunity against influenza viruses is lost ~4–6 months post infection (18). Moreover, this loss of anti-influenza immunity is coordinate with substantial loss in $CD8^+$ T_{mem} cells of the airways, despite stable numbers in the spleen (19) and the continual recruitment of cells from the circulation into the airways (40, 75). While these former studies did not directly assess the role of T_{RM} cells, recent evidence suggests that protective heterosubtypic immunity against influenza infection is mediated solely by T_{RM}, as the ability to control viral titers and protect from severe disease is gradually lost along with T_{RM} cells in the airways (20). Yet, the question of why T_{RM} cells do not persist in the lung and lung airways to the extent that they do in other tissues remains unanswered. Interestingly, following influenza infection lung T_{RM} cells retain expression of interferon-induced transmembrane protein IFITM3, which imparts cells with a survival advantage in the face of viral infection (76). This increased survival mechanism

Table 1 | Factors associated with the positioning and survival of defined pools of memory CD8$^+$ T cells in specific anatomical sites.

	CD127	CD122	PD-1	CD103	CXCR3	IFITM3	CD69	CD27	VLA-1
T_{EM}	+++	+++	−	−		−/+	−	+	
T_{CM}	+++	+++	−	−		−/+	−	++	
T_{RM} Lung	−/+	+	++	−/+	+++	+++	+++	++/+++	+++
T_{RM} Gut	+/++	+		+++			+++	+	++*
T_{RM} Skin	+	+		+++	+++		+++		+*
T_{RM} Brain	++	+	++	+++		+++	+++		−

−absent, +low levels, ++moderate levels, +++high levels, blank = no data for this tissue.

**Indicates data is from human studies, all other data in table obtained from mouse models.*

may be particularly important at this site, due to the regularity at which respiratory infections are acquired. The unique properties of respiratory T_{RM} cells have provided some insight into why their persistence in the lungs is limited (**Table 1**).

The cytokines IL-7 and IL-15 are requisite for the development and maintenance of memory CD8$^+$ T cells after systemic infection (35, 86). However, what role, if any, these cytokines play in the maintenance of T_{RM} cells in the lung has not been defined. In most sites assessed to date, T_{RM} cells express reduced levels of CD127, as compared to T_{CM} and T_{EM} cells. Concurrently, CD8$^+$ T cells in the lung airways express reduced levels of CD127 (11, 87, 88) as do cells in the lung parenchyma, although to a lesser extent (56). Like CD11a, it is possible that CD127 is cleaved from CD8$^+$ T cells in the airways, leaving these cells incapable of receiving proliferative or survival signals, either from IL-7 or from TSLP, which has been shown to be produced constitutively in the gut (89), and in the lung during both resting conditions and after inflammatory stimuli (56). IL-15 has been shown to be dispensable for the development and maintenance of memory cells that develop from respiratory infections and CD122 is lost from CD8$^+$ T cells within the respiratory tract (87). Furthermore, CD122 or the beta chain of the IL-15R, which signals to memory CD8$^+$ T cells is expressed at lower levels on T_{RM} isolated from the epithelium of the small intestine (90). A recently described pool of T_{RM} isolated from secondary lymphoid organs are maintained independently of IL-15 and even found in increased numbers in mice lacking IL-15 (91). Therefore, IL-15 appears to be uniformly dispensable for the maintenance of T_{RM} cells, and while levels of CD127 on T_{RM} cells is more variable, the near complete loss of this receptor in the respiratory tract may provide one mechanism in which CD8$^+$ T cells at this site have decreased sustainability. However, it should be noted that T_{RM} cells from the brain do not respond to IL-7 or IL-15 *ex vivo*, unlike splenic memory cells, which show increased survival upon exposure to these cytokines (16), indicating that perhaps the survival of T_{RM} cells is completely independent of classical cytokine memory signals.

The maintenance of CD8$^+$ T cells in the lungs has also been attributed to residual antigen found in the MdLN for ~2 months post influenza infection (92). Influenza antigens have also been detected in the lung tissue itself for 30 days within focal inflammatory structures (93), reminiscent of inducible bronchus associated lymphoid tissue (iBALT). iBALT develops following influenza infection and has similar structure to lymph node tissue, such as defined B cell follicles and the formation of germinal centers surrounding DCs; this structure contributes to the proliferation of B and T cells during primary influenza infection and can be protective in mice where other lymphoid organs are lacking (94). As the timing of loss of residual antigen coincides with the loss of protective heterosubtypic immunity, it has been hypothesized that antigen is necessary for the persistence of T_{mem} in the lung and lung airways. In support of this possibility, T_{RM} cells in the lung express PD-1 (20), which may indicate continued exposure to antigen. While certain T_{RM} populations have been shown to persist in the absence of antigen (8, 31) definitive studies have not been carried out for T_{RM} cells in the lung to rule this out as a mechanism for maintenance.

It is likely in humans that the maintenance and survival of T_{RM} cells may be much different than what is observed in mice. As previously mentioned, constant antigenic stimulation, allergic inflammation, and relatively common airway disorders such as asthma will influence the lung environment in ways that will affect many indigenous respiratory cells. In addition, the regularity of respiratory infections in humans will result in the accumulation of many pools of clonally diverse antigen-specific cells, recognizing a plethora of pathogens. de Bree et al. showed that influenza and respiratory syncytial virus-specific CD8$^+$ T cells were enriched in the human lung compared to the circulation (95). In direct contrast, antigen-specific CD8$^+$ T cells that developed from the blood-borne pathogens cytomegalovirus and Epstein-Barr virus equilibrated between the blood and lung of these patients (95). The accumulation of CD8$^+$ T cells in the lung due to respiratory infection would certainly lead to large numbers of T_{RM} cells populating the human lung during steady state conditions. Indeed, studies have determined that CD103$^+\alpha\beta$ TCR CD8$^+$ T cells comprise about 1/3 of the total CD8$^+$ T cell population in the human lung (96), or over 10 billion total cells (97). However, the history of human lung T_{RM} (when developed/how long maintained) and how the history of individual clones correlates with acquisition of specific infections is difficult to determine. Furthermore, in humans, the survival of these pools may be affected by attrition resulting from heterologous infections. In these scenarios, either competition for resources in distinct environmental niches or by bystander apoptosis via cytotoxic factors present at the time of the new viral infection may deplete previously existent T_{RM} pools (98).

CONCLUDING REMARKS

The study of T_{RM} cells is in its infancy. As we continue to analyze this unique lineage of memory cells, we will certainly deepen our understanding of T_{RM} biology in unique sites such as the respiratory tract and perhaps better understand how to selectively manipulate this pool for development of vaccines. While the defining characteristic of what makes a cell a T_{RM} cell is quite clear (i.e., long-term residence at a site), some of the markers currently used to distinguish T_{RM} cells, most notably CD103, only recognize a subset of T_{RM} cells localized to the (respiratory) epithelium. This leaves a large population (anywhere from 50 to 90% of T_{RM} cells in the lung) excluded from studies. Thus, overall T_{RM} frequency can only be confirmed using complicated transfer and cell tracking experiments, warranting the need for more definitive phenotypic markers to readily identify T_{RM}. Moreover, understanding the environment in which T_{RM} cells at specific sites reside will be key to developing phenotypic definitions of these cells, as markers vary between anatomical locations. In the case of the lung, this particular environment has many mechanisms in place to suppress inflammation and any inadvertent immunopathology. Thus, while higher numbers of T_{RM} cells at the site of infection may be ideal for protection against disease, tight regulation of the number, and longevity of T_{RM} cells at this site may be essential for tissue function. This may be especially relevant in the context of human disease, where respiratory infections are commonplace and populations of T_{RM} are not only numerically enhanced but very likely dynamically regulated.

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Tissue-resident T cells: dynamic players in skin immunity

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The skin is a large and complex organ that acts as a critical barrier protecting the body from pathogens in the environment. Numerous heterogeneous populations of immune cells are found within skin, including some that remain resident and others that can enter and exit the skin as part of their migration program. Pathogen-specific $CD8^+$ T cells that persist in the epidermis following infection are a unique population of memory cells with important roles in immune surveillance and protective responses to reinfection. How these tissue-resident memory T cells form in the skin, the signals controlling their persistence and behavior, and the mechanisms by which they mediate local recall responses are just beginning to be elucidated. Here, we discuss recent progress in understanding the roles of these skin-resident T cells and also highlight some of the key unanswered questions that need addressing.

Keywords: skin immunity, tissue-resident memory T cell, intravital imaging, two-photon microscopy, DETC, cell migration

IMMUNE CELL SUBSETS IN SKIN

The skin is a complex organ with critical roles in defense against pathogens. The epidermis forms a physical barrier that limits entry of microorganisms that make up the substantial microbiome on the skin (1), as well as pathogens and substances in the environment. The stratified layers of the epidermis are composed of specialized epithelial cells: the keratinocytes. The outermost layer of the epidermis, the stratum corneum, is composed of dead keratinocytes (corneocytes) that perform the main barrier functions. Keratinocytes in the basal layer of the epidermis are responsible for establishing the upper layers of the epidermis through cell division, and progeny of these cells migrate upwards as they differentiate and eventually die (2). Keratinocytes have key roles in immune defense via the production of cytokines, chemokines, and antimicrobial proteins in response to environmental or pathogenic stimuli. Cytokines and chemokines produced by keratinocytes alert cells in the dermis and in lymph nodes (LN) draining the skin of potential danger as well as recruit cells of the immune system (including neutrophils, monocytes, and T cells) to the skin.

The dermis is separated from the epidermis by a continuous basement membrane. The epidermis is interspersed with invaginations for hair follicles that are themselves, also lined by basement membrane and separated from the dermis. The dermis is composed of a network of fibroblasts that produce a collagen-rich extracellular matrix. In addition, blood vessels and lymphatic vessels are distributed throughout the dermis, facilitating entry of immune cells from the blood and exit to the LN, respectively.

A variety of immune cells are present in normal skin (**Figure 1**), including subsets of dendritic cells (DC) and lymphocytes, as well as macrophages, mast cells, and neutrophils (3). DC resident in the dermis can be divided into two main subsets: $CD103^+CD11b^-$ (that can be further divided into langerin +/−) and $CD11b^+$ (that can either be derived from steady-state precursors or from monocytes recruited during inflammation) (4). These DC migrate throughout the dermis before egressing via the afferent lymphatics to the LN where they either directly present antigen to T cells or transfer antigens to DC resident in the LN (5). In the epidermis, Langerhans cells (LC) form a dense network of DC capable of capturing antigen and migrating to the LN after traversing the basement membrane into the dermis. In mice, LC appear to be particularly efficient at tolerance induction and the formation of regulatory T cells (Tregs) (6, 7), whereas they are dispensable for induction of CD8 T cell responses to infections (8–10). Skin macrophages also populate the dermis and include perivascular macrophages that are distributed along post-capillary venules and can assist with the recruitment of neutrophils from the blood (11). Furthermore, a number of innate $CD3^-$ lymphocytes (ILC) have been described in the skin, including $NKp46^+$ ILC1 NK cells (12), $CD90^{hi}$ ILC2 cells that produce IL-13 (13), and more recently $NKp44^+$ ILC3 cells in human skin with psoriatic lesions (14). These ILC appear to reside in the dermis where they can interact with resident cells such as mast cells (13).

Lymphocytes are present in significant numbers in healthy skin, in particular $CD4^+$ T cells, which populate the dermis (15). In contrast, B cells are rare in healthy skin. Tregs are also found in substantial numbers in healthy mouse dermis and their contribution to immunity or inflammation appears regulated by skin commensals (16). Skin Tregs display a much slower migrational velocity compared with effector $CD4^+$ T cells although acute inflammation results in a rapid increase in their motility (17). $CD8^+$ T cells

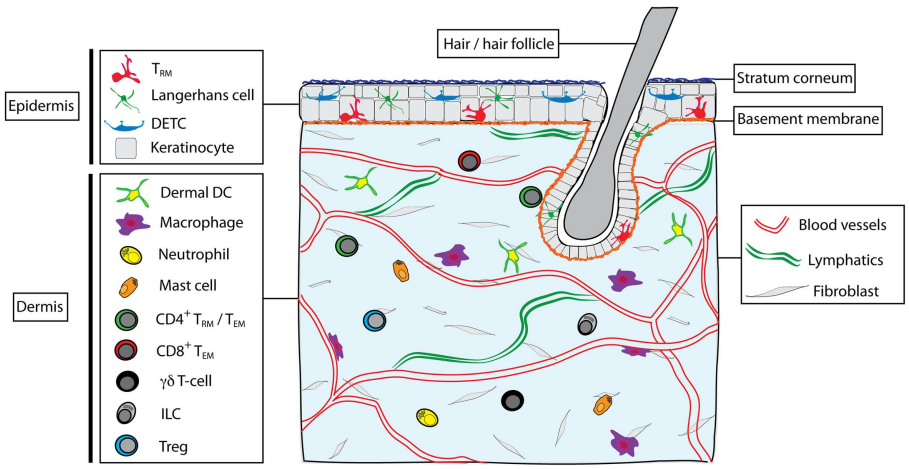

FIGURE 1 | Skin structure and immune cell types found in skin. The skin is composed of epidermis and dermis, interspersed with hair follicles. Dead keratinocytes construct the stratum corneum in the outer epidermis. The dermis and epidermis are separated by a basement membrane. Blood vessels and lymphatic vessels and a network of fibroblasts are found in the dermis, as well as nerves, sebaceous glands, sweat glands (not shown). Multiple immune cells types are found within skin, including Langerhans cells, dendritic epidermal γδT cells (DETC), and memory αβT cells (T_{RM}) in the epidermis. In the steady state, the dermis contains a heterogeneous mix of immune cells, including subsets of dendritic cells (including $CD11b^+$ and $CD103^+$ DC), macrophages (including dermal and perivascular macrophages), neutrophils, mast cells, γδT cells, ILC, $CD4^+$ T cells (both T_{EM} and possibly T_{RM} subsets), T regulatory cells (Treg), and $CD8^+$ T_{EM}.

are found in the skin in mice and humans, and are predominantly localized to the epidermis, in contrast to the predominantly dermal $CD4^+$ T cells. This dichotomy is striking following herpes simplex virus infection (HSV) where antigen-specific $CD4^+$ and $CD8^+$ memory T cells localized to the dermis and epidermis, respectively, after clearance of the pathogen (18) (**Figure 2A**). The epidermal $CD8^+$ T cells persist for long periods in this anatomical compartment (19) and are now commonly referred to as tissue-resident memory T cells (T_{RM}).

Finally, populations of γδT cells are found in the dermis and epidermis, where they can contribute to wound healing and immune responses (20–22). Dendritic epidermal γδT cells (DETC) form a prominent network in the skin in mice where they appear to monitor the integrity of the epidermal layer. DETC form polarized immunological synapses that anchor at keratinocyte tight junctions (23). In response to infection or wounding, DETC upregulate molecules including NKG2D, JAML, and CD100 that contribute to inflammation and assist in wound closure (21, 24–26). DETC can also be infected by viruses such as HSV-1, which may influence their survival and functions during skin infections (27).

SKIN TISSUE-RESIDENT MEMORY T CELLS

$CD8^+$ T_{RM} cells that reside within the epidermis are retained in this compartment for very long periods without reentering the circulation (19, 28). Populations of T_{RM} have also been described in other tissues including the small intestine (29, 30), vaginal mucosa (18, 31), brain (32, 33), lung (34), salivary glands (35), and thymus (36). These cells can be identified by high expression of the α_E integrin chain (CD103) and the marker CD69 (19). T cells expressing this canonical T_{RM} phenotype have also been observed in other tissues such as the kidney, pancreas, and heart (32), while $CD69^+$ T cells may also reside in LN and spleen for extended periods and provide a unique pool of cells that could guard against systemic pathogen entry (37).

Tissue-resident memory T cells that form in the skin, intestine, and lungs were recently shown to express a core set of genes that may facilitate accurate dissection of this memory T cell subset at a molecular level (38). This transcriptional signature suggests T_{RM} undergo a similar developmental program in different tissues. Elucidating the molecular pathways critical for T_{RM} development from tissue-derived signals will be important for future therapeutic approaches.

In addition to $CD8^+$ T cells, some $CD4^+$ T cells may also form a T_{RM} population in the lungs after respiratory viral infection (39). Although a proportion of the memory $CD4^+$ T cells found within the dermis appear to be capable of entering the circulation (18), it is not yet clear whether the remaining cells permanently or semi-permanently reside in this site (i.e., could be designated T_{RM}) and might thus be distinguished from circulating T effector memory cells (T_{EM}).

During infection or inflammation of the skin, effector $CD8^+$ T cells enter the dermis from the blood, and can then be recruited into the epidermis. This process is dependent on chemokine receptor signals, including CXCR3 (38). Whether other chemokine receptors are also required for $CD8^+$ T cell entry into the epidermis is unclear, though this is likely since T_{RM} formation was only partially blocked when effector $CD8^+$ T cells lacked CXCR3 expression. In contrast, in order to exit the skin via lymphatics T cells need to upregulate expression of CCR7, and blocking this step can promote increased T_{RM} formation. T_{RM} in skin develop from $KLRG1^-$ effector cells that also give rise to classical central memory T cells (T_{CM}) in the circulation (38). Once they enter the skin, these precursors migrate more effectively to CXCR3 ligands

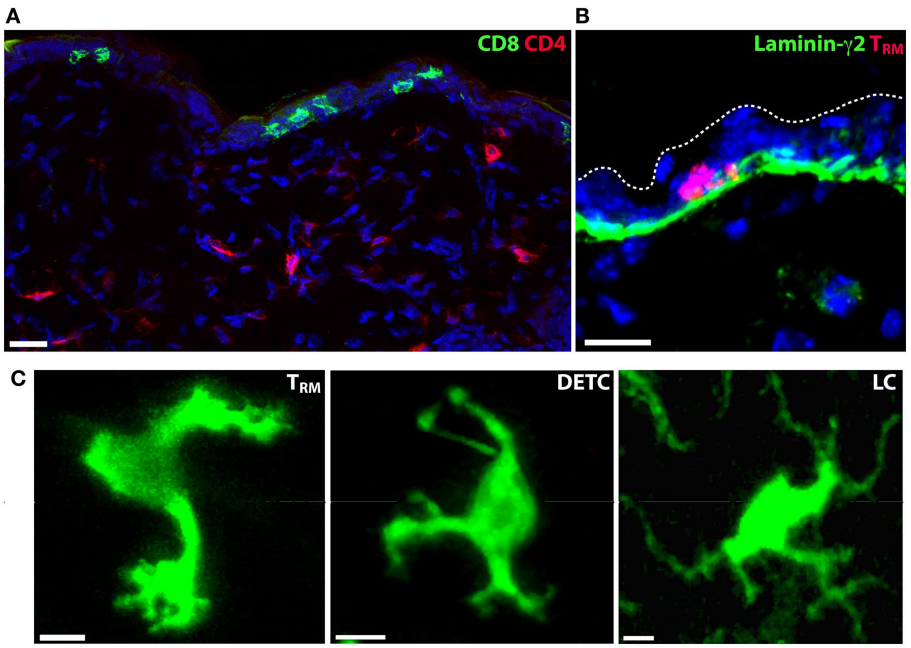

FIGURE 2 | Tissue-resident immune cells in the epidermis. **(A)** $CD4^+$ and $CD8^+$ T cell localization in the skin of mice following clearance of HSV-1 infection. $CD4^+$ T cells (red) localize to the dermis, while $CD8^+$ T_{RM} persist in the epidermis. Nuclei are stained blue with DAPI. **(B)** Skin T_{RM} localize to the basal epidermis in contact with the basement membrane that separates dermis from epidermis. $CD8^+$ T_{RM}, red; laminin-γ2, green; DAPI, blue. **(C)** The morphology of epidermis-resident T_{RM}, LC and DETC is distinct. Scale bars: A, B: 20 μm; C: 5 μm.

including CXCL10. Signals found within the epidermis instruct $CD8^+$ T cells to develop into T_{RM} via upregulation of molecules involved in the persistence of these cells (CD103 and CD69), and downregulation of S1PR1 that is required for tissue egress (38, 40). Our recent data also revealed that T_{RM} express high levels of regulator of G protein signaling-1 (RGS1) and RGS2 (38). RGS1 expression has been shown to reduce T cell migration in response to CXCL12 and CCL19 (41), suggesting that these molecules may also contribute to the migration and persistence of T_{RM} within the skin as well as other tissues.

MIGRATION BY SKIN T_{RM}

$CD8^+$ T_{RM} localize to the basal layers of the epidermis in mice and appear to be in regular contact with the basement membrane that separates the epidermis from the dermis (42) (**Figure 2B**). Whether T_{RM} use this as a substrate for migration and adhesion is not known. Although the epidermal layer is considerably thinner in mice than in humans, $CD8^+$ T cells also appear to localize to the border between the epidermis and the dermis in humans following HSV-2 infection (43) and in healthy or psoriatic skin (44). A unique feature of skin T_{RM} is their highly dynamic dendritic morphology (18, 42, 45) (**Figure 2C**). In contrast, T cells in the dermis consistently display a more amoeboid shape that is typical of T cells observed in all other tissues thus far. Whether T_{RM} in other tissues display a similar morphology and slow mode of migration is yet to be determined.

The immediate tissue environment appears to dictate the morphology and locomotion of T cells. This is supported by our observations in mice that both $CD4^+$ and $CD8^+$ T cells displayed a pronounced dendritic morphology when present within the epidermis, irrespective of whether the T cells were activated effector cells or memory cells (42). In addition, epidermal LC and DETC both adopt dendritic shapes. Notably, each of these cells (T_{RM}, DETC, and LC) can be distinguished from each other by key differences in cell shape (**Figure 2C**). Whereas T_{RM} form many amorphous shapes marked by short dendrites and many small projections similar to filopodia, DETC produce a relatively consistent number of long dendrites and are mostly immotile because they are anchored in the upper epidermis. LC are immotile and produce multiple long, branched dendrites. While both DETC and LC project their dendrites upwards toward the stratum corneum, T_{RM} were only observed to extend projections laterally (42).

T cells migrating within the epidermis reduce their speed upon resolution of inflammation (42), suggesting that tight connections between keratinocytes present a difficult environment for T cells to navigate. It will be important to determine whether T_{RM} regulate unique molecules that facilitate digestion of the surrounding matrix and cell–cell adhesions to allow them to move relatively freely. The mechanisms used by T_{RM} to navigate the epidermis, including the molecules and pathways regulating the actin cytoskeleton to induce the unique cell shape are unclear. Since T cells migrating within tissues do not typically generate substantial protrusions such as lamellipodia or blebs (46), the way in which the actomyosin network generates force to propel T cells in the epidermis may differ from that in the dermis and other tissues. Moreover, the roles of adhesion molecules such as integrins and chemotactic factors including chemokines in controlling T cell migration in the epidermis is not known. The integrin CD103 is involved in the

attachment of DETC dendrites to the keratinocytes (23). We found that CD103 expression by T_{RM} is important for their long-term retention in skin (38). This is unlikely to involve stable dendrite attachment due to the motile nature of T_{RM}, though persistent adhesion to the keratinocytes via E-cadherin may facilitate the retention of T_{RM} in this site. T_{RM} also show increased expression of E-cadherin, the ligand for integrin $\alpha_E\beta_7$, as well as the integrin $\alpha_1\beta_1$ that binds collagen and laminin, both major components of the basement membrane separating epidermis from dermis. T_{RM} in skin have increased expression of the chemokine receptor CCR8 compared with memory T cells in other tissues (38). Expression of CCR8 is programed by the epidermis (47) suggesting that expression of this receptor is important for αβT cell residence in this site. Together, these receptors potentially contribute to adhesion, morphology, and survival of T cells in the epidermis.

IMMUNOSURVEILLANCE AND LOCAL PERSISTENCE OF SKIN T_{RM}

Although T_{RM} localization in the skin can be relatively dispersed (28), they predominate at sites of infection or inflammation (19). This concentration of memory cells in mouse skin remains remarkably constant for >1 year after infection, despite their sustained motility (42). *In silico* simulation of the migration of T_{RM} in the skin over long periods revealed that T_{RM} move by random brownian motion and persist within the region of the epidermis in which they form simply as a result of this slow migration. These experiments suggest that T_{RM} induced by infection or vaccination should persist for very long periods in the immediate environment where they were formed and provide robust site-specific immunity. While such site-specific immunity may be of little use against subsequent infections at remote sites, repeated infections can induce T_{RM} in non-involved regions of skin (28), potentially providing more widespread protection at least in this tissue. Whether this is the case with many infections or tissues and the protective efficacy of these more dispersed T_{RM} needs to be investigated further.

Skin T_{RM} display a persistent mode of random migration that can facilitate surveillance of skin against reinfection or the recrudescence of latent viruses such as HSV (42, 45). Although T_{RM} migrate considerably slower in the epidermis of mice than T cells in the dermis or in lymphoid tissues, the cells are trapped within the constrained epidermal environment and move largely two-dimensionally. How T_{RM} survey the epidermis in humans remains to be visualized, although the location of these cells in the basal epidermis in human skin samples suggests that the mechanism and efficiency of immunosurveillance may be very similar to that observed in mice. Importantly, in addition to the shape and motility of T_{RM}, the density of cells present in the epidermis will likely influence the efficiency of their surveillance, as suggested in experiments modeling T_{RM} migration (45). Therefore, novel vaccine strategies designed to induce T_{RM} in the skin or other sites in the body may need to reach a certain threshold of T_{RM} density in the tissues for effective protection against disease.

EPIDERMAL NICHE

As mentioned above, large numbers of γδT cells (DETC) exist in the epidermis in mice, where they contribute to homeostasis, would repair and inflammation. In humans, γδT cells are present in the epidermis, though in lower numbers than αβT cells. The reason for this difference is unclear, though both T cell subtypes present in human epidermis can contribute to wound repair (21), suggesting that this may reflect a functional specialization of all T cells that persist in this tissue, as opposed to only γδT cells. DETC are the first T cells that form in mice very early in life. After migrating to the skin, they persist for life and are maintained by homeostatic turnover. Examination of DETC in mouse skin after the clearance of HSV infection revealed a substantial and sustained decrease in DETC numbers around the site of infection, and a corresponding increase in numbers of virus-specific T_{RM} (42). This inverse relationship between DETC and T_{RM} was maintained for months, suggesting that DETC were unable to repopulate regions of skin containing considerable numbers of T_{RM}. These findings indicate the existence of a T cell-specific niche within the epidermis that regulates the total number of T cells in this site, irrespective of TCR usage or specificity. Both DETC and T_{RM} rely upon the cytokine IL-15 and signals via the aryl hydrocarbon receptor (AhR) for persistence in the skin (38, 42, 48, 49). AhR is a transcription factor that can regulate a large number of genes, including c-kit and various cell cycle genes, suggesting that this pathway may influence T cell proliferation and homeostasis in the epidermis (50). Ligands for AhR are produced in the epidermis via metabolism of tryptophan or from microbiota such as yeast. Nevertheless, AhR ligands are abundant in the skin, suggesting that other mechanisms likely also contribute to the regulation of T cell numbers in the epidermis.

If the epidermis constitutes a privileged niche with limited space for populations of T cells, this may have implications for T_{RM} persistence following subsequent infection or inflammation where new populations of effector $CD8^+$ T cells are recruited to the skin. Therefore, whether there is a maximum number of T cells capable of persisting in the epidermal niche remains a key unanswered question. If so, we would expect that competition for space in this niche would restrict numbers of T_{RM} that can persist in regions of skin prone to multiple infections. Moreover, if effective protection from infection requires a certain density of T_{RM} in skin to rapidly respond, then competition for niche may influence such immunity. This also raises the intriguing question of whether low numbers of γδT cells in the epidermis of adult humans, and correspondingly higher numbers of αβT cells is, at least in part, the result of replacement of DETC via competition for space by T_{RM} that are generated by infections and environmental antigens. Developing a better understanding of the mechanisms of T cell homeostasis within the epidermis is critical for the design of strategies to boost immunity to infections as well as potentially reducing unwanted T cell responses.

PROTECTION BY SKIN T_{RM}

Reinfection with a previously encountered pathogen results in recruitment of circulating memory T cells to the inflamed tissues where they function to eradicate the infection. $CD8^+$ T cells recruited to tissues then clear the pathogen by killing infected cells and releasing cytokines. This process is still relatively slow, yet appears to be significantly enhanced by the presence of T_{RM} within the infected tissues. Notably, T_{RM} present within mucosal tissue

epithelia were found to rapidly produce interferon-γ upon peptide stimulation, resulting in the non-specific recruitment of circulating memory T cells into the tissue within hours (51). Thus, it has been suggested that T_{RM} function as an antigen-specific sensor and rapidly respond by producing signals that induce local inflammation and recruit memory T cells from the blood. Though it is not yet clear whether T_{RM} in different tissues behave the same way, it will be important to determine what signals are released by T_{RM} in response to stimulation and the effects that these have on the subsequent response. For example, we found that T_{RM} express high levels of the chemokine XCL1, which may allow them to recruit $XCR1^+$ cells such as dermal $CD103^+$ DC (52) and facilitate local recall responses.

In addition to an alarm function, T_{RM} presumably also contribute directly to the clearance of pathogens in tissues. Whether they do this via the killing of target cells or production or cytokines, or both, has not yet been determined. Moreover, the relative contribution of T_{RM} versus memory T cells recruited from the circulation is not known. Thus, examination of whether skin T_{RM} have the capacity to eradicate a local infection without the assistance of circulating memory $CD8^+$ T cells will provide insight into the role of resident memory in protective immunity. The relative roles of T_{RM} in raising the alarm versus directly clearing an infection may be influenced by their density with the tissue. We would predict that a high local density of T_{RM} could protect against viral infection and possibly provide sterile immunity. There is some evidence to suggest that T_{RM} also proliferate locally in response to challenge (53, 54), although the extent and widespread nature of this proliferation remains far from clear. Finally, given the restricted localization of T_{RM} to epithelial layers such as the skin epidermis, we might predict that these memory cells are terminally differentiated and highly dependent on their environment to survive. Experiments suggest this is the case, since isolation of T_{RM} from the brain followed by adoptive transfer into mice demonstrated poor survival and responses to challenge (33). Nevertheless, it remains unclear whether T_{RM} can exit the epithelial layers upon recall and migrate through tissues or enter the circulation, and if they do whether they can survive. Finally, experiments are needed to determine if T_{RM} form secondary memory in the tissues following restimulation, or are replaced by memory cells recruited from the circulation.

PERSPECTIVES

There is considerable complexity in the immune cell content of the skin. While this content includes populations such as T cells and DCs, it is now clear that these are heterogeneous, comprised of a number of phenotypically and functionally distinct subsets. In the case of T cells in particular, their action is predominantly local, affording regional protection against skin-invading pathogens or promoting tissue repair after injury. Given the need for such restricted action, it is not surprising that the skin contains skin-resident populations. Despite this, the relative contribution of resident versus migrating cells still remains unclear in many instances. The existence of such uncertainty highlights the need for clear demarcation between resident and migrating populations in future studies of the skin immune system.

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Visualizing T cell migration *in situ*

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Mounting a protective immune response is critically dependent on the orchestrated movement of cells within lymphoid tissues. The structure of secondary lymphoid organs regulates immune responses by promoting optimal cell–cell and cell–extracellular matrix interactions. Naïve T cells are initially activated by antigen presenting cells in secondary lymphoid organs. Following priming, effector T cells migrate to the site of infection to exert their functions. Majority of the effector cells die while a small population of antigen-specific T cells persists as memory cells in distinct anatomical locations. The persistence and location of memory cells in lymphoid and non-lymphoid tissues is critical to protect the host from re-infection. The localization of memory T cells is carefully regulated by several factors including the highly organized secondary lymphoid structure, the cellular expression of chemokine receptors and compartmentalized secretion of their cognate ligands. This balance between the anatomy and the ordered expression of cell surface and soluble proteins regulates the subtle choreography of T cell migration. In recent years, our understanding of cellular dynamics of T cells has been advanced by the development of new imaging techniques allowing *in situ* visualization of T cell responses. Here, we review the past and more recent studies that have utilized sophisticated imaging technologies to investigate the migration dynamics of naïve, effector, and memory T cells.

Keywords: CD8 T cells, imaging techniques, intravital microscopy, migration, T cells, infections

IMAGING TECHNOLOGY

The ability to image the dynamics of T cell immune responses *in situ* has undergone significant advances over the past decade. For over a century, bright field transillumination or epifluorescence microscopy was the only technology utilized to image excised organ sections or to visualize cellular processes *in vivo*. These techniques were useful for visualizing leukocyte interactions with the endothelium (1–3). The introduction of immunohistochemistry and immunofluorescence coupled with the use of monoclonal antibodies introduced specificity to the staining of lymphocytes. More recently, the advent of integrated fluorescent probes (e.g., CFSE) or natural fluorescent proteins (e.g., green fluorescent protein) permitted investigators to tag specific cell populations *in vivo*. These fluorescently labeled cells could now be tracked in real-time by directly imaging organs in explant preparations or directly intravitally in live animals. An overview of the techniques used for dynamic imaging of T cells is shown in **Figure 1**.

A significant technological advance was achieved with the laser scanning confocal microscope (LSCM). This type of microscope uses a lens to focus the laser excitation light on the specimen and the emitted light from the focal plane is refocused trough the same lens to the center of an open detector aperture (pinhole). This innovation obstructs the light coming from above and below the focal plane and thus increases the resolution. Sharp optical sectioning through a specimen at different depths can be performed to produce a 3 dimensional reconstruction of the sample. However, single photon confocal microcopy does not allow imaging at great depth ($>100\,\mu m$) due to light scattering, photobleaching of stained tissue that is outside of the focal plane, and slow speed of data acquisition. Thus, it is very suitable for imaging thin tissues sections. Real-time dynamic imaging using LSCM is limited to the surface of the organ and for shorter periods of time. However, recent modifications to the standard single photon confocal microscope such as the addition of a microlens high speed spinning disk prevents cell damage and allows for rapid acquisition of imaging data of very large surfaces (approximately $870\,\mu m \times 660\mu m$) (6). Thus, if deep tissue imaging is not required, the spinning disk confocal microscope can be very effective for performing dynamic imaging of large areas of various tissues. Several groups have recently used this technology for *in vivo* imaging, since it allows superior resolution (7). In a recent study, Cockburn and colleagues described the antigen-specific CD8+ T cell mediated killing of liver stage malaria parasites using a high speed spinning disk confocal microscope (7). In this case, even a superficial penetration of the laser beam was sufficient to observe the morphology of the liver parenchyma.

Compared to conventional lower wavelength and single photon excitation, the use of near-infrared two-photon (2P) excitation permits imaging of tissues at substantially greater depth ($>300\,\mu m$). Moreover, the fact that the excitation of fluorescent proteins is confined to the focal plane significantly minimizes the problem of photobleaching. Consequently, by using 2P microscopy it is now possible to visualize the dynamics of immune cells in real-time, and at greater depths in intact explanted tissues or in live animals without causing overt cellular damage (8). Readily available tissues like the skin and the associated draining lymph nodes (dLN) were among the first tissues that were imaged intravitally using elegant surgical techniques (**Figure 1**). More recently,

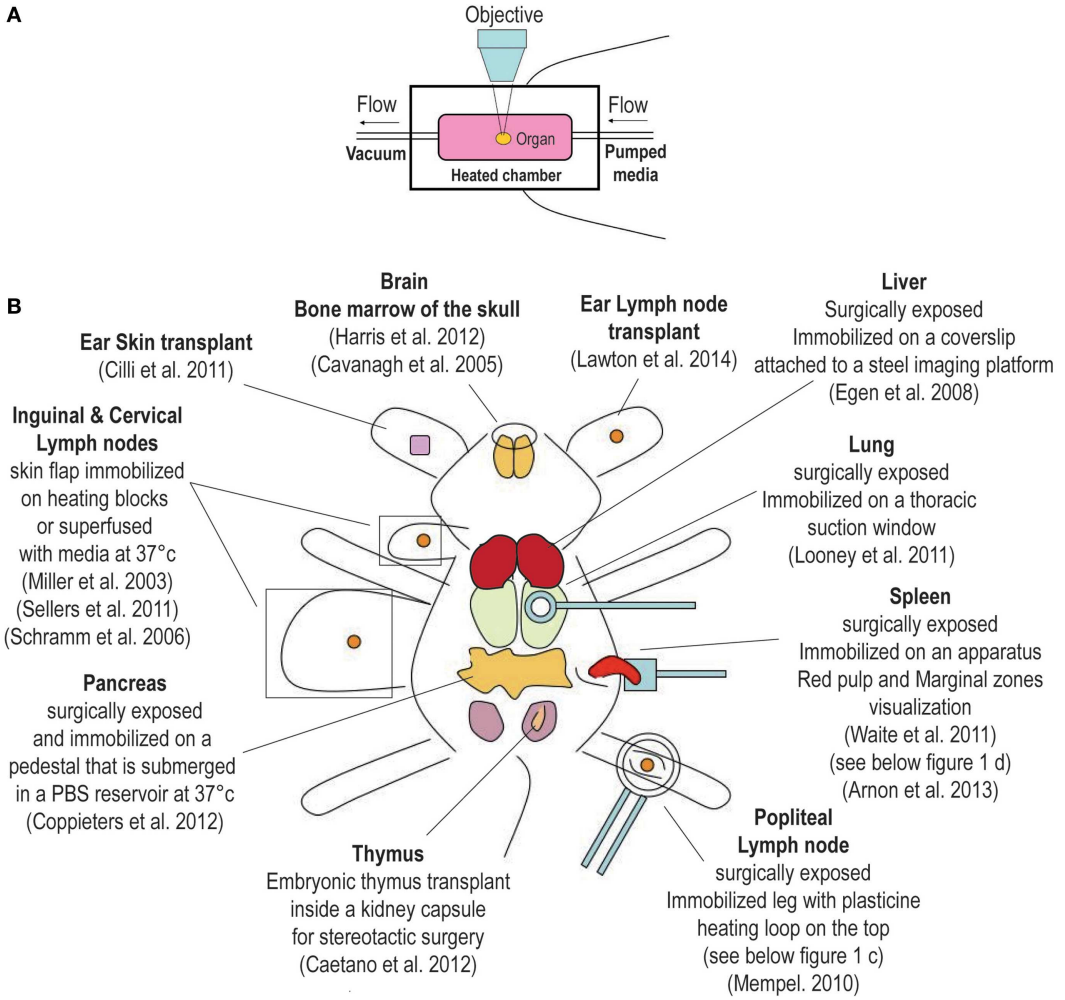

FIGURE 1 | Overview of surgical techniques for studying T cell dynamics. **(A)** Explant system, the organ is kept in a heated chamber. Constant flow of warm media (bubble with 95% oxygen and 5% carbon dioxide) is maintained by a peristaltic pump. **(B)** Examples of intravital imaging methods previously used to observed T cell dynamic *in vivo*. **(C,D)** Examples of custom built stages used to immobilize the mouse for intravital imaging. **(C)** This stage has been used to image the popliteal lymph node; for a detailed description of this method, please refer to a publication by Murooka and Mempel (4). **(D)** The second stage is designed for imaging the spleen (5).

2P microscopes have been modified and used to image several non-lymphoid tissues such as the lung, the intestines, the brain, and the liver (**Figure 1**) (9–12). 2P microscopy can also be used to visualize non-centrosymmetric structures such as collagen fibers (13). Non-linear optical effect called second harmonic generation (SHG) can be used to image collagen bundles in muscle and in bone tissues. When using a 2P laser, the emission of the SHG signal is exactly half of the excitation wavelength and can be very useful

for providing structural reference of most tissues being imaged *in vivo* (14).

T cells are constantly moving inside and between organs, they are among the most motile cells in the body (an average of 10 μm/min, with peak velocity as high as 25 μm/min in the LN) (15). For this reason, the use of 2P microscopy has been a critical tool that has significantly increased our understanding of the dynamics of T cell responses *in vivo* (8, 16, 17). The disadvantages of this technique are the cost, and the limitation of the available fluorescent reporter mice or fluorescent probes.

SURGICAL TECHNIQUES TO STUDY T CELL DYNAMICS *IN SITU*

Among the first techniques used for observing T cell dynamics *in situ* was the organ explant system (**Figure 1A**) (18). It consists of a heated imaging chamber in which an organ such as a LN is immobilized and the chamber is then perfused with heated oxygenated media. This method offers greater stability and is suitable for imaging number of lymphoid and non-lymphoid tissues (11, 15, 19–21). However, excised organs that are submerged in a media filled chamber lack major vascular innervations such lymphatics and blood vessels. Moreover, chemokine production and distribution within the organ may be completely disrupted, and thus, the milieu in the excise organ may not reflect the tissue environment that exists *in vivo* in live animals. Moreover, in certain situations the dynamics of T cell behavior depends on the forces exerted by the fluid circulation. The best example is leukocytes extravasation from blood circulation into the underlying tissues where shear forces play an important role (22). Thus, intravital microscopic techniques to image myriad of different organs have been developed by several investigators (an overview is shown in **Figure 1B**) (23–25). As noted earlier, any studies that investigate the role of chemokines in regulating T cell migration will benefit from intravital microcopy since chemokine and the cytokine milieu can change drastically after an organ is removed. However, intravital microscopy involves complicated surgical techniques that can be invasive and cause vascular damage. As a result, several controls have to be performed and the experiments have to be repeated many times. In addition, other issues associated with intravital imaging must be considered; for example, the protracted anesthesia induced unconsciousness can decrease the heart rate impacting normal levels of blood circulation and ambient body temperature (26). However, it is possible to detect vascular leakage within the tissue being imaged by the systemic injection of fluorescent quantum dots. Local body temperature can be measured by the use of portable thermometers and constant temperature can be maintained by the use of a heated stage (**Figures 1C,D**) (5, 24). Certain organs (i.e., thymus) within their normal bodily context simply cannot be accessed for intravital imaging. For this reason, several groups have developed transplantation methods to provide better access of the organ for imaging (27, 28). For instance, a recent report described a thymic transplant on the kidney capsule of a nude mouse (that lacked an endogenous thymus) enabling better access of the organ for intravital imaging (27). Another group designed a thoracic suction window that stabilized the lung tissue in a live mouse without overtly disrupting the function (9). Although these surgical approaches for *in situ* imaging of lymphocytes are technically demanding, they have greatly facilitated our ability to observe T cell behavior directly *in vivo*.

VISUALIZING ANTIGEN-SPECIFIC T CELLS *IN SITU*

The interaction of the T cell receptor (TCR) expressed on CD4+ and CD8+ T cells with a cognate peptide bound to a major histocompatibility complex (MHC) on an antigen presenting cell (APC) is essential to initiate the signaling cascade that eventually leads to T cell activation. Since, at steady state the precursor frequency of a naïve antigen-specific T cell population for a given epitope is extremely low (29, 30), adoptive transfer of labeled or congenically mismatched antigen-specific T cells isolated from TCR transgenic mice into a wild-type host has been a very useful tool for visualizing T cell dynamics *in vivo* (31–33). However, studies using adoptive transfer of TCR transgenic T cells are associated with certain caveats. Transfer of large numbers of naïve TCR transgenic T cells do not reflect the physiological precursor frequency, and will likely fail to mimic normal T cell responses (34). In addition, transferred TCR transgenic T cells express TCRs that exhibit identical affinity or avidity for a particular antigen, and thus, may not reflect a more physiological polyclonal endogenous T cell response to a pathogen (35). Nevertheless, in the absence of alternative technologies, in order to visualize the initial T cell activation (within minutes to hours after immunization), transfer of large numbers of TCR transgenic T cells is required.

Another major advance in detecting antigen-specific T cells was the development of MHC-multimers (36). An MHC monomer binds poorly to a specific TCR, while a multimeric MHC molecule binds stably to TCRs expressed on T cells and thus can be used effectively to stain antigen-specific TCRs allowing the detection of endogenous antigen-specific T cells. MHC class I tetramers have largely been used in flow-cytometric analysis, however, our group and others have effectively used *in situ* MHC-I tetramer staining for static imaging studies (37–39). Using this technique, we have previously visualized the anatomical program followed by endogenous antigen-specific CD8 T cells during a primary and a memory immune response against *Listeria monocytogenes* (LM) in the spleen (38). For a list of seminal publications that have contributed to the advancement of T cell imaging *in situ* please refer to **Table 1**.

ANALYSIS AND DATA INTERPRETATION

Observing the orchestrated movement of immune cells within intact organs without disrupting intricate organ structure is a powerful benefit of using 2P microscopy. However, imaging techniques described above offer a full spectrum of parameters that have to be effectively analyzed to obtain physiologically relevant and reliable data. Real-time imaging requires the acquisition of four dimensional data (x, y, z, t; time), which can be used effectively to quantify cellular dynamics such as cell–cell interactions, cellular velocity, cellular contact time, chemotactic and shape index, and much more. For a more thorough review of this topic readers should refer to previously published reviews (8, 40). However, it is noteworthy that two groups recently combined flow cytometry and *in situ* imaging (41, 42) to develop a novel way to analyze imaging data. The first group published the "histo-cytometry" method, which was applied to investigate dendritic cell (DC) subset localization

Table 1 | Advances in *in situ* T cell imaging.

Category	Year	Advancement	Imaging technique	Method	Organ imaged	Reference
Imaging T-cells – the beginning	1839	First *in vivo* imaging	Bright field	Intravital	N/A	(1)
	1994	First TCR transgenic adoptive transfer	Bright field	Immuno-histochemistry	Brachial LN	(31)
	1996	Intravital video microscopy	Bright field/EF	Intravital	Inguinal LN	(3)
Naïve T-cells in lymphoid tissues	2002	Real-time imaging of thymocytes positive selection	2P	Thymic organ culture	Thymus	(46)
	2002	T/B cell random walk in the lymph node cortex	2P	Explant	Inguinal LN	(15)
	2003	Intravital imaging of T cell trafficking	2P	Intravital	Inguinal LN	(109)
	2006	Lymphocyte migration along FRC in the LN cortex	2P EM LSCM	Fixative perfusion IF	Popliteal LN	(50)
	2009	T cell egress from the LN	2P	Explant	Inguinal LN	(56)
T-cell priming/effector T cells	2002	Static imaging APC–T cell priming	LSCM	IF	Popliteal LN	(32)
	2002	Dynamic imaging of APC–T cell interaction at the LN surface	LSCM	Explant	Popliteal LN	(110)
	2003	Dynamic APC–CD8 T cell interactions in the LN cortex	2P	Explant	Inguinal LN	(58)
	2004	Intravital imaging of APC–CD8 T cell interaction in the LN cortex	2P	Intravital	Popliteal LN	(33)
	2005	Dynamic imaging of T/B cell conjugates in the LN	2P	Explant	Inguinal LN	(111)
	2006	Chemokine-driven non-random cell–cell interactions, initiating priming	2P	Intravital	Popliteal LN	(62)
	2007	Endogenous CD8 T cell activation following infection *in situ*	LSCM	Whole-mount, MHC-I tetramer staining	Spleen	(38)
	2008	Dynamic imaging of APC–CD8 T cell interactions in the splenic whit pulp	2P	Vibratome-cut explant	Spleen	(21)
	2011	Intravital APC–CD8 T cell interaction after LM infection	LSCM	Intravital	Spleen	(72)
	2011	Chemokine-induced optimization of CD8 T cell–APC interaction	2P	Intravital	Inguinal LN	(65)
	2012	Intranodal migration control T helper 1 differentiation	2P	Intravital	Popliteal LN	(61)
	2013	T cell–T cell interaction drive protective CD8 T differentiation	2P	Intravital	Popliteal LN	(66)
Naïve and effector T cells in non-lymphoid tissues	2008	Effector T cell dynamics in mycobacterial granulomas	2P	Intravital	Liver	(12)
	2011	Naïve and effector T cell dynamics in intact lung	2P	Intravital	Lung	(9)
	2011	Dynamics of primed CD8 T cell response during allograft rejection	2P	Intravital	Skin transplant	(100)
	2012	Effector T cell migration in *T. gondii* infected brain	2P	Explant and intravital	Brain	(11)

(Continued)

Table 1 | Continued

Category	Year	Advancement	Imaging technique	Method	Organ imaged	Reference
Memory T cells	2001	Generation of memory T cells in whole mouse body	EF	Sections	Whole body	(112)
	2011	Dynamic imaging of memory CD4 and CD8 T cells in skin	2P	Intravital	Skin	(96)
	2012	Chemokine-guided response of Central Memory T cells (T_{CM}) to antigenic	LSCM 2P	Tissue sections and intravital	Popliteal LN	(71)
	2013	Chemokine-dependent peripheral localization of CD8 memory T cells in lymph node	LSCM 2P	Tissue sections and intravital	Popliteal LN	(64)

The table lists important advances in imaging of T cells in lymphoid and non-lymphoid tissues. The references represent to the best of our knowledge, the initial seminal research article published on the particular topic and the technique listed. 2P, two-photon microscopy; APC, antigen presenting cell; EF, epifluorescence; LN, lymph node; LSCM, laser scanning confocal microscopy; IF, immunofluorescence; FRC, fibroblastic reticular cell network.

in the LN. DCs represent a highly heterogeneous population of cells and thus it is necessary to stain for at least five markers to identify several specific subsets. Using this novel method they were able to gate on a specific DC subset and simultaneously analyze the localization of the particular population directly within the LN section (42). The second group, Moreau et al. developed the so-called "dynamic *in situ* cytometry" (DISC), by combining 2P imaging with direct *in vivo* staining by injecting Fab fragments of antibodies against cell surface molecules of interest. By converting files that represent imaging data into the regular FCS format, data were easily analyzed using a flow-cytometric software. As a consequence, cell phenotype was effectively linked to *in situ* cell behavior (41). In addition, another elegant method termed, the "intravital dynamics-immunosignal correlative microscopy" linked dynamic behavior of T cells with static antibody stained imaging. Chodaczek and colleagues fixed the whole tissue immediately after dynamic imaging and proceeded to stain the fixed tissue with antibodies to specific proteins. By using tissue landmarks they were able to realign the T cell movements with static immunofluorescent images, and thus, single cell dynamic behavior was effectively linked to the location of TCRs and signaling molecules *in situ* (43).

VISUALIZATION OF T CELL RESPONSES IN LYMPHOID ORGANS

THYMUS

Early histological studies using fixed thymic sections revealed the geographical location of developing thymocytes *in situ* (44). It was demonstrated that double-negative (DN) thymocytes spent an average period of 14 days before becoming double-positive (DP) cells at the corticomedullary junction. In the next 3–5 days these DP cells were shown to migrate to the cortex where they interacted with the cortical thymic epithelial cells (cTEC) and underwent positive selection and matured into single-positive (SP) thymocytes. The final process of negative selection occurred in the thymic medulla where SP thymocytes interacted with the medullary thymic epithelial cells (mTECs), before exiting to the periphery (45).

Recent studies using 2P dynamic imaging have significantly increased our understanding of the T cell developmental process by defining thymocyte–APC interactions and trafficking patterns within the thymus. The first real-time imaging study utilized reaggregate thymic organ cultures to characterize the dynamics of thymocyte behavior during the process of positive selection (46). The same group further extended their findings by using thymic explants and 2P microscopy (19). They showed that T cells located in the thymic cortex exhibited the same stochastic migration pattern previously observed in the LN cortex (15), but the cells moved at a relatively low speed of 3–8 μm/min. However, after undergoing positive selection, T cells exhibited significantly higher motility of 10–25 μm/min as they migrated toward the medulla. Since the thymic medulla is located at greater depth, dynamic 2P imaging of this region required the use of vibratome-cut thymic explants (47). Intriguingly, SP thymocytes undergoing negative selection were confined to a specific area of the thymic medullary region and exhibited an average velocity of 10 μm/min. By using a mouse model that expressed the ovalbumin (OVA) antigen in mTECs, Le Borgne et al. visualized the process of negative selection of OVA specific TCR transgenic CD8 T cells (OTI) *in situ*, and showed that negatively selected T cells surprisingly continued to stay motile but were confined to a restricted area of the medulla. This observation implied that SP thymocytes needed continuous cellular interactions and integrations of signals before undergoing apoptosis (47).

LYMPH NODE

The emerging data indicate that mounting a protective immune response against pathogens or tumors is critically dependent on the orchestrated movement of cells within lymphoid organs. The lymph node structure is one of the underlying regulators of immune responses against mucosal infections or following vaccination by promoting interactions between different cell types. Thus, understanding the dynamics of T cell behavior *in situ* within the LN is essential. Skin draining LN can be accessed for intravital imaging, therefore, several previous studies have reported intranodal T cell behavior in several different contexts (48).

Naïve T cell trafficking

T cell trafficking patterns even under steady state conditions is a highly regulated process and several recent elegant studies have helped illuminate the processes that control this complex behavior of T cells *in situ*. Naïve T cells access the LN via the blood and enter the LN cortex through the high endothelial venules (HEV). Once in the cortex, T cells scan the DC networks (49) for antigen as they follow the fibroblastic reticular network within the LN (50). Factors that govern this migration pattern are not completely understood (48); however, G-protein coupled receptors (GPCRs) are very important in regulating this process. By abrogating global GPCRs signaling with pertussis toxin (PTX) treatment, Cyster and colleagues showed that PTX treated T cells showed a 50% reduction in median velocity, and a 90% decrease in mean motility coefficient in the LNs when compared to untreated T cells (51). Among the different GPCR, CCR7 was shown to be important for the localization of T cells in the paracortex. Indeed, modification of the CCR7 ligand (CCL19 and CCL21) distribution by subcutaneous injection distracted the lymphocytes from the T cell zone (52). The absence of the CCR7 signaling on naïve T cells significantly reduced the intranodal normal T cell velocity. However, this deficiency did not introduce any directional biases (52, 53), and thus, the "random walk" behavior exhibited by T cells was unchanged. Although both ligands for CCR7, CCL21, and CCL19 are produced by FRCs (54) only surface bound CCL21 is require for the random T cell motility (55) within the LN. These observations suggest that T cells follow a haptotactic (immobilized ligand) instead of a chemotactic (soluble ligand) gradient.

Egress of naïve T cells is also regulated by GPCRs. Upon LN entry, naïve T cells spend on average 6–12 h in the LN cortex, before using the cortical lymphatic sinuses to exit the LNs. Cyster and colleagues elegantly visualized this process, and showed that naïve T cells first probed the cortical lymphatic sinuses, and subsequently entered the lymphatic vessels by a sphingosine-1-phosphate receptor-1 (S1PR1) dependent mechanism (56). The S1PR1 ligand shingosine-1-phosphate (S1P) is present at high concentrations in the blood and in the lymph, but virtually absent in the tissues due to the degradation by the enzyme S1P lyase (57). S1PR1 is rapidly desensitized after S1P ligation, thus newly arrived T cells in the LN cortex fail to express the receptor on their surface. Once S1PR1 is recycled back to the surface, T cells are able to respond to the S1P gradient and return back to the circulation following exit from the LNs.

Naïve T cell priming

Visualizing T cell activation *in situ* has considerably enhanced our understanding of the mechanisms that regulate T cell and APC interactions *in vivo*. Three major experimental models have been utilized to image T cell priming; subcutaneous antigen delivery coupled with adjuvant, transfer of antigen pulsed DCs or direct infection of animals with pathogens.

Early 2P microscopy studies using antigen pulsed DCs to prime T cells revealed the dynamics of T cells–DC interactions *in situ* (33, 58) during antigen presentation. Antigen-specific T cells formed protracted interactions with DCs that lasted not minutes but hours (33, 58). Mempel et al. described a more complex process that characterized the dynamics of T cell activation. They demonstrated that T cell priming occurred in distinct phases where initial repeated brief encounters with DCs was followed by long-lived stable DC–T cell conjugates that in some instances lasted for more than 20 h in the LNs (33). The stability of these interactions depends on the antigen dose and TCR–MHC affinity (59, 60). Chemokines are also important for promoting T cell–APC interactions. CD4 T cell that are deficient in CXCR3 display fewer and shorter interactions with DCs that expressed the cognate ligand CXCL10, which resulted in poor Th1 differentiation as well as misplaced intranodal migration of primed CD4 T cells (61). In addition, collaboration between lymphocyte subsets was shown to facilitate antigen recognition of rare antigen presenting DCs at early stages of an immune response in the LN, resulting in non-random cell–cell interactions (62). Early during an immune response, the interaction of CD4 T cells with antigen bearing DCs resulted in the production of chemokines CCL3 and CCL4. This in turn lead to the recruitment of CCR5 expressing CD8 T cells to these rare sites of antigen depot in the LN, allowing for optimized T cell priming and memory cell generation (62).

Thus, the emerging data suggest that the "dwell time" (the length of initial T cell–DC conjugate formation) and the subsequent T cell motility and migration within defined compartments of secondary lymphoid organs are important parameters directing optimal T cell activation *in vivo* and these parameters are sensitive *in situ* indicators of antigen recognition.

In limited number of cases T cell dynamics in secondary lymphoid organs has been investigated following an infection. Although imaging studies using simple antigens have improved our knowledge regarding the mechanics of T cell activation, observing T cell responses to live replicating pathogens adds new layers of complexity. Since naïve T cells are largely located in the LN cortex where they continuously scan DCs, it was tempting to assume that following infection, T cell priming would occur deep in the LN cortex. However, *in vivo* visualization of early T cell–APC interactions immediately following viral infections revealed that T cell priming in fact occurred near the cortical ridge or at the interfollicular region of the dLN (32, 63–65). Subcutaneous infection with vaccinia virus or vesicular stomatitis virus (VSV) resulted in the infection of macrophages and DCs present in the LN subcapsular sinus (SCS), however, only DCs that expressed virus encoded protein appeared to present antigen directly to transferred TCR transgenic CD8 T cells (32, 63, 65). Interestingly, acquisition of effector functions and proper differentiation of primed CD8 T cells during the later phases of the T cell priming process depends on T cell–T cell interactions rather than just the early DC–T cell interactions (66). Integrin (CD11a) mediated T cell homotypic interactions was shown to be essential for the ability of antigen-specific CD8 T cells to secrete interferon-γ and subsequently differentiate into memory T cells following infection.

Following infection, effector T cells proliferate briefly in the LNs before exiting via the efferent lymphatics into the circulation. Most studies have focused on early events using TCR transgenic adoptive transfer methods but the subsequent intranodal migration of newly primed T cells, or the mechanisms that drive the egress kinetics from the LN following a localized infection have not been investigated adequately. However, a recent study used antigen

pulsed DCs to immunize mice and demonstrated that stromal cells and DCs in the interfollicular area express CXCL9 and CXCL10, respectively, and attract newly primed CXCR3 expressing CD4 T cells to the interfollicular and medullary areas of the LN (61). In this case, cell–cell interactions in the periphery of the LN were important for the proper intranodal T cell positioning as well as adequate Th1 cell differentiation.

Thus, these observations clearly demonstrate the complexity of the T cell activation process that requires several types of dynamic interactions between immune as well as stromal cells. Clarification of these elaborate processes has only been made possible by the use of *in situ* imaging methods.

Central memory T cells

Following the resolution of an acute infection, a heterogeneous population of memory T cells are generated that exhibit differential tissue tropism (67–70). Central memory T cells (T_{CM}) that express the lymphoid homing receptors CCR7 and CD62L preferentially localize to the secondary lymphoid organs, while effector memory T cells (T_{EM}) that fail to express lymph node homing receptors migrate to non-lymphoid tissues where they may upregulate CD69 and CD103 and form a stable resident memory T cell population. Central memory CD4+ and CD8+ T cells (64, 71) are largely located in the periphery of the LNs in close proximity to the lymphatic sinuses where a potential re-infection may occur. This peripheralization was shown to be dependent on the expression of the chemokine receptor CXCR3. Thus, this "pre-positioning" of memory T cells allowed for a more rapid response to a challenge infection.

SPLEEN

Following a blood borne infection, the spleen plays an essential role in the initiation of an anti-microbial immune response. A systemic infection with the intracellular Gram-positive bacteria LM is a widely used model to study the immune responses in this important lymphoid organ. Contrary to the LN, the spleen is more difficult to access for intravital imaging. Moreover, light scattering and absorption by red blood cells makes multi-photon microscopy of the spleen challenging. Additionally, the splenic white pulp, which plays host to T and B cells, is too deep to be imaged directly by 2P microscopy. However, using a standard confocal microscope the splenic red pulp was imaged intravitally following LM infection (72). During early time points after infection, DCs in the splenic red pulp established static LM depots, which were subsequently swarmed by neutrophils and monocytes, as well as CD8+ T cells (72). At later times after infection, infected DCs migrate to the splenic white pulp where they form stable interactions with CD8+ T cells, resulting in T cell activation (21, 73).

Using *in situ* tetramer staining and static whole-mount confocal microscopy with thick spleen sections, we have previously mapped the entire anatomical program followed by *endogenous* antigen-specific CD8+ T cells in the spleen after a primary and secondary LM infection (38). At day 3 after infection tetramer positive (tet+) cells were readily detected in small clusters at the T/B cell border and in the splenic marginal zone in close contact with DCs. After a brief period of expansion in the T cell zones, by 6 days after infection, virtually all antigen-specific effector CD8+ T cells had exited the white pulp using the bridging channels (lymphatic vessels are absent in the spleen). Similar to what was observed in the LN (64, 71), we found that early memory T cells were preferentially localized to the periphery of the splenic T cell zones and surprisingly even in the B cell follicles. Interestingly, another study showed that memory CD8 T cells beyond 55 days post LCMV infection were primarily located in splenic T cells zones (74), suggesting that as memory T cells mature their anatomical localization may change. This process will likely depend on the differential expression of homing and chemokine receptors that are involved in memory cell localization and migration.

IMAGING T CELL RESPONSES IN NON-LYMPHOID ORGANS

Following an infection, effector T cells, apart from proliferating in lymphoid organs, also migrate to peripheral inflamed tissues via the vasculature. Several adhesion molecules and chemokines are involved in the migration and entry of effector T cells into peripheral tissues. Receptors like E-selectin and P-selectin expressed on endothelial cells within tissues and molecules like CD44, P-selectin glycoprotein ligand 1 (PSGL1) and α4β1 integrin on antigen-challenged T cells enable non-selective localization of T cells to various peripheral organs (75). However, tissue-specific chemokines and receptors expressed on T cells specifically direct the homing of activated T cells to a particular non-lymphoid organ such as the skin, brain, or the gut (76). E-selectin ligands and the chemokine receptors CCR4 and CCR10 direct T cell homing specifically to the skin, while CXCL10 and CXCR3 regulate recruitment to the brain (11). At mucosal sites such as the intestinal mucosa, DCs in the Peyer's patches or mesenteric LNs imprint T cells to specifically migrate to the intestinal tissue (77). These mucosal activated T cells upregulate CCR9 and α4β7, which allow the T cells to home to the gut mucosa. α4β7 binds to the mucosal addressin cell adhesion molecule-1 (MADCAM-1), which is expressed heavily on vascular endothelial cells in the intestinal tissue (78), while CCL25 (CCR9 ligand) is produced by the intestinal epithelial cells (79).

EFFECTOR T CELLS

Once effector T cells enter peripheral tissues, they search for their cognate antigen, which is presented in context with MHC molecules by APCs or tissue stromal cells. Advanced imaging techniques like intravital imaging and multi-photon microscopy have attempted to shed light on the behavior of T cells navigating peripheral tissues (11, 16, 80). 2P imaging in the brain showed CD8+ T cells undergoing a non-random, directed mode of migration (11). The authors speculated that this unusual pattern of migration called a "Levy walk" enabled T cells to find rare APCs faster. In this particular study, the observed Levy walk helped T cells control *Toxoplasma gondii* infection in the brains of infected mice. Analysis of cell motility in non-lymphoid tissues revealed that T cells move at an average velocity that is significantly lower than their counterparts in the LNs. Intravital imaging in the lung was used to image naïve and activated T cells. Naïve T cells maintained an average track speed of around 2.5 μm/s, whereas activated T cells moved at a substantially lower speed of 0.4 μm/s (9). In the liver, granuloma formation following infection with *Mycobacterium bovis* (BCG) has been used as a model system to image the dynamics of innate and adaptive immune cells (12, 81). The

study revealed fast but restricted movement of CD4+ T cells inside granulomas (12). The same group later demonstrated that antigen presentation within granulomas determined T cell motility and cytokine production (81), which in turn influenced pathogen clearance. Thus, it is clear that tissue microenvironment likely influences T cell motility. There are several factors that can influence the differential dynamic behavior of T cells in non-lymphoid tissues. These factors include but not limited to: (i) nature of the replicating pathogen with regards to cellular tropism and the inflammatory milieu the pathogen may induce; (ii) the production of chemokines and their receptors expressed on T cells (62, 82); and (iii) the tissue resident cells including stromal or APCs that T cells may interact with (83). Determining the role each of these factors play in regulating T cell dynamics in non-lymphoid tissues will be essential for closing the large gaps that exist in our knowledge regarding T cell behavior in non-lymphoid tissues.

Non-T cell populations like neutrophils and DCs in the lung and DCs in the gut lumen have also been studied using 2P imaging (9, 84–88). However, as the focus here is on T cells, a detailed review of non-T cell populations is beyond the scope of this article.

MEMORY T CELLS

After effector T cells perform their function of antigen recognition, interaction, and clearance, a subpopulation of T cells persist as memory cells. A hallmark of protective immunity is that these cells help mount a rapid immune response following a secondary infection. As noted earlier, memory cells can be divided into T_{CM} and T_{EM} cells. T_{CM} re-circulate predominantly between secondary lymphoid organs and the blood, whereas T_{EM} re-circulate through peripheral non-lymphoid tissues (68). A third category of memory cells are called resident memory cells (T_{RM}) that remain in peripheral tissues and fail to re-circulate (89–93). Understanding the receptors, chemokines, and other factors that influence formation of a T_{RM} population in non-lymphoid tissues is an area of active research. Intra-epithelial lymphocytes (IELs), the T_{RM} cells in the gut epithelium, downregulate expression of homing receptors α4β7 and CCR9 and upregulate CD69 and CD103 to establish residence (89, 94). Decrease in the expression of KLF2, the transcriptional activator of the gene that codes for S1PR1 was recently shown to be important for establishment of resident memory CD8+ T cells (95) in non-lymphoid tissues. Since S1PR1 plays an important role in naïve T cell egress from lymph nodes (56), transcriptional downregulation of this gene for the generation of resident memory population is an important and interesting observation. 2P microscopy has helped address previously unappreciated questions about the nuances of memory T cell dynamics in non-lymphoid tissues following infection. After HSV infection in the skin, memory CD4+ and CD8+ T cell populations adopt different rates of migration based on tissue localization. CD8+ memory T cells remain in the epidermis close to the initial site of infection and migrate at a slower rate (2–3 μm/min) than dermis-localized CD4+ T memory cells (5–6 μm/min) (96). Interestingly, CD4+ T cells could re-enter the blood circulation, whereas CD8+ cells did not, suggesting that CD4 and CD8 T cells are regulated differently with regards to tissue residence. The mechanisms responsible for these differences are not known. As a follow-up, recent published work using multi-photon imaging and computer modeling showed that the slower velocities of CD8+ T_{RM} cells in the epidermis enabled these cells to remain on site throughout the life of the mouse (97). The observed cell velocities obtained *in vivo* over 2–3 h were used to model migration patterns over longer periods of time. The mathematical modeling showed that percentages of CD8+ T cells within a 5-μm region of skin at day 100 (49.8%) and day 365 (49.4%) after infection were not significantly different from day 0 (50%). Furthermore, two-photon imaging also demonstrated that the CD8+ T_{RM} population interacted with and were influenced by other cell populations like Langerhans cells (LCs) and dendritic epidermal γδ T cells in the epidermis.

AUTOIMMUNITY, TISSUE REJECTION, AND TUMOR IMMUNITY

The immune system can sometimes backfire, leading to autoimmunity and medical complications like graft rejection. Using mouse models and novel imaging tools, efforts are being made to understand these processes better. In a diabetic mouse model, 2P microscopic study showed that cytotoxic T lymphocytes (CTLs) underwent random walks in the exocrine tissues of the pancreas. On encountering β-cells in the pancreatic islets, the CTL motility slowed and led to the eventual death of β-cells, causing diabetes (98). In experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis, a molecular sensor that combines fluorescent nuclear factor of activated T cells (NFAT) with the histone protein H2B was used to detect intravital T cell activation (99). In activated T cells, NFAT is dephosphorylated and translocates into the nucleus. Tagging NFAT with fluorescent histone 2B enables simultaneously tracking NFAT nuclear translocation and cell mitosis, a powerful tool to follow activation of individual T cells *in vivo* following CNS invasion during development of EAE. Initially, T cells that traversed the leptomeningeal blood vessels in the CNS were activated following transient contacts with resident macrophages. During disease progression, activated T cells spread to the CNS parenchyma. In another study, the step-wise host tissue destruction was studied in a mouse model of allograft rejection, where ear skin grafts were imaged using intravital 2P microscopy (100). Donor dermal DCs were destroyed within 3–5 days after transplant. Rapid infiltration of host CD11b+ cells, initially neutrophils replaced by monocytes, could act as APCs that transported antigen from graft to the dLN, thereby activating CD8+ T cells. The above-mentioned two studies, therefore, demonstrated how intravital imaging and multi-photon microscopy could provide crucial temporal information on important processes like disease progression and graft rejection.

T cell homing and infiltration of tumors play an important role in improved cancer prognosis. Receptors like CXCR3, CCR5, CCR4, and adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) have been implicated in T cell infiltration to various types of tumors (101, 102). Cytotoxic T lymphocytes (CTLs) infiltrate tumors and mediate tumor cell destruction. Multi-photon microscopy has been used effectively to image the dynamics of CTLs within solid tumors. In a mouse thymoma model, E.G7-OVA tumor cells (modified to express CD8 T cell antigen OVA) were injected into mice subcutaneously (s.c), and 2P microscopy was utilized to assess OVA specific CD8 T cells (OTI) dynamics during early and late phases of tumor rejection.

OTI arrest coefficient was higher, while the mean velocities were decreased in the OVA-expressing tumors during early stage of rejection, when compared to non-OVA bearing tumors, suggesting antigen-specific recognition by CD8 T cells was necessary for T cell arrest and the eventual destruction of tumor cells. (103). To better understand if CD8 T cells directly mediate tumor cell lysis, a combination of 2P microscopy and a fluorescence resonance energy transfer (FRET) based caspase-3 activity reporter system was used to track apoptosis of tumor cells following adoptive transfer of activated CD8 T cells (104). Indeed, this study revealed that CD8 T cells directly kill individual tumor cells, however, the time required for tumor cell lysis was unusually lengthy (6 h on average), which may explain why T cell mediated tumor regression is often inefficient. Other immune cell types that target tumor antigens, like natural killer (NK) cells, have also been imaged using multi-photon microscopy and were shown to have very different dynamics as compared to CTLs (105).

Real-time *in situ* imaging of tumor tissue has also shed light on potential mechanisms that can restrict CTLs from carrying out their antitumor function within solid tumors. Myeloid-derived suppressor cells (MDSCs) are heterogeneous populations of APCs such as tumor dendritic cells (TuDCs) or tumor-associated macrophages (TAMs), which can subvert the antitumor activity of CTLs (106). 2P imaging of tumor tissue showed that following chemotherapy, tumor-infiltrating lymphocytes (TILs) made increased contacts with TuDCs but were trapped in TuDC-rich areas in the tumor parenchyma, restricting their infiltration deeper into tumor tissue (107). Furthermore, in a mouse model of adoptive T cell transfer, intratumoral regulatory T cells (Tregs) in an antigen-dependent manner, induced a functional anergic state in tumor-infiltrating CTLs, which resulted in poor tumor regression (108). Thus, using 2P microscopy to image solid tumors has been a valuable tool for gaining critical information regarding the dynamic behavior of TILs *in vivo*.

CONCLUSION

Two-photon microscopy and intravital imaging have helped make significant strides in increasing our understanding of the spatial and temporal behavior of T cells and APCs *in situ* during an immune response and subsequent steps of memory cell generation. With lesser restraints on the type and thickness of peripheral live tissues that can be imaged in real-time, the possibilities are limitless to address further unanswered questions regarding the biology of the immune response to a tumor or following an infection, and thus the development of effective vaccines and therapeutics.

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Mucosal resident memory CD4 T cells in protection and immunopathology

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Tissue-resident memory T cells (TRM) comprise a newly defined subset, which comprises a major component of lymphocyte populations in diverse peripheral tissue sites, including mucosal tissues, barrier surfaces, and in other non-lymphoid and lymphoid sites in humans and mice. Many studies have focused on the role of CD8 TRM in protection; however, there is now accumulating evidence that CD4 TRM predominate in tissue sites, and are integral for *in situ* protective immunity, particularly in mucosal sites. New evidence suggests that mucosal CD4 TRM populations differentiate at tissue sites following the recruitment of effector T cells by local inflammation or infection. The resulting TRM populations are enriched in T-cell specificities associated with the inducing pathogen/antigen. This compartmentalization of memory T cells at specific tissue sites may provide an optimal design for future vaccination strategies. In addition, emerging evidence suggests that CD4 TRM may also play a role in immunoregulation and immunopathology, and therefore, targeting TRM may be a viable therapeutic approach to treat inflammatory diseases in mucosal sites. This review will summarize our current understanding of CD4 TRM in diverse tissues, with an emphasis on their role in protective immunity and the mechanisms by which these populations are established and maintained in diverse mucosal sites.

Keywords: mucosal immunity, T-cell memory, intestine, lung, tissue homing

INTRODUCTION

The anatomic complexity of vertebrates necessitates an immune defense system, which provides protection at diverse sites of pathogen encounter. Earlier views of the immune system as a circulating, surveilling defense force have been supplanted by more recent evidence that the immune response is both localized and adapted to specific anatomic compartments. For T lymphocytes, seminal work by Leo Lefrançois and colleagues first revealed that virus-specific CD8 T cells that were generated and maintained as long-lived memory T cells after infection could be maintained in multiple tissue sites throughout the body (1). Subsequent studies using parabiosis models provided early evidence that certain tissues such as intestines contained populations of memory CD8 T cells that did not readily circulate (2). In recent years, non-circulating populations of memory CD8 T cells have been identified in skin, lung, vaginal mucosa, brain, and even in lymphoid tissues (3–7), which are collectively referred to as "Tissue-resident" memory CD8 T cells (CD8 TRM) (8, 9). TRM are populations of clonally expanded memory T cells that permanently reside in peripheral tissues, are maintained independently of lymphoid and circulating memory T-cell populations, and have the ability to respond rapidly to re-exposure to cognate antigen.

While most studies in mouse models of infection have focused on memory CD8 T-cell generation and maintenance to virus infection, less is understood about memory CD4 T cells and their role in protection and in tissue-specific responses. In both mice and humans, CD4 T cells are the most abundant lymphocytes throughout the body; they predominate in lymphoid tissue and memory CD4 T cells also outnumber memory CD8 T cells in mucosal tissues and barrier surfaces (10–12). Tissue-resident CD4 TRM have been identified in the lung, skin, and mucosal surfaces, and function to direct protective responses and coordinate recruitment of immune cells to tissues sites (7, 12–15). In addition to protective responses, there is also potential in any *in situ* immune response for collateral tissue damage, resulting in immunopathology. Since tissue-specific inflammatory disease can be driven by CD4 T-cell responses, the contribution of tissue-resident memory T-cell responses in these contexts is important to consider. In this review, we will focus on the role of CD4 TRM in immune responses, both protective and pathogenic and discuss current research and models for their generation and maintenance.

ANATOMIC HETEROGENEITY OF MEMORY CD4 T CELLS: EARLY STUDIES

The effectiveness of T-cell mediated immunity against pathogens is partly derived from the wide distribution throughout the body of a large repertoire of individual T-cell clones with the ability to recognize and mount an effector response to a large number of pathogen-associated antigenic signatures. Naïve T cells express chemokine receptors such as CCR7 and L-selectin (CD62L) that target their migration from circulation through lymphoid tissue. This circulatory pattern provides the greatest probability of

encounter of naïve T cells with their cognate antigens, which are presented by mature antigen presenting cells (APC) that ferry antigen from peripheral tissue to lymph nodes. Upon activation by antigen, naïve cells clonally expand and acquire effector properties, and in the process, upregulate expression of integrins and chemokine receptors that direct migration and access to inflamed peripheral tissues. During the ongoing immune response, effector cells are thus present in both lymphoid organs and peripheral tissues. While the majority of these activated and effector T cells die after antigen clearance, a proportion persists and develops into long-lived memory T cells.

The identification of memory CD4 T-cell heterogeneity in humans and mice based on homing receptor expression 15 years ago provided the initial evidence that T-cell memory was anatomically diverse. In humans, heterogeneity in CCR7 expression was identified among CD45RO$^+$ memory CD4 T cells in blood in a landmark study, which designated the CCR7^{hi} memory subset as central-memory (TCM) and the CCR7^{lo} memory subset as effector-memory (TEM) (16, 17). There were also early indications of memory T-cell heterogeneity in mice based on CD62L expression in antigen-specific memory CD4 T cells generated from virus infection or peptide-specific priming, giving rise to CD62L^{lo} and CD62L^{hi} memory subsets (18–20).

Anatomic heterogeneity of memory CD4 T cells was subsequently demonstrated in mouse models and some human studies. Jenkins and colleagues showed in whole mouse studies that memory CD4 T cells generated in response to peptide immunization were found in both lymphoid and non-lymphoid sites, including in lung, liver, intestines, and salivary glands (21). Other studies identified antigen-specific memory CD4 T cells in mouse lungs following respiratory virus infection (22), or from adoptive transfer of effector cells (23). Similarly, memory CD4 T cells were identified in mouse bone marrow (24), female reproductive tract (FRT) (25), and skin (26). Similarly, early studies in human tissue identified memory CD4 T cells in tonsils and non-lymphoid tissues isolated from surgical explants (27). Additional populations of human memory CD4 T cells were also identified in skin (28) and cerebrospinal fluid (29). These initial findings suggested that memory T cells may circulate through multiple and diverse sites. However, early evidence of phenotypic and functional distinction between memory CD4 T cells in tissues compared to those in spleen or circulation (23, 28), suggested that these tissue memory populations may be maintained independent of their counterparts in circulation.

Several new technological approaches were subsequently implemented to study whether memory T cells could take up residence and be retained in tissue sites as well as to distinguish circulating from tissue-resident memory T cells. Parabiosis experiments in which mouse pairs are surgically conjoined to create shared circulations provided direct evidence for memory CD4 T cells retained in lung tissues (13), and for memory CD8 T cells resident in intestines and skin (2, 3). Imaging via confocal or intravital microscopy also demonstrated that specific T cells are localized in niches within tissues (12, 30, 31). However, it is still difficult to assess whether immune cells isolated from peripheral tissues are present within microcapillaries of the tissues or are resident within the tissue. To overcome this problem, an increasing number of studies have used *in vivo* antibody labeling of T cells with a fluorescently labeled antibody prior to tissue harvest, such that T-cell accessible to circulation become labeled *in vivo* with antibody, while those within tissues and not in circulation are protected by *in vivo* labeling (6, 12, 13, 32). In response to infection, memory CD4 T cells that are protected from *in vivo* antibody labeling have been identified in lungs following respiratory infection with influenza virus, *Mycobacterium tuberculosis* (Mtb), and systemic infection with LCMV (12, 13, 15, 32). When combined with imaging approaches, both circulating and resident memory CD4 T cells can be identified in mouse lungs and spleen. In the following sections, we present the current state of knowledge about CD4 TRM in general and the specific role of CD4 TRM in mucosal sites.

RESIDENT MEMORY CD4 T CELLS AND PROTECTIVE IMMUNITY

CD4 TRM: GENERAL PROPERTIES

CD4 TRM are defined as non-circulating, memory CD4 T cells that are not readily accessible to the vasculature and are retained locally in specific tissue sites. Phenotypically, mouse CD4 TRM are distinguished from circulating TEM populations based on upregulated expression of the early activation marker CD69 and the integrin CD11a (12, 13, 33). CD69$^+$ memory CD4 T cells have been identified in mouse lungs, skin, and intestine, while spleen contains only a minority proportion of CD69$^+$ memory CD4 T cells (12, 13). In humans, CD4 TEM phenotype cells in lungs, intestines, lymph nodes, and bone marrow express CD69, with 50–60% of spleen CD4 TEM expressing CD69, while TEM circulating in blood uniformly lack CD69 expression (10, 34, 35). The specific upregulation of CD69 by tissue memory CD4 T cells suggests that memory CD4 T cells in human tissues perceive distinct signals compared to those circulating in blood (36). While CD8 TRM are also characterized by upregulation of the α_E integrin, CD103 (9), CD4 TRM in mucosal and lymphoid sites in human and mice generally do not express CD103 (12, 36), except for a proportion of skin memory CD4 T cells (31). Whether CD4 TRM in specific sites express other tissue-specific or TRM-specific integrins or adhesion markers is not known, although expression of the collagen-binding integrins VLA-1 and α2 are associated with lung effector CD4 T-cell responses and bone marrow memory CD4 T cells, respectively (37, 38). Functionally, CD4 TRM exhibit rapid recall function and can produce IFN-γ and IL-17 in mucosal sites, although the extent to which their functional profile differs from circulating memory populations is not well characterized.

These observations support the general concept that TRM are an effective first line of defense against invading pathogens due to their localization in mucosal tissues that are frequently the sites of infection. TRM populations are likely derived from clonally expanded populations of effector T cells responding to an infection, and therefore, contain relatively high frequencies of T-cell clones specific for pathogens that target individual tissue sites. This emerging hypothesis postulates that while TRM provide an immediate *in situ* immune response to infection, TCM and TEM located in lymphoid organs provide a delayed response due to their reliance on migration of APCs for the initiation of the response. The relative contribution of each component to

conferring protective immunity will probably differ based on the tissue(s) that is infected and the nature of the pathogen; however, this is currently a major research focus. Our knowledge of CD4 TRM and their properties is quickly expanding and it is likely that they will be identified in additional tissues and implicated in immune protection against a variety of tissue-tropic pathogens. A summary of current observations of CD4 TRM in mucosal sites and their protective capacities in different pathogen models is presented in **Table 1**.

LUNG CD4 TRM

The lung or respiratory tract is a major site for entry of viral and bacterial pathogens, with respiratory infections constituting the most prevalent cause of illness globally and throughout an individual's lifetime. It has been known for some time that respiratory viral infections induce TEM populations within the lung that display an activated phenotype (47), and that these populations persist within the lung tissue and the lung airways following infection (22). Due to the possible inclusion of cells within the microcapillaries of the lung, these previous studies found phenotypic heterogeneity among lung memory CD4 T-cell isolated from digested tissue (48). Introduction of virus-specific memory CD4 T cells directly into the respiratory tract by intranasal delivery, provided protection to secondary virus challenge (22); however, it was not established whether these protective subsets were circulating or remained resident in lung tissue.

CD4 TRM in the lung were the first resident memory CD4 T-cell population to be extensively characterized and demonstrated to exhibit protective function. Using the *in vivo* labeling technique to analyze lung memory T-cell populations following influenza virus infection, we found that CD4 TRM were phenotypically distinct from circulating TEM populations in their expression of high levels of CD69 and CD11a, and in their residence in a distinct niche of the lung near airways (12). Further evidence of distinct properties of lung effector-memory T cells come from adoptive transfer and parabiosis experiments. These studies showed that lung memory CD4 T cells specifically migrate back to the lung following adoptive transfer into congenic hosts while spleen-derived memory CD4 T cells migrate into multiple tissues (13). Parabiosis further revealed that lung memory CD4 T cells were specifically retained in lungs while spleen-derived memory CD4 T cells freely recirculated among multiple lymphoid tissues and entered the lung, but were not retained there (13). Moreover, lung CD4 TRM generated following influenza infection were maintained longterm and were unperturbed in the presence of inhibitors of lymphoid egress and inducers of lymphopenia (12). Similarly, Mtb infection in mice resulted in generation of lung-tropic and retentive CD4 TRM as well as circulating TEM cells (15). Moreover, human memory CD4 T cells in lung are predominantly a TEM phenotype with upregulated expression of CD69 (10, 34). Together, these studies identified a new subset of lung CD4 TRM with distinct phenotypic, migration, retention, and maintenance properties.

In experimental models of respiratory infection with influenza, parainfluenza virus, and Mtb, the resulting lung TRM population is enriched with pathogen-specific CD4 (12, 15, 49) and CD8 T cells (12). Likewise, the lungs of human subjects that had been exposed to Mtb contain resident memory CD4 T cells that were specific for Mtb antigens (50). CD8 T cells specific for influenza and respiratory syncytial virus are found in higher frequencies within human lungs than in the spleen, blood, and skin (12, 34, 39). While it is possible to determine Mtb exposure by a PPD skin test, it is difficult to document the history of influenza and parainfluenza virus infection in human subjects. The high prevalence of IAV infection among the population, however, suggests that the

Table 1 | Observations of CD4 TRM in mucosal tissues.

Tissue	Pathogen/antigen	Features	Reference
Lung	Influenza virus	$CD69^+$, cluster around airways Unaffected by FTY720 treatment Lung-tropic, protect against second infection	(12, 13)
	Influenza virus (humans)	Virus-specific memory CD4 T cells enriched in lung, $CD69^+$, VLA-1^+	(34, 39)
	Mycobacterium tuberculosis	$CD69^+$, $CXCR3^{hi}$, PD-1^{hi}, KLRG1^{lo}, lung-tropic. Protect against second infection	(15)
	Mycobacterium tuberculosis	Lung CD4 TRM generated by BCG vaccination CD4 TRM enhances MHC II on lung macrophages during 2° challenge	(40)
	Nippostrongylus brasiliensis	Pathogen-specific production of IL-4 and IL-13 Lung TRM unaffected by FTY720 treatment Protect against second infection	(41)
Female genital tract	Herpes simplex virus (humans)	Enrichment of antigen-specific CD4 T-cell clones in cervical cytobrush specimens and genital lesions	(42, 43)
	Herpes simplex virus (mice)	CD4 TRM generated in vaginal mucosa (no CD8) IFN-γ-mediated protection against 2° HSV challenge	(44)
Gut	*Listeria monocytogenes*	Primary and second oral infection *Listeria* generates long-lived antigen-specific T-cell population in LP	(45)
	N/A	Homeostatic proliferation of naïve CD4 T cells in MLN generates gut-tropic, $\alpha_4\beta_7^+$, T_H17 cells	(46)

compartmentalization of IAV-specific T cells within the lung is likely a consequence of local infection.

The elevated precursor frequency of pathogen-specific cells in the lung is thought to direct an early *in situ* immune response against secondary infection. In support of this hypothesis, it has been reported that there is local activation and expansion of memory CD4 T cells in the lung upon secondary IAV challenge (49). We have likewise found that lung CD4 TRM can produce effector cytokines at early time points following secondary viral infection (33). Rapid recall of memory CD4 T cells in the lung has also been suggested as being integral for protection against Mtb in both mouse and human studies (50–52). Lung CD4 TRM in mice were found to mediate superior protective responses to influenza virus challenge compared to spleen-derived memory CD4 T cells (13). Interestingly, influenza-specific lung CD4 TRM protected from morbidity of infection while also mediating rapid viral clearance, and carried out these functions *in situ* without extensive proliferative expansion or migration to other sites (13). In a mouse Mtb infection model, CD4 TRM cells conferred better protection from secondary Mtb infection in susceptible hosts than their circulating intravascular counterparts (15). The mechanisms for protection by CD4 TRM in the lung have not yet been elucidated. While IFN-γ is important for memory CD4 T-cell-mediated recall responses to influenza (53, 54), protection for Mtb was not associated with IFN-γ production (15).

In humans, protection due to resident T cells is difficult to assess. One group has used the novel approach of bronchoscopic antigen challenge with purified protein derivative of Mtb (PPD) to assess the role that local lung memory T cells play in the secondary immune response to Mtb infection. By comparing the local lung immune response (after bronchoscopic challenge) of healthy individuals with a positive PPD skin test to healthy PPD negative controls they observed rapid mobilization of CD4 T cells into the lung airways (48 h) resulting in a significant increase in antigen-specific T cells (55). These early responding cells did not undergo proliferative expansion as assessed by Ki67 staining, suggesting that they may represent lung TRM cells that migrate into the airways in response to antigen challenge (55). Together, these findings indicate the importance of lung TRM in protecting against respiratory infections, suggesting that targeting generation of persisting CD4 TRM in the lung would provide optimal protection.

REPRODUCTIVE TRACT MUCOSAL CD4 TRM

The mucosal surfaces of the male and FRT are major sites of entry for sexual transmitted diseases such as herpes simplex virus (HSV), *Neisseria gonorrhoeae*, human papillomavirus, and human immunodeficiency virus (HIV) – all of great public health concern. The reproductive tract is also prone to opportunistic fungal and bacterial infections with increased incidence in immunocompromised (56) and immunosuppressed patients (57), indicating a role for T-cell mediated immunity in preventing these infections. CD4 T cells are thought to be especially important in controlling genital HSV-2 infection, with mouse studies showing that CD8 deficient mice can be successfully vaccinated against disease while CD4 deficient strains are not (58, 59). The importance of CD4 T cells in protection against HSV-2 was supported by the finding that intravaginal HSV-2 infection generates CD4 TRM but little CD8 TRM. These vaginal mucosal memory CD4 T cells in the FRT are sufficient for protective responses to HSV (44) even in the absence of CD8 T cells. In humans, CD4 T cells specific for multiple viral epitopes localize to the uterine cervix (42, 43, 60) and this resident population is thought to limit the severity of recurrent HSV infections (43). As is the case with HSV-2, pre-existing CD4 TRM cells in the RT may be important for conferring protection against other infections of the urogenital tract such as *N. gonorrhoeae*, *Chlamydia muridarum*, and *Candida* infections (61–64).

The relative contribution of CD4 and CD8 T cells in providing protective immunity in the reproductive tract can vary based on the nature of the invading pathogens; however, new studies indicate that CD4 and CD8 TRM can provide early *in situ* immune responses to infection of the FRT. CD8 TRM have been targeted in the quest to develop a vaccine against HIV because CTLs are thought to be most important for killing virally infected cells. Non-human primate models reveal that the simian immunodeficiency virus (SIV) establishes a small founder population of infected cells in the local tissue after infection (65, 66). This founder population serves as an expanding source of virus that contributes to virus dissemination (66), and presents an opportunity for total elimination of mucosal viral infections during a narrow window of time early after infection. This task may require early *in situ* immune responses mounted by local TRM populations.

CD4 TRM IN THE INTESTINES

The intestinal mucosa is a major interface where the body is exposed to environmental antigens, including benign food antigens, beneficial commensal microorganisms as well as dangerous pathogens. Within the intestine are multiple specialized populations of adaptive and innate immune cells that contribute to various immune functions including: oral tolerance to food antigens, tolerance of commensals, and protective immunity against enteric pathogens (67). These populations include memory CD4 T cells, some of which are permanently resident CD4 TRM. Gut T cells are distributed throughout the organized lymphoid tissues that are found throughout the intestines including: Peyers patches, gut-associated lymphoid tissue (GALT), and isolated lymphoid follicles (68, 69). Additionally, gut T cells are also found diffused throughout the lamina propria (LP) and within the intraepithelial (IEL) compartment. The majority of the IEL T cells are $CD8^+$ T cells that also express CD103 (70–72) with a lower proportion of CD4 T cells in the IEL compartment. However, CD4 T cells comprise the majority of T cells in the LP and they express an effector-memory phenotype ($CD62L^{lo}CD44^{hi}$) (67). In humans, the vast majority of memory CD4 T cells in healthy small and large intestines express CD69, the putative TRM marker (10).

Intestinal resident memory CD4 T-cell populations are shaped by commensal bacterial species. One particular commensal microbe, segmented filamentous bacteria (SFB), was recently shown to induce T_H17 cells in the LP of mice (73, 74). T_H17 cells provide mucosal immunity against bacterial pathogens through the production of IL-17 and IL-22 (73, 74). In addition to T_H17 cells, commensal bacteria induce resident T-cell populations with regulatory function. Studies have shown that a significant proportion of T_{regs} in the intestines are conventional T cells that are converted to a regulatory phenotype in response to the commensal

bacterium of the intestinal microbiota (75). Further research revealed specific strains of *Clostridium*, within mouse intestinal commensals, which were sufficient to induce gut resident T_{regs} in mice (76). This group further showed that a selected mixture of *Clostridia* strains from the human microbiota also induced T_{regs} in mice after colonization of the intestines (77). Gut infections with pathogenic bacteria, likewise, induce CD4 TRM populations within the LP. In experimental systems, memory CD4 T cells in the LP are induced by oral infection with bacterial pathogens like *Listeria monocytogenes* (45).

Studies have employed parabiosis and tissue-grafting approaches to show that gut T-cell populations are maintained independently of systemic populations (2, 78, 79). The mucosal immune system of the gastrointestinal tract is a compartmentalized division, including resident memory T-cell populations with both pro- and anti-inflammatory functions, which provide important functions for the physiology of the intestines. It has been shown that gut APCs acquire antigen and migrate to the draining mesenteric lymph nodes where they activate T cells, imprinting the resulting effector and memory T cells to migrate specifically back into the intestines from circulation (80). This migration tropism of gut memory CD4 T cells is similar to that observed with lung CD4 TRM, and may be a distinguishing feature of mucosal CD4 TRM.

TRM IN CHRONIC INFLAMMATORY DISEASES

CD4 TRM have been investigated mainly for their role in providing protective immunity to pathogens that target specific tissues. However, there has been emerging evidence that this population may play a significant role in the pathogenesis of certain autoimmune, allergic, and atopic diseases. In mucosal sites, aberrant immune function and cross-reactivity of CD4 TRM in peripheral tissues are being investigated in inflammatory bowel disease (IBD) and asthma as possible causes of chronic or remitting immunopathology. In addition, there is evidence that CD4 TRM may play deleterious roles in inflammatory disorders of barrier surfaces such as skin. Understanding how CD4 TRM can promote undesirable inflammatory effects in the tissues is important to develop more targeted strategies for therapeutic control of inflammatory diseases.

Allergen-specific TRM populations can be established within lungs following local immune responses induced by exposure to allergens. As is the case following pathogen infection of barrier surfaces, a subset of the effector cells responding to the allergen is imprinted with a TRM phenotype and retained within the tissue. Memory T cells, particularly T_H2 cells, are strongly involved in the pathogenesis of the chronic manifestations of allergic and atopic diseases (81–83); therefore, their localization at particular tissues make them prone to being reactivated and causing chronic disease. It will be interesting to determine whether CD4 TRM cells are established and maintained within the lung in mouse models of allergic asthma and their role in asthma pathogenesis and also in maintaining the hyper-responsive condition in the tissues. Pathogenic functions of lung CD4 TRM could involve immune cell recruitment into the lung airway upon secondary and chronic allergen exposure.

Inflammatory bowel disease is a chronic inflammatory disease of the gastrointestinal tract characterized by persistent inflammation of the gut or, in some cases, is manifested as a relapsing–remitting syndrome with flare-ups and resolution (84, 85). The chronic recurrence of disease and the restriction of the inflammation to the gastrointestinal tract suggest a role for resident memory T cells in the pathogenesis of IBD. T_H1 and T_H17 cells have both been implicated in the pathogenesis of the disease. In experimental models of IBD circulating colitogenic memory CD4 T cells required the presence of gut commensals to induce inflammation and IBD pathogenesis (86). In mouse models of IBD transfer experiments of gut CD4 TRM transferred disease to RAG2$^{-/-}$ mice (87). This demonstrates resident memory CD4 T-cell populations in the gut can propagate local inflammation leading to chronic IBD symptoms.

Psoriasis is another chronic inflammatory disease caused by T-cell responses at a barrier surface (88), with pathogenesis of relevance to inflammation in mucosal sites. Disease pathogenesis is driven by T-cell migration into the epidermis and local production of inflammatory cytokines. T_H1 and T_H17 cells in particular have been linked to disease pathogenesis (89, 90). Skin resident memory T cells that are pathological are thought to influence disease recurrence (91). It was recently found that there were elevated numbers of CD8 and CD4 T cells in the dermis of resolved psoriasis lesions. These cells expressed markers associated with TRM (92), including CD103 and the α1β1 integrins expressed on epidermal CD4 T cells (88, 92). It is thus possible that chronic inflammation induces expression of integrins, which mediate cell–cell interactions involved in T-cell retention in the epidermis and establishment of CD4 TRM. The harmful effects of TRM in cases of tissue-specific chronic inflammation, as seen in asthma and psoriasis, make TRM ideal targets for therapeutic interventions.

ESTABLISHMENT AND MAINTENANCE OF CD4 TRM

The factors involved in the establishment and maintenance of TRM populations within non-lymphoid tissues are not clearly understood. Entry of effector T cells into non-lymphoid mucosal sites is controlled by the expression of certain chemokine receptors, selectins, and integrins, which are universally upregulated after T-cell activation, regardless of the secondary lymphoid tissue where cells were activated (93). It has also been shown, however, that T cells primed by dendritic cells in certain lymphoid sites are programed to home specifically to certain tissues (94, 95). This tissue-specific homing is mediated through the expression of various integrins and chemokine receptors, which are involved in cell migration into specific tissues. For example, chemokine receptor chemokine receptor 9 (CCR9) and integrin α4β7 target T cells to the intestines (96, 97), cutaneous leukocyte antigen (CLA) targets cells to the skin (98), and lung DC promote effector T-cell homing to the lung through upregulation of CCR4 (99) (**Figure 1**). Whether these specific chemokine receptors persist in TRM remains to be established, and identifying specific tissue signatures for TRM in distinct sites is an active area of study in the field.

Effector T cells responding to infection/inflammation within non-lymphoid or mucosal tissues may further respond to inflammatory and/or tissue-specific environmental factors, which impart them with a resident memory phenotype. For CD8 T cells, expression of transforming growth factor-beta (TGF-β) within certain

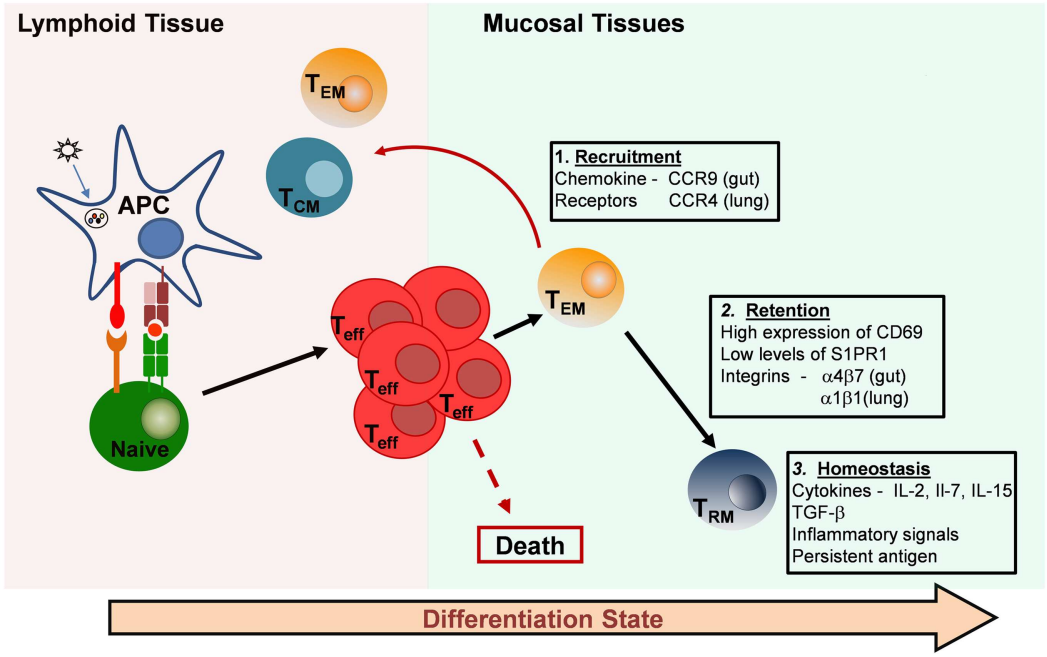

FIGURE 1 | Generation and maintenance of resident memory T-cell subsets. Resident memory T cells in mucosal tissues are likely derived from recruited effector T cells that originate in lymphoid organs. Effector cells can be imprinted with specific chemokine receptors that direct migration to individual tissues (Box 1). Most of the effector cells die, but a proportion of the effector or primed T cells differentiates into long-lived resting memory T cells. There are three major types: central-memory T cells (TCM), which migrate back to lymphoid tissue, effector-memory T cells (TEM), which circulate through peripheral tissues and tissue-resident memory T cells (TRM), which are retained in mucosal tissue sites and take up long-term residence there without recirculating. Retention of TRM in peripheral tissues is thought to be mediated by the inhibition of egress through S1PR1 and by cell–cell interactions facilitated by integrin expression (Box 2). Maintenance and homeostasis of TRM in mucosal tissues may depend on pro-survival cytokines, constitutive low-level inflammation, and the persistence of antigen at the site (Box 3).

tissues (100, 101) induces the expression of the mucosal integrin, αE(CD103)β7 (102, 103), which is responsible for retention of CD8 TRM in non-lymphoid tissues. CD4 TRM at mucosal sites express CD103 at a much lower frequency compared to CD8 TRM (10, 12) and may be maintained by other, unknown mechanisms. Other integrins may be involved in CD4 TRM retention and residence, which may represent a major difference between CD4 and CD8 TRM in the same tissue. It has been found that the vast majority of CD4 T cells persisting in the lung airways following influenza virus infection express the α1β1 integrin (VLA-1) while virus-specific cells in lymphoid sites have low expression of VLA-1 (37). Secondary infection with IAV revealed that these VLA-1^+ cells represented 80% of the early producers of IFN-γ (37) suggesting that the α1β1 integrin might be a marker of lung CD4 TRM cells. CD11a or LFA-1 is also expressed at higher levels in lung CD4 TRM compared to circulating CD4 TEM (12, 13), and may also contribute to tissue retention.

The lectin CD69 is constitutively expressed on CD4 and CD8 TRM in all the tissues that have been described (3, 13, 78, 104). Traditionally, CD69 has been thought of as an early activation marker of T cells, being transiently upregulated early after activation through the T-cell receptor (105) or in response to proinflammatory cytokines, including type I interferons (IFN-α and IFN-β) and tumor necrosis factor-α (106, 107). TRM cells in the lung constitutively express elevated levels of CD69 while T cells of the same specificities express low levels of CD69 in the lymph node and spleen (12). This local expression of CD69 by TRM may be the result of continued stimulation through encounters with persistent antigen at tissue sites, which has been observed following influenza virus (108, 109). We found that acquisition of TRM properties by effector cells adoptively transferred into congenic hosts in a manner that is independent of antigen (13). Induction of CD69 expression by T cells within tissues may therefore be the result of the environmental milieu associated with mucosal tissues, which is likely to be quite different from that of lymphoid organs.

Tonic signaling, through low levels of cytokines produced in response to environmental antigens, may also be involved in the differentiation of effector and effector-memory T cells into TRM. CD69 is thought to play a functional role in T-cell retention within tissues because of its regulation of sphingosine-1-phosphate receptor 1 (S1PR1) (110), which play a role in the egress of lymphocytes from certain tissues (111). A summary of processes involved in the recruitment, retention, and homeostasis of TRM in peripheral tissues is provided in **Figure 1**. Further studies are needed to define the exact molecular determinants of CD4 TRM establishment and maintenance. Defining the differences and similarities between the requirements for CD4 and CD8 TRM development and maintenance in tissues is also of utmost importance for the targeting of these new subsets by vaccines and therapeutics.

IMPLICATIONS FOR VACCINES

As outlined in **Table 1**, there is now evidence for the presence CD4 TRM in multiple mucosal sites and roles for this subset in protection against pathogenic infections (**Table 1**). These findings present important implications for future therapeutic developments for promoting protective responses *in situ*. In the lung, generation of TRM populations targeting respiratory pathogens may significantly reduce the mortality and morbidity associated with these infections. In the case of influenza virus, the more common subunit vaccine is administered by intramuscular immunization, while the live attenuated influenza virus vaccine (LIAV), which is more commonly used for younger individuals, is administered intranasally. Both types of vaccine have been optimized for the generation of protective antibodies; however, both vaccines can induce circulating virus-specific T cells (112, 113) with the LIAV vaccine thought to generate more tissue-tropic T cells (114). A vaccine that induces memory T cells that recognize conserved epitopes from internal viral proteins could form the basis of a universal influenza virus vaccine. It may also be important that such a vaccine is administered in a manner that generates protective memory T-cell populations resident in the lung for optimal protection, likely via the intranasal route.

In the case of Mtb, current intramuscular bacille Calmette–Guérin (BCG) vaccination protocols show reliable protection during childhood but protection wanes during adulthood (115). This protection is mediated by T_H1 memory cells; however, the exact effector mechanisms by which Th1 memory protect is not fully understood. Recent attempts to boost BCG protection by parenteral vaccination have yielded disappointing results. For example, clinical trials of the recombinant vaccinia virus booster vaccine, MVA85A, did not show better efficacy than the BCG vaccine (116) even though the new vaccine generates highly durable Mtb-specific T_H1 responses (117). This result may have been foreshadowed by mouse experiments showing that parenteral boost with MVA85A after BCG priming showed no improvement in protection (118–121), compared with BCG vaccination alone, even with each vaccine showing high immunogenicity. Improved protection over BCG alone is only observed after multiple immunizations, which induce entry of cells into non-lymphoid tissues (122). These results suggest that memory T-cell mediated protection against respiratory Mtb infection may depend on the early *in situ* effector functions of TRM populations. Optimal protection may require both parenteral and mucosal administration of vaccines, which will generate both TRM and lymphoid memory populations.

The prevalence and protective capacities of TRM in the FRT has encouraged efforts for generating *in situ* vaccines for protection against sexually transmitted diseases. A new strategy for generating TRM in the FRT involves a "prime and pull" technique in which parenteral vaccination (prime) is combined with recruitment of activated T cells into the genital tract by local application of a chemokine (pull). When applied to the mouse HSV-2 infection model, this approach resulted in the recruitment but not retention of CD4 memory T cells, although HSV-2-specific CD8 TRM were generated (7). These results suggest that the establishment of CD4 TRM in the reproductive tract may require additional signals, such as those present during HSV infection (44, 123, 124). In other studies for HIV vaccines, intranasal vaccination was found to generate higher anti-SIV T-cell responses in the colorectal mucosa, increased numbers of gut-tropic $\alpha4\beta7$ cells in circulation, and a longer disease-free period compared to vaccination via the intramuscular route (125). These findings suggest some connections between mucosal sites important for assessing the optimal route of administration, and perhaps suggesting that a pull step may not be necessary. Further studies are needed to define the signals necessary for the local differentiation of CD4 T cells into TRM in order to develop vaccination and therapeutic protocols that harness the unique properties of these cells to prevent and fight site-specific infections.

CONCLUDING REMARKS

Compartmentalization of immunological memory in diverse non-lymphoid and mucosal tissues may be a central mechanism underlying the long-term persistence and efficacy of T-cell memory to systemic and site-specific pathogens. CD4 TRM in mucosal tissues may be optimally poised to orchestrate the immune response to recurring tissue-tropic infections. Developing vaccines that therefore generate this important population in targeted tissues should be a major focus of future research; however, greater understanding of the mechanisms involved in imprinting tissue-resident CD4 T cells is needed. Elucidating strategies to target TRM in mucosal and tissues will also allow for the development of therapeutics that reduce TRM populations in various tissues in instances of aberrant immune responses and immunopathology.

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The role of non-cognate T cell stimulation during intracellular bacterial infection

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Intra-macrophage bacterial infections cause significant morbidity and mortality in both the developed and developing world. Protective host immune responses to these infections initially requires the activation and expansion of pathogen-specific CD4 Th1 cells within lymphoid tissues and subsequent relocation of these effector cells to sites of infection. After entering infected tissues, the elicitation of Th1 bactericidal activity can be triggered by cognate or non-cognate signals that are delivered by locally infected antigen-presenting cells and innate cells. However, the contribution of non-cognate stimulation to the resolution of bacterial infection remains poorly understood, especially in the context of a Th1 response. Here, we review the current data on Th1 cell activation and expansion in mouse models of *Salmonella* and *Chlamydia* infection and discuss the potential role of non-cognate Th1 cell stimulation in these disease models. Greater understanding of this pathway of T cell activation may lead to the design of therapeutics or vaccines to combat intra-macrophage pathogens.

Keywords: T cell, bacterial infections, protective immunity, IFN-gamma, *Salmonella*

INTRODUCTION

The mammalian immune system contains a variety of cell types that respond in a highly coordinated fashion to eradicate microbial pathogens. The different cell populations that mediate this host defense capability are conveniently assigned to innate or adaptive immune compartments depending a variety of factors, including the tempo of the effector function produced, the use of certain pathogen recognition receptors, and whether these cells have an inherent capacity to confer immune memory. Innate immune responses typically invoke an immediate effector response, make use of germ-line encoded receptors with a restricted capacity for pathogen recognition, and lack the ability to confer a stronger response to secondary infection (1). In contrast, adaptive immune responses require a period of maturation before effector functions are elaborated, utilize complex, rearranged receptors that allow a wider range of specificities, and confer a modified host response to re-infection (2). This general compartmentalization of cells into innate and adaptive arms of the immune system is useful since it provides a conceptual framework that reduces complexity in understanding the dynamics of host–pathogen interactions. However, as might be expected, this model is an oversimplification and some cells of the innate immune system can display characteristics of the adaptive response, and vice versa (3–6). In this review, we will discuss the capacity of adaptive Th1 cells to elaborate effector functions in response to innate stimuli and thus under these conditions appear to function as a component of the innate immune response. The ability of these expanded effector lymphocytes to blur the lines between innate and adaptive immunity may be a critical component of protective immunity to *Salmonella*, *Chlamydia*, and other intracellular bacteria.

GLOBAL IMPACT OF *SALMONELLA* AND *CHLAMYDIA* INFECTIONS

Salmonella can cause different clinical diseases in a human host, depending upon the genome of the infecting *Salmonella* serovar and the immune competence of the infected host (7, 8). Typhoid fever is caused by human transmission of *Salmonella enterica* serovar Typhi or serovar Paratyphi and this disease remains prevalent in parts of Africa and Asia (9). Current estimates suggest that typhoid causes 217,000 deaths globally every year, the impact of which is felt predominantly in geographical regions with limited access to clean water or basic sanitation infrastructure (10). Although typhoidal serovars enter the human host via the intestine, much of the *in vivo* bacterial replication occurs in the systemic tissues of the liver, spleen, and bone marrow. In contrast, many other *Salmonella* serovars can cause local gastro-intestinal infections that are often self-limiting but are a major cause of food-borne infection in the US and other developed nations (11, 12). Thus, *Salmonella* infection has a global footprint and largely affects developed and developing nations with different patterns of systemic or localized disease. A third disease caused by *Salmonella* has emerged in sub-Saharan Africa and primarily affects patients with an immature or compromised immune system, either due to age, co-infection, or nutritional status (13, 14). These *Salmonella* infections can be systemic and are caused by non-typhoidal serovars and therefore this disease is collectively referred to as invasive non-typhoidal Salmonellosis (NTS). While vaccines are currently available for typhoid, these are not widely used in typhoid endemic areas due to concerns about efficacy, safety, or cost (8). The development of improved vaccines for typhoid and NTS therefore remains a priority. Greater understanding of host protective

immune mechanisms during *Salmonella* infection will be required in order to meet this important goal.

While *Salmonella* is a facultative intracellular pathogen that can grow inside and outside host cells, *Chlamydia* is an obligate intracellular organism and is only metabolically active within host cells (15). *Chlamydia trachomatis* causes a sexually transmitted infection in humans that is now the most common notifiable disease in the US (16). The 1.4 million *Chlamydia* cases reported in 2011 represent an 8% increase over 2010 and is the largest number of cases ever reported to the Centers for Disease Control (CDC) for any single condition (16). Overall, the CDC reports an 8.3% positivity rate among young women screened at family planning clinics, making *Chlamydia* one of the most prevalent bacterial infections in the US (17). Although most *Chlamydia* infections are initially asymptomatic, they cause serious pelvic inflammatory disease (PID) in 5–15% of untreated female patients (18, 19). Approximately one in six women who develop PID become infertile, while many others develop chronic pelvic pain, ectopic pregnancy, and if exposed to HIV, *Chlamydia*-infected women are five times more likely to acquire the virus (18–20). Thus, *Chlamydia* infection represents a growing healthcare problem in the US and greater understanding of protective immunity in the female reproductive tract will be required to develop an effective vaccine.

ROLE OF CD4 Th1 CELLS IN PROTECTIVE IMMUNITY TO *SALMONELLA* AND *CHLAMYDIA*

Given the location of *Chlamydia* infection in the reproductive tract and *Salmonella* infection in the intestine, the immune response to these infections will undoubtedly contain unique tissue-specific components. However, in both mouse models of *Salmonella* and *Chlamydia* infection, pathogen-specific CD4 Th1 cells have been found to be essential for successful resolution of primary infection (21, 22). In the *Salmonella* model, oral infection of C57BL/6 mice with attenuated bacteria generates a systemic infection that eventually resolves over a period of several weeks (23). The ability to resolve this infection is absent in mice lacking MHC class-II-restricted T cells, IFN-γ, or the Th1 transcription factor T-bet (24, 25). Furthermore, successful resolution of *Salmonella* infection correlates with the expansion of *Salmonella*-specific Th1 cells in systemic tissues (23, 26).

Genital inoculation of C57BL/6 mice with *Chlamydia muridarum* generates a self-limiting ascending infection of the upper reproductive tract (21). Similar to *Salmonella* infection, the resolution of primary *C. muridarum* infection requires the presence of MHC class-II restricted T cells and IFN-γ (27). The *Chlamydia*-specific T cell response has been visualized using antigen-specific reagents and the predominant T helper subset detected in draining lymph nodes and spleen consists of a Th1 population that expresses T-bet and secretes IFN-γ (28, 29). In both infection models, the contribution of CD8 T cells and B cells in resolving primary infection is thought to be limited (27, 30–33), although recent data suggest a requirement for B cells in preventing bacterial dissemination to systemic tissues following *Chlamydia* genital challenge (28). It is not yet clear whether this implies a requirement for B cells in antigen presentation to CD4 T cells or simply a requirement for early antibody production.

Secondary responses to *Salmonella* and *Chlamydia* infection have also been examined and the data suggest a wider range of lymphocyte responses that can contribute to bacterial clearance (21, 34). Despite the fact that *Salmonella* and *Chlamydia* replicate intracellularly in an infected host, B cells and antibody can contribute to the resolution of secondary infection (30–32, 35, 36). A role for B cells is evident in experiments examining acquired immunity in B cell-deficient mice or by examining the protective immunity mediated by the transfer of immune serum (31, 32, 36–38). Similarly, CD8 T cells have been reported to contribute to secondary protection against both *Salmonella* and *Chlamydia* (24, 27, 39), although a recent report examining *Salmonella* infection of MHC class-I, perforin-, and granzyme-deficient mice did not detect an impaired protective response to secondary infection (33). Despite the expanded contribution of antibody and CD8 T cells in secondary protective immunity, CD4 Th1 cells are still thought to be the primary cell type involved in the resolution of secondary infection (21, 22). Thus, the development of pathogen-specific CD4 Th1 cells is essential for the development of protective immunity in mouse models of *Salmonella* and *Chlamydia* infection.

COGNATE SIGNALS DRIVING T CELL ACTIVATION AND REACTIVATION

Naïve pathogen-specific CD4 T cells are activated in secondary lymphoid tissues by dendritic cells expressing CD80/86 and displaying microbial peptides on surface MHC class-II (40). In both *Salmonella* and *Chlamydia* infection models, TCR transgenic mice and MHC class-II tetramers have been used to visualize naïve T cell activation, expansion, and acquisition of effector functions *in vivo* (28, 41–43). Initial T cell expansion occurs in the Peyer's patch and mesenteric lymph nodes after oral infection with *Salmonella* (41, 44). However, systemic expansion of CD4 T cells can also occur in the spleen and recent evidence suggests that these mucosal and systemic responses are functionally and antigenically distinct (43). Thus, while flagellin-specific CD4 Th17 cells expand in the intestine of *Salmonella*-infected mice, CD4 Th1 cells specific for components of the *Salmonella* Pathogenicity Island 2 (SPI2) Type III Secretion System were expanded in the spleen (43). Genital infection of mice with *C. muridarum* initially drives *Chlamydia*-specific T cell expansion in the draining ileac lymph, before systemic expansion occurs in the spleen (28). Unlike *Salmonella* infection, the antigenic targets of the CD4 response appear to be similar in mucosal and systemic locations and Th1 cells were primarily detected both locally and systemically. The most prominent feature of the immune response in both *Salmonella* and *Chlamydia* infection models is that a large pool of expanded pathogen-specific Th1 cells is generated. The activation and clonal expansion of *Salmonella*-specific T cells is strictly dependent on cognate stimulation since flagellin-specific T cells remain unactivated after infection of mice with flagellin-deficient *Salmonella* (41, 44). Thus, Th1 cells arise from a relatively infrequent pool of naïve pathogen-specific T cells in response to cognate (TCR-dependent) signals that are delivered in lymphoid tissues and these signals eventually lead to clonal expansion and effector development.

NON-COGNATE ACTIVATION OF EFFECTOR T CELLS

When Th1 cells relocate to an infected non-lymphoid tissue, they can produce IFN-γ locally in order to restrain intracellular bacterial replication (45). The stimulatory signals required to elicit local IFN-γ from effector T cells in tissues could potentially involve cognate stimulation via peptide/MHC complexes on the surface of infected cells or resident dendritic cells (**Figure 1**). However, many intracellular pathogens have evolved strategies that prevent MHC presentation of microbial peptides or down-regulate surface MHC expression on infected cells (46, 47). While down-regulation of MHC class-I is often discussed as a viral evasion strategy (48), *Salmonella* have also been reported to reduce expression of MHC class-II of antigen-presenting cells (49). Thus, in the absence of cognate ligands, Th1 cells may simply recognize inflammatory cues such as cytokines and TLR ligands in infected tissues to secrete IFN-γ (**Figure 1**). However, the relative contribution of cognate versus non-cognate signals in the eradication of intracellular pathogens is not fully understood.

Effector CD4 T cells that have relocated to non-lymphoid tissues retain the ability to respond to cognate signals in that location. Indeed, in a non-infectious model system, antibody that effectively blocked the peptide/MHC complex reduced the ability of CD4 T cells to produce effector cytokines (50). Similarly, recent experiments with bone marrow chimeras containing MHC class-II-deficient and MHC class-II sufficient myeloid cells demonstrated an increased burden of *M. tuberculosis* in host cells lacking MHC class-II (51). These data support the idea that Th1 cells scan infected tissues and can respond to local cognate signals to produce cytokines. However, the ability to respond to cognate signals may not always be required for the elaboration of effector functions. Studies of CD8 T cell effector function have demonstrated that expanded pathogen-specific T cells can secrete IFN-γ in response to a variety of inflammatory cytokines including IL-12, IL-18, and IL-15 (52–54). In a similar manner, CD4 cells have been shown to produce cytokines after direct ligation of surface TLRs by microbial products (3). Thus, non-cognate stimulation of Th1 cells could potentially be a major contributing factor to bacterial clearance from tissues during intracellular infections.

In a mouse model of *Salmonella* infection, a large proportion of CD4 T cells can be rapidly induced to secrete IFN-γ following intravenous injection of heat-killed bacteria (23). It was initially assumed that this complex mixture of bacterial antigens was able to efficiently activate *Salmonella*-specific Th1 cells via cognate signals delivered after antigen presentation of heat-killed bacteria. However, it was subsequently demonstrated that this large response from Th1 cells could also be induced following the injection of TLR ligands and more importantly also occurred in the absence of host MHC class-II (55, 56). Recently, this response was shown to be due to the induction of IL-18 and IL-33 in response to both TLR and inflammasome stimulation (57). The primary inflammasome components involved in recognition of *Salmonella* infection are NLRC4 and NLRP3 (58). Although NLRC4 can be activated in response to flagellin, bacteria that lacked flagellin expression were still able to induce non-cognate T cell activation suggesting that other components also participate in this response. Overall, these data suggest that inflammasome activation combines with TLR ligation to induce IL-18 and IL-33 production and that these cytokines drive T cell stimulation. Indeed, optimal IFN-γ production required T cell expression of IL-18R and IL-33R and mice containing a T cell-specific deficiency in Myd88 were less able to control the growth of *Salmonella* (57). A very similar pathway of non-cognate T cell activation has been reported following the injection of bacterial flagellin, although activation of CD8 T cells in this case was thought to require direct flagellin recognition by NLRC4 expressed by dendritic cells (59). Together, these data suggest that, during *Salmonella* infection, non-cognate signals may be vitally important for driving CD4 Th1 and CD8 T cells to produce IFN-γ and that mice lacking these particular pathways may be unable to generate an effective adaptive response. Interestingly, a similar non-cognate response was detected from Th1 cells in *Chlamydia*-infected mice (57), suggesting that non-cognate activation of CD4 T cells may be a common feature of the host immunity to intracellular bacteria. Future experiments examining other intracellular pathogens will be important to determine how ubiquitous this pathway is for eliciting protective Th1 responses to microbial pathogens. However, the finding that clearance of *M. tuberculosis* from individual myeloid cells requires direct cognate stimulation implies that an appropriate balance of cognate and non-cognate signals in infected tissues will be important for Th1 responses to different intracellular pathogens (51). Indeed, it is possible that cognate and non-cognate signals are each responsible for Th1 cytokine production at different stages of the host response, in different anatomical locations, or simply depending on the overall bacterial load within an infected tissue.

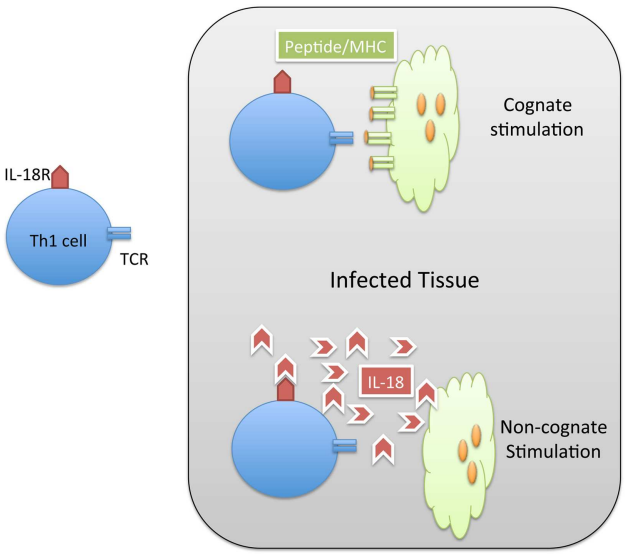

FIGURE 1 | Th1 cells can be activated by cognate and non-cognate stimuli in infected tissue. Naïve CD4 T cells are activated in lymphoid tissues to generate Th1 cells specific for *Salmonella*. These *Salmonella*-specific T cells can enter infected tissue and be stimulated by cognate (MHC/peptide) stimuli (TOP) or non-cognate (IL-18) stimuli. In both cases, the result of this stimulation is the production of IFN-gamma and resolution of the infection.

CONTRIBUTION OF NON-COGNATE T CELL ACTIVATION TO PATHOGEN CLEARANCE

The non-cognate elicitation of an effector response from expanded T cells may be required to specifically deal with pathogens that are able to alter host MHC expression or affect the presentation of microbial peptides in infected tissues. Any Th1 cell that enters an infected tissue would therefore retain some capacity to produce IFN-γ in response to local inflammation. Indeed, it has been shown that IFN-γ produced locally can induce iNOS expression from locally infected macrophages, even an individual macrophage happens to lack expression of MHC class-II (45). Thus, there is a degree of non-specificity in the function of Th1 cells within infected tissues. The ability of these same T cells to respond to non-cognate signals may simply further decrease the activation threshold for eliciting bactericidal response. Although it has not been directly examined *in vivo*, the contribution of non-cognate Th1 cell stimulation may be directly related to the overall pathogen burden in the infected tissue. Thus, if the overall tissue burden is low, then PAMP-elicited cytokines such as IL-18 and IL-33 would also be expected to be at low concentrations, leaving Th1 cells to seek out cognate stimulation and thus constraining T cell activation to a very localized radius around the few infected cells in the tissue. In contrast, if a Th1 cell encounters high concentrations of inflammatory cytokines, the threshold for T cell stimulation would effectively be lowered, allowing immediate and widespread production of IFN-γ. Such a lower threshold of activation may be particularly important when an infected host is combating a rapidly dividing or rapidly spreading pathogen such as *Salmonella*, but conversely may be less important for immunity to a slow growing pathogen such as *M. tuberculosis*.

Another potential role for non-cognate T cell activation could occur in situations of bacterial co-infection. Indeed, a role for non-cognate T cell activation in driving pathology has been examined in the context of influenza and bacterial co-infections (60). In this case, an expanded pool of virus-specific CD8 T cells could be rapidly activated to produce harmful pathology in response to inflammatory cytokines elicited by bacterial infection. Conversely, persistent viral stimulation of macrophages can sometimes provide protection against some intracellular bacterial infections (61). In the case of Th1 cells, a pathway of non-cognate activation could be a primary driver of protective immunity during a co-infection. For example, if an individual is infected with an intracellular pathogen and therefore has invested in the expansion and functional maturation of a pool of Th1 cells, the simultaneous encounter with an unrelated secondary infection may well recruit and activate these Th1 cells in a non-cognate manner. Indeed, the original discovery of macrophage activation was surprising because the efferent phase of the adaptive response involved a relatively non-specific mechanism and was demonstrated using a co-infection model where *Brucella* infection prevented productive infection with *Listeria*. However, a role for non-cognate T cell activation in the elicitation of protective immunity during co-infections has not yet been described. Overall, it seems most likely that non-cognate mechanisms of Th1 cell activation could have evolved to help the host combat bacterial evasions of host immunity, superior bacterial cell division, or co-infections. Future research in this area is required to examine each of these possibilities.

CONCLUSION

Naïve CD4 T cells are activated by cognate signals leading to the expansion of an effector pool of pathogen-specific T cells that can migrate to infected tissues and deliver local anti-microbial effects. Recent data have demonstrated that Th1 cells can be activated within infected tissues in response to cognate and/or non-cognate signals that arise from TLR and inflammasome activation. Thus, although the adaptive response is regulated by highly specific antigen-specific surface receptors, an expanded pool of effector cells retains the ability to respond immediately to inflammatory cues that are normally associated with the innate arm of the immune system. This functional capability reinforces our growing understanding that innate and adaptive immune systems are not completely separate entities but instead work in a coordinated fashion to resolve infection with microbial pathogens. This ability of expanded effector lymphocytes to blur the lines between innate and adaptive immunity may be a critical component of protective immunity to *Salmonella*, *Chlamydia*, and other intracellular bacteria.

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Organ-specific and memory Treg cells: specificity, development, function, and maintenance

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Foxp3$^+$ regulatory T cells (Treg cells) are essential for establishing and maintaining self-tolerance, and also inhibit immune responses to innocuous environmental antigens. Imbalances and dysfunction in Treg cells lead to a variety of immune-mediated diseases, as deficits in Treg cell function contribute to the development autoimmune disease and pathological tissue damage, whereas overabundance of Treg cells can promote chronic infection and tumorigenesis. Recent studies have highlighted the fact that Treg cells themselves are a diverse collection of phenotypically and functionally specialized populations, with distinct developmental origins, antigen-specificities, tissue-tropisms, and homeostatic requirements. The signals directing the differentiation of these populations, their specificities and the mechanisms by which they combine to promote organ-specific and systemic tolerance, and how they embody the emerging property of regulatory memory are the focus of this review.

Keywords: Foxp3, immune tolerance, immune memory, regulatory T cells, T cell homeostasis

INTRODUCTION

It has become increasingly accepted that most individuals have self-reactive lymphocytes circulating throughout their peripheral tissues. In the wrong context, these cells may be capable of mediating pathogenic autoimmune responses. By contrast, in healthy individuals, these cells are counterbalanced by regulatory cells, which act to stably suppress the pathogenic potential of self-reactive cells. Regulatory T (Treg) cells, a subset of CD4$^+$ T cells defined by their expression of the transcription factor Foxp3, constitute a major immune-regulatory cell population in the body. The majority of Treg cells arise during T cell development in the thymus, where moderate- to high-avidity recognition of self-antigen leads to the development of Foxp3$^+$ thymic Treg (tTreg). The second pathway of Treg generation is in the periphery, where mature, naïve CD4$^+$ T cells develop into peripheral Treg (pTreg) cells upon antigen encounter under certain conditions (1). The choice between tolerance (i.e., control of inflammation) and autoimmunity is determined to a significant extent, by the relative generation and maintenance of pathologic effector T cells (Teff) and protective Treg cells specific for self-antigens. An imbalance in this number or activity of Treg cells is thought to underlie many inflammatory and autoimmune disorders. When Treg cells are absent or rendered non-functional, both mice and human beings develop fulminant and life-threatening autoimmunity (2). Additionally, genome-wide association studies have identified several genes involved in the development, maintenance, or function of Treg cells that are linked to autoimmune disease susceptibility (3). In addition to preventing autoimmunity and maintaining immune homeostasis, Treg cells are required to minimize tissue damage in inflammatory settings such as viral infection (4) or mediate tolerance to allografts (5). However, Treg cell-mediated suppression can also have undesirable effects such as the development of chronic infection or suppression of anti-tumor responses. Indeed, Treg cells are now considered a promising target in cancer therapy (6).

In order to therapeutically manipulate Treg cell numbers or function, a multitude of studies have defined the factors required to generate and maintain these cells, and characterized the mechanisms of how they mediate their regulatory functions in various settings. An emerging concept is that Treg cells are a phenotypically and functionally heterogeneous population, with specific subsets requiring different factors for their differentiation, maintenance, and function in different inflammatory contexts or tissues. In this review, we discuss the diversity of Treg cells in peripheral tissues and identify some of the key open questions in Treg biology that present potential opportunities and roadblocks for the therapeutic manipulation of Treg cells. These include the development, specificity, and maintenance of specialized Treg cell populations, a better understanding of the effector mechanisms Treg cells employ, and how they manage to discriminate between potentially harmful and beneficial responses. We also discuss the

emerging concept of regulatory memory, and how Treg cells may also fulfill non-immune tissue-support functions.

PHENOTYPIC DIVERSITY OF Treg CELLS

When initially described in the mid-1990s, Treg cells were identified based on their constitutive expression of the CD25 component of the high-affinity IL-2 receptor complex (7). However, the identification of Foxp3 as the specific transcription factor that drives Treg cell development and function (8, 9), and the generation of experimental tools for analysis of Foxp3 expression allowed for more thorough examination of the phenotypic diversity of Treg cells (10). It quickly became apparent that like conventional $CD4^+$ effector cells that can be divided into functionally distinct effector populations based on differential expression of adhesion and chemoattractant receptors, Treg cells could also be extensively sub-divided based on expression of homing receptors expected to target them to both lymphoid and non-lymphoid tissues (11). Indeed, Treg cells can be found in many tissues even in the absence of any strong ongoing immune responses. Moreover, many studies over the last decade have demonstrated that Treg cells function in both lymphoid and non-lymphoid tissues in order to prevent inflammatory disease and maintain normal immune homeostasis (12–18). Additionally, Treg cells are rapidly recruited to inflamed tissues, where they dampen autoimmunity and prevent collateral tissue damage during ongoing inflammation, but may also promote pathogen persistence and tumor development/growth. In this section, we will briefly summarize the current understanding of tissue- and inflammation-specific Treg cells.

ORGAN-SPECIFIC Treg CELLS ASSOCIATED WITH THE INTESTINES AND SKIN

Because of their important barrier function, exposure to benign commensal micro-organisms and food-derived antigens, and frequent pathogen encounter, the intestines are immunologically active organs that need to maintain a fine balance between pro- and anti-inflammatory responses. Although this balance is the result of a coordinated effort between many cell types, including intestinal epithelial cells, dendritic cells (DCs), innate lymphoid cells and conventional T cells, it is clear that $Foxp3^+$ Treg cells have a central role in maintaining normal intestinal immune homeostasis. This is best exemplified by the fact that defects in Treg cell differentiation or function result in development of intestinal inflammation in both humans and mice (2, 19, 20). Additionally, one of the most commonly used *in vivo* mouse models of Treg cell function measures their ability to block T cell-mediated inflammatory colitis following adoptive transfer into lymphopenic mice (21). Consistent with this, the intestines harbor a large population of $Foxp3^+$ Treg cells. Migration of T cells to the intestine requires expression of high levels of the intestinal homing integrin α4β7. Given the importance of Treg cells in maintaining intestinal immune homeostasis, it may seem somewhat surprising that very few Treg cells in adult peripheral blood are $\alpha4\beta7^+$ (22, 23). However, studies with parabiotic mice have demonstrated that in adults, most intestinal T cells, including Treg cells, are tissue-resident and do not actively recirculate (24, 25). Moreover, α4β7-expressing Treg cells are abundant in umbilical cord blood (26), and together this suggests that after initial development and seeding early in life, intestinal Treg cells maintain themselves as a stable, self-renewing population with little input from the periphery.

Because of the unique immunological challenges posed by the intestine, intestinal Treg cells display several phenotypic and functional properties distinct from other Treg cell populations. First, given the large burden of benign, non-self-antigens that the intestines are exposed to through the commensal microflora and ingestion of food-derived antigens, it is not surprising that a large fraction of the Treg cell population in the intestines, and especially in the colon, display phenotypic features consistent with a peripheral origin (27–29). Indeed, feeding model antigens such as ovalbumin to mice in their drinking water leads to efficient generation of antigen-specific pTreg cells in the gut-associated lymphoid tissues (30, 31). This is due to the presence of a specialized population of $CD103^+$ DCs in the intestines and their associated lymphoid tissues that can produce active TGF-β and retinoic acid (RA), which together promote pTreg cell development (30, 32). pTreg cell differentiation was also observed in cells expressing cloned T cell receptors (TCRs) derived from intestinal Treg cells, which had been generated in response to specific components of the intestinal microflora (33). Interestingly, effector T cells expressing these TCRs induced colitis in immunodeficient mice, indicating that pTreg induction is an important mechanism by which T cells specific for commensal antigens are tolerized *in vivo*. However, it is important to note that not all commensal-specific T cells undergo pTreg cell conversion, as T cells specific for flagellin expressed by *Clostridium* bacterial species are potently activated and undergo effector differentiation in mice when the epithelial barrier is compromised during infection with the inflammatory parasite *Toxoplasma gondii* (34). However, consistent with the unique array of antigens they are exposed to, the TCR repertoire of colonic Treg cells is distinct from that of colonic effector T cells, and from Treg cells in other tissue sites (33).

In addition to their unique specificity, intestinal Treg cells are also exposed to an environment rich in commensal and host metabolites that can influence their development and function. For instance, as mentioned above, RA (derived primarily from dietary vitamin A) augments pTreg cell development in the intestine, and also drives T cell expression of intestinal homing receptors such as α4β7 integrin and the chemokine receptor, CCR9 (35). Additionally, the intestine contains a high concentration of commensal-derived toll-like receptor (TLR) ligands that may directly influence the abundance and function of Treg cells. For instance, stimulation of Treg cells with TLR2 ligands can augment Treg cell proliferation but inhibit their suppressive activity (36). Additionally, TLR ligands can impact Treg cell generation and abundance in the intestine indirectly by altering cytokine production and activation of other cell types. In this context, activation of TLR9 by DNA from commensal organisms enhances inflammatory cytokine production that limits TGF-β-driven Treg cell differentiation *in vitro*, and accordingly TLR9-deficient mice have increased Treg cell abundance in intestinal tissues (37). Similarly, IL-6 produced upon TLR ligation can both block pTreg cell development (promoting Th17 cell differentiation instead) (38), and inhibit the suppressive function of existing Treg cells (39). Finally, a series of recent papers have demonstrated that certain metabolites of the commensal flora can dramatically influence the

development and maintenance of intestinal Treg cells. Specifically, short-chain fatty acids (SCFA), such as butyrate, that are produced by intestinal bacteria during the breakdown of dietary fiber promote pTreg cell differentiation in the intestine, and augment the proliferation of existing intestinal Treg cells (40–42). The effects of SCFA of intestinal Treg cells were dependent on the expression of the free fatty acid receptor, GPR43, and are at least partially due to the ability of SCFA to directly promote Foxp3 expression. Accordingly, GPR43-deficient mice were highly sensitive to disease development and showed impaired recovery in a model of chronic inflammatory colitis (43). Interestingly, the effects of SCFA range beyond the intestine, as GPR43-deficient animals were also more sensitive to development of inflammatory arthritis and asthma. However, GPR43 is also expressed by a range of myeloid cells, and the specific contribution of impaired Treg cell function to these inflammatory phenotypes has not been precisely delineated.

Like the intestine, the skin is a barrier tissue with a large commensal microbial community that is frequently a site of pathogen encounter/entry. Additionally, the skin is exposed to environmental irritants and damage from ultraviolet light exposure, and undergoes frequent traumatic injury and wound repair. Dysregulated immune responses in the skin result in a number of inflammatory disorders, including contact hypersensitivity, atopic dermatitis, psoriasis, and *Pemphigus vulgaris*, and it is therefore not surprising that as in the intestines, there is a large population of Treg cells in both mouse and human skin even in the absence of overt inflammation (12, 22, 44). In human peripheral blood, most Treg cells express functional skin-homing receptors such as the functional E-selectin ligand cutaneous lymphocyte antigen (CLA) and CCR4 (22, 23), and skin-tropic Treg cells in mouse have been defined based on their expression of P- and E-selectin ligands, CCR4 and CD103 (12, 13, 17). Additionally, multiple studies have demonstrated that Treg cell migration to the skin is essential for their ability to prevent inflammatory disease in the skin (12, 17), and to regulate cutaneous immunity in the contexts of delayed-type hypersensitivity responses and viral or parasitic infection (13, 45, 46). Furthermore, both mice and humans with impaired Treg cell activity display severe skin inflammation (47, 48).

The size of the Treg pool in the skin may be controlled by keratinocyte-derived IL-7, an essential factor for their maintenance in murine skin (49). In addition to the production of IL-7 (50), keratinocytes may indirectly regulate inflammation via expression of the TNF-family molecule, RANKL. Skin inflammation (triggered by UV-light and prostanoids) increases RANKL production by keratinocytes. RANK/RANKL interactions lead to activation of skin-resident DCs and preferential expansion of Treg cells in skin-draining lymph nodes (51). Moreover, similar to the gut, RA-producing skin-derived DCs are capable of triggering the generation of Treg cells. However, in the skin, RA production is restricted to $CD103^-$ DCs (52). Interestingly, TLR triggering or the presence of a commensal microflora was not essential to induce RA production.

Despite their clear importance in regulating immune responses in the skin, far less is known regarding the developmental origin, specificity, and function of cutaneous Treg cells as compared with Treg cells in the intestines. The notion that Treg cells in the skin have a unique specificity profile is supported by data indicating that the TCR repertoire of Treg cells in the skin-draining inguinal and axillary lymph nodes of mice differs substantially from that of Treg cells found in the spleen or mesenteric lymph nodes (53). However, the fine specificity of cutaneous Treg cells is almost entirely uncharacterized. Given the complex microbial communities resident on the skin, one would expect that as in the intestine many cutaneous Treg cells would recognize these foreign antigens. However, to date the limited data available regarding Treg cell specificity in the skin suggest that cutaneous Treg cells are largely specific for self-antigens. For instance, Treg cells with a skin-tropic phenotype were found in transgenic mice expressing a TCR cloned from skin-reactive $CD4^+$ T cells found in Foxp3-deficient mice (54). Although the precise antigen recognized by these cells was not defined, they reacted equally well to DCs from the skin-draining lymph nodes of specific-pathogen free and germ-free mice, indicating that they are not specific for cutaneous commensals. Additionally, Treg cells specific for an inducible, transgenic self-antigen rapidly accumulated in the skin when antigen expression was activated (55).

Interestingly, Treg cells appear to occupy a specialized anatomic niche in the skin, accumulating in and around the epithelial invaginations associated with hair follicles (44, 49). Recent data have indicated that hair follicles can act as specialized immune structures that coordinate immune cell migration and function in the skin. This may relate to the fact that skin appendages such as hair follicles, sweat glands, and sebaceous glands house diverse and unique microbial communities that interact with and shape the cutaneous immune system (56). Indeed, there was a pronounced increase in the frequency and number of Treg cells in the skin of germ-free mice, indicating that interactions with cutaneous commensal flora help regulate Treg cell abundance in the skin (57). Moreover, this study demonstrated that commensal-dependent production of IL-1 in the skin is essential for inflammatory immune responses to the parasite *Leishmania major*, and this may in part be due to the ability of IL-1 to suppress Treg cell function (58).

Aside from the skin and intestine, other non-lymphoid tissues with large numbers of Treg cells in the steady-state include the lungs, liver, adipose tissue, and skeletal muscle. Like the skin, the lungs and the liver are major targets of the organ-specific inflammatory disease that develops in Foxp3-deficient mice (48), suggesting that Treg cells in these organs have an important function in maintaining hepatic and pulmonary immune homeostasis. The function of Treg cells in other tissues, including potential "tissue-support" functions in the adipose tissue and muscle will be addressed later in this review.

INFLAMMATION-SPECIFIC Treg CELLS

In addition to Treg cells that constitutively reside in tissues such as the skin and intestine, Treg cells are rapidly recruited to sites of inflammation. In many sites, Treg cells recruited during inflammation accumulate over time and persist even after inflammation has resolved. For instance, skin inflammation in an autoimmune setting results in the generation and recruitment of Treg cells to the skin where they steadily increase in abundance to make up 60–80% of the skin-resident $CD4^+$ T cell population, and help resolve the inflammatory response (55, 59). T cell recruitment

to inflamed tissues is the result of dramatic changes in expression of chemokines, adhesion molecules, and extracellular matrix components that occur during tissue inflammatory responses. Importantly, these changes often act to amplify the inflammatory response in feed-forward loops. For instance, during inflammatory responses dominated by IFN-γ-producing Th1 cells, IFN-γ induces the expression of the chemokines CXCL9 and CXCL10 by tissue-resident cells, which act to further the recruitment of $CXCR3^+$ Th1 cells (60). Similarly, IL-17A and IL-17F produced by Th17 cells can amplify the recruitment of $CCR6^+$ Th17 cells by inducing expression of the chemokine, CCL20 (61). Moreover, expression of CXCR3 and CCR6 is controlled by the Th1 and Th17 lineage-specifying transcription factors, T-bet and RORγt, respectively, and this links the functionality of these cells to their ability to access different inflammatory sites (61, 62). The realization that distinct populations of both human and mouse Treg cells express these and other inflammatory homing receptors raised the possibility that specialized populations of Treg cells are recruited to different types of inflammatory responses, and that these may share molecular characteristics with pro-inflammatory helper T cell populations. In fact, several recent studies have demonstrated that regulation of Th1, Th2, and Th17 responses by Treg cells has distinct molecular requirements (63–65). Moreover, populations of Treg cells that phenotypically mirror effector T cell subsets and share expression of key transcription factors such as T-bet and RORγt have been identified in both mouse and human (63, 66–69). In addition to these "lineage-specific" transcription factors, in mice the function of these effector Treg cell populations was dependent on upregulation of the transcription factor Blimp-1 following Treg cell activation (70).

The ability of Treg cells to be rapidly mobilized to inflamed tissues has led to the somewhat paradoxical observation that the number of Treg cells is often elevated in target tissues during autoimmune and inflammatory diseases, including inflammatory bowel disease, multiple sclerosis and rheumatoid arthritis (71–73). Similar studies have also observed Treg cell accumulation in multiple mouse models of autoimmune disease (74, 75). Although this likely represents an effort by the immune system to re-establish proper control of the autoimmune response, the inability of these tissue-infiltrating Treg cells to effectively modulate disease suggests that they are somehow functionally compromised *in vivo*. This can occur as the result of inflammatory cytokines that either directly inhibit Treg cells or render effector T cells and other immune cells resistant to Treg cell-mediated suppression (76).

The formation of stable Treg cells requires two independent processes: the expression of Foxp3 and the establishment of a Treg cell-specific CpG hypomethylation pattern, both of which require TCR stimulation (77). This hypomethylation is the basis for Treg-specific gene expression, lineage stability, and full suppressive activity. A recent study has found that the main function of Foxp3 is to act as a transcriptional repressor. Importantly, Foxp3 binding alone was not sufficient to establish suppression in resting Treg where Foxp3-bound regulatory elements are only poised for repression. An inflammatory stimulus was then required to incorporate the polycomb-group histone methyltransferase Ezh2 into the complex and deposit repressive chromatin modifications at Foxp3-bound loci (78). This approach used systemic inflammation caused by Treg cell depletion as inflammatory stimulus and more research is required to identify the exact inflammatory signals that were sensed and led to chromatin remodeling. However, this cross-talk between tissue inflammation and Treg cell stability and function may serve to ensure that Treg cells that have undergone an inflammatory response that they successfully resolved are stable and more suppressive than resting Treg cells.

CRITICAL ISSUES IN Treg CELL BIOLOGY

Overall, the phenotypic diversity of Treg cells allows them to access multiple tissue sites, where they maintain immune homeostasis by both preventing initiation of immune responses in secondary lymphoid tissues and dampening ongoing inflammatory responses in non-lymphoid organs. Their potent anti-inflammatory function has led to efforts to boost Treg cell activity for treating autoimmunity and chronic inflammation and preventing graft rejection (79, 80). Conversely, transient inhibition of Treg cell function may allow for more effective immune responses in the contexts of vaccination, persistent infection, and cancer. However, several key questions regarding the development, specificity, function, and maintenance of different Treg cell populations remain as key barriers to clinical success. In this section, we will discuss some of these issues, and how their resolution may contribute to successful implementation of Treg cell-based immunotherapies.

Treg CELL SPECIFICITY

Like other $CD4^+$ T cells, it is clear that Treg cell development depends on expression of MHC class II molecules in the thymus, against which they are positively and negatively selected (81). Additionally, abundant evidence indicates that at least a large fraction of Treg cells are self-antigen-specific. However, current knowledge of the precise antigen-specificities of Treg cells is extremely limited. As a result, some of the biggest unanswered questions regarding Treg cells relate to their antigen specificity, and understanding how this influences their differentiation and homeostasis, as well as their migratory and functional characteristics.

That Treg cells are largely autoreactive was initially inferred based on the fact that they shared phenotypic features of activated T cells. For instance, in mice, most Treg cells display a $CD44^{hi}CD45RB^{lo}CD25^+$ phenotype resembling activated conventional T cells. Additionally, large (and somewhat overlapping) subsets of Treg cells express other activation markers such as CD69, ICOS, and CD38, and consistent with chronic antigen stimulation Treg cells undergo a rapid rate of steady-state proliferation *in vivo* (82). Analysis of the TCR repertoire of Treg cells demonstrated that there is little overlap between the TCRs expressed by Treg cells and conventional $Foxp3^-$ T cells, indicating that antigen specificity is a key determinant in Treg cell differentiation (83). Additionally, this study showed that when expressed in effector T cells, TCRs from Treg cells can induce a wasting/autoimmune disease upon transfer into lymphopenic recipients, further supporting the notion that many Treg cells are indeed autoreactive. A key advance in understanding the self-reactivity of Treg cells came from analyses of TCR transgenic mice. Although most TCR transgenic mice expressing MHC class II restricted TCRs do develop a population of Treg cells, this is usually dependent on rearrangement of endogenous TCR genes and is therefore abrogated in RAG-deficient mice. However,

in several cases providing their cognate antigen as either a tissue-restricted or systemic transgene drives efficient Treg cell development even in RAG-deficient TCR transgenic mice, definitively demonstrating that recognition of self-antigens promotes Treg cell differentiation (84–86). Accordingly, it has been postulated that expression of AIRE, a transcription factor that promotes expression of tissue-restricted antigens in thymic medullary epithelial cells, can influence Treg cell development (87, 88). However, the extent to which AIRE influences the Treg cell repertoire remains somewhat controversial (89). Nonetheless, the preponderance of evidence clearly indicates that the vast majority of tTreg cells are selected on the basis of self-antigen recognition in thymus, and that this autoreactivity has dramatic consequences on their phenotype and behavior in the periphery.

Although the self-reactivity of tTreg is well-accepted, the precise autoantigens recognized by Treg cells are almost completely unknown. Classically, presentation of antigens by MHC class II molecules was thought to be restricted to exogenous antigens taken up into cells via the endocytic or phagocytic pathways. However, it has become clear that the MHC class II antigen-processing pathway can access almost any cellular protein either through uptake of apoptotic cells or through autophagy of cellular contents. Indeed, many self-peptides eluted from MHC class II molecules expressed by activated B cells and macrophages were actually derived from cytosolic proteins (90). Thus, the number of potential peptide–MHC complexes that could drive Treg cell differentiation in the thymus is likely very large. However, the fraction of these antigens actually recognized by thymic and peripheral Treg cells is unknown. The diverse TCR repertoire of Treg cells suggests that they have broad reactivity (83). Interestingly, this may be enforced during thymic development of Treg cells, as the efficiency of Treg cell development for thymocytes of any given TCR is governed by readily saturable "niches" that likely relate to antigen availability (91), and this may help ensure that Treg cells specific for a wide range of self-antigens are generated in the thymus. Similarly, in the periphery competition for limited peptide–MHC niches could help ensure that the Treg cell repertoire remains sufficiently broad to maintain self-tolerance to the vast array of potential tissue-specific and systemic autoantigens (92).

Further adding to the confusion regarding the differentiation and specificity of Treg cells are the recent findings that Treg cells specific for some pathogens expand during infection, and can contribute to immune dysregulation and impaired pathogen clearance (93). Surprisingly, unlike pTreg cells specific for commensal microbes and other environmental antigens, in many cases the pathogen-specific Treg cells were actually present in the pre-infection Treg cell repertoire. For instance, in murine infection with *Mycobacterium tuberculosis*, Treg cells specific for the immunodominant epitope $ESAT6_{4–17}$ were identified in the lung-draining lymph nodes using peptide:MHC class II tetramers (94). Interestingly, TCR Vβ utilization was distinct in the $Foxp3^+$ vs. $Foxp3^-$ ESAT6-specific cells, which suggested that they have different developmental origins. Indeed, adoptive transfer studies definitively established that ESAT6-specific Treg cells were derived from pre-existing tTreg cells, and were not the product of pTreg cell differentiation from naïve precursors. Similarly, Treg cells specific for epitopes of mouse hepatitis virus were found in the pre-infection Treg cell pool (95), as were Treg cells reactive to *Leishmania major* (although the precise epitopes in this case have not been identified) (96). These studies raise several interesting questions regarding Treg cell development, specificity, and function. Because Treg cell differentiation in the thymus depends on high-affinity TCR triggering, what are the nature of the antigens that drive the differentiation and maintenance of these pathogen-specific Treg cells? How does expansion of pathogen-specific Treg cells impact the outcome of subsequent pathogen encounters? Is Treg cell specificity a virulence factor of pathogens that were evolutionary selected to be recognized by Treg (i.e., are pathogens that express peptides capable of triggering Treg cells more successful)? Additionally, are TCRs expressed by Treg cells likely to recognize multiple ligands due to the fact that their selection in the thymus requires high-affinity interactions with self-MHC? In this regard, despite the fact that most Treg cells are thought to develop in response to recognition of self-antigen in the thymus, broad reactivity to foreign antigens in Treg cells has also been observed (97).

The relationship between TCR specificity and development of the phenotypically and functionally specialized Treg cell populations discussed previously is also poorly understood. The fact that Treg cells in different tissue sites have distinct TCR repertoires is strong evidence that Treg cell specificity impacts their phenotype, homing receptor expression and tissue distribution (53). Indeed, Treg cells in mice expressing a TCR specific for a skin-expressed self-antigen acquire a skin-tropic P-/E-selectin $ligand^+CCR4^+$ phenotype (54), likely through interaction with skin-derived DCs in peripheral lymph nodes (98). Thus, efficient Treg cell migration to the skin only happens when the antigen is expressed at that site (55). In addition to indirectly controlling Treg localization by influencing homing receptor expression, TCR triggering also induces potent "stop" signals that act to retain antigen-specific T cells in tissues (99). The TCR may also control Treg cell localization by directly mediating interactions between Treg cells and vascular endothelial cells that promote cellular exit from the blood into antigen-bearing tissues (100).

DEVELOPMENT OF SPECIALIZED Treg CELL SUBSETS

The existence of tissue- and inflammation type-specific Treg cell subsets with specialized functions implies that Treg cell-based immunotherapies must target correct Treg cell populations in order to successfully modulate different types of immune responses in distinct tissue sites. Additionally, the diversity of tissue Treg cells suggests that they alter their migratory, functional, and homeostatic properties in response to contextual cues from the immune environment (101). However, the mechanisms guiding the development of specialized Treg cell subsets, and the ways in which they mirror and diverge from the comparatively well-characterized pathways of effector T cell differentiation have not been extensively explored.

Development of specialized effector T cell subsets such as Th1, Th2, Th17, and Tfh cells from naïve precursors is believed to be driven primarily by the presence or absence of specific cytokines in the local environment at the time of priming. These cytokines are primarily derived from innate immune cells upon pathogen recognition, and in this way the innate immune system

can instruct antigen-specific $CD4^+$ T cells to differentiate into effector cells with functions appropriate for eliminating different types of pathogens. The relatively stable phenotypes of these cells are believed to be the result of subset-specific expression of "master" transcriptional regulators that control many of the phenotypic and functional characteristics of these cells (102). Because distinct populations of Treg cells share expression of these key transcription factors and often develop in parallel with their effector cell counterparts, it is tempting to speculate that the same factors induce the differentiation of phenotypically similar effector and regulatory T cell (Treg) subsets. However, cases in which this has been examined in detail have revealed important differences in the differentiation of effector and Treg subsets.

The parallel development of Th1 cells and T-bet^+ Treg cells exemplifies the different ways in which effector and Tregs respond to cytokine signals. Differentiation of IFN-γ-producing Th1 cells is initiated by activation of the signaling adaptor and transcription factor Stat1, which is phosphorylated following activation of naïve conventional T cells through cytokines such as the type-1 IFNs, IFN-γ, or IL-27. Stat1 activates low-level expression of the Th1-associated master transcription factor T-bet, which renders cells sensitive to IL-12 by inducing expression of the IL-12 receptor component IL-12Rβ2. IL-12-mediated activation of Stat4 then drives the high-level T-bet expression required for full Th1 cell differentiation. Similarly, Treg cells upregulated T-bet in response to Stat1 activation following either IFN-γ or IL-27 stimulation *in vitro*, and T-bet expression in Treg cells is dramatically reduced in either Stat1- or IFN-γ-deficient mice (103, 104). However, unlike IFN-γ stimulated effector T cells, Treg cells transiently stimulated with Stat1 activating cytokines failed to efficiently upregulate IL-12Rβ2 expression, and therefore could not complete IL-12-dependent Th1 differentiation (103). The delayed induction of IL-12Rβ2 was associated with the presence of inhibitory H3K27 tri-methyl histone methylation marks at the *Il12rb2* promoter in Treg cells. However, Treg cells did upregulate IL-12Rβ2 during dysregulated inflammatory responses *in vivo* or prolonged activation *in vitro* and these cells were then rendered susceptible to IL-12-mediated functional "reprograming," losing their suppressive function and upregulating expression of IFN-γ (103, 105). Thus, differential sensitivity to IL-12 appears to be a major factor underlying the relative ability of effector and Tregs to differentiate into IFN-γ-producing cells. Additionally, during *Mycobacterium tuberculosis* infection, pathogen-specific Treg cells are selectively eliminated at later stages of infection in an IL-12-dependent manner (94). Interestingly, unlike mice, in which it is difficult to detect any IL-12-responsive or IFN-γ-producing Treg cells in the absence of overt inflammatory pathology (95, 103, 104), $Foxp3^+IFN\text{-}\gamma^+$ Treg cells are readily identified in the peripheral blood of healthy humans (27, 68). Although IFN-γ production by Treg cells can be protective in the context of graft-versus-host disease (106), both type-1 diabetes and multiple sclerosis have been associated with an increase in IFN-γ–producing Treg cells, suggesting that redirected Treg cells may contribute to autoimmune pathogenesis (66, 67).

Similar to these T-bet-expressing Treg cells that express CXCR3, a large population of human and mouse Treg cells expresses the Th17-associated chemokine receptor, CCR6 (107, 108), and in human it is clear that many of these cells also express the key transcriptional regulator of Th17 development RORγt (68, 69). CCR6 can direct Treg cell migration to sites of Th17-mediated inflammation, indicating that these $CCR6^+$ Treg cells may be particularly potent suppressors of Th17 responses (109). $CCR6^+ROR\gamma t^+$ cells were generated *in vitro* from Treg cells stimulated in the presence of Th17 polarizing cytokines such as IL-1, IL-23, IL-6, and TGF-β, but this was also associated with downregulation of Foxp3 and loss of suppressor function (110), and this differs from the highly suppressive $CCR6^+$ Treg cells found *in vivo*. Interestingly, Treg cell expression of the signaling adaptor and transcription factor Stat3 was found to be essential for their ability to properly regulate Th17 cell responses *in vivo*, and loss of Stat3 resulted in decreased CCR6 expression by Treg cells and impaired their migration to the intestines (64). Surprisingly, rather than the pro-inflammatory Stat3 activating cytokine IL-6 that drives Th17 cell differentiation, it was the anti-inflammatory cytokine IL-10 that promoted the Stat3 phosphorylation in Treg cells required for suppression of Th17-mediated autoimmune disease (111). Thus, as with Th1-associated Treg cells, the development of Th17-associated $CCR6^+$ Treg cells appears to be molecularly distinct from canonical Th17 cell differentiation.

Aside from the aforementioned studies on the development of the Th1- and Th17-associated Treg cells, the differentiation of other specialized Treg cell populations has not been extensively studied. These include Bcl-6^+ T "follicular regulatory" (Tfr) cells that express the B cell-associated chemokine receptor CXCR5, localize to B cell follicles and germinal centers in the secondary lymphoid tissues and regulate the magnitude and output of the germinal center response (112, 113). These Tfr cells develop in parallel to Bcl-6^+ T follicular helper (Tfh) cells that promote humoral immunity, and share some of their developmental requirements such as CD28 mediated co-stimulation and signaling lymphocytic activation molecule-associated protein (SAP)-dependent interaction with B cells. However, a recent study found that the transcription factor NFAT2 was required for CXCR5 expression in Tfr, but not Tfh (114), further supporting the notion that effector and Tregs use distinct molecular pathways to achieve similar phenotypes.

In addition to signals regulating their functional differentiation, responding T cells also receive anatomical directions so that they are targeted to the appropriate non-lymphoid tissue sites. This has been best explored in the skin and intestines, where it seems that signals from distinct tissue DCs program the migratory behavior of the responding T cells in either the skin- or intestine-draining lymphoid tissues (98, 115). This is, at least in part, due to the presence of specific vitamin metabolites in these different tissue sites. Whereas $CD103^+$ DCs in the intestine convert dietary vitamin A to RA that induces expression of the intestinal homing receptors α4β7 integrin and CCR9 on responding T cells (116), skin DCs can convert sunlight-derived vitamin D into the active $1,25(OH)_2D_3$ form, which induces T cell expression of CCR10, the receptor for the epithelial chemokine CCL27 that is produced in abundance by skin keratinocytes (117). Although many of these tissue signals are likely sensed by both effector and Tregs (118), Treg cells display some unique tissue-migratory characteristics. For example,

Treg cells selectively express the orphan G-protein-coupled receptor, GPR15, and loss of this receptor resulted in impaired Treg cell migration to the large intestinal lamina propria and dysregulated intestinal immune responses (119). GPR15 expression in Treg cells was dependent on TGF-β1 signaling and on the presence of intestinal commensal bacteria, indicating that Treg cells can adopt unique tissue-specific phenotypes based on sensing local environmental stimuli.

SUPPRESSIVE MECHANISMS OF Treg CELLS

Although Treg cells clearly have an important role in maintaining immune tolerance and preventing autoimmune disease development, the functional mechanisms by which Treg cell accomplish these tasks *in vivo* are still not well understood. A key concept that has emerged, however, is that Treg cells are functionally heterogeneous, and that the importance of any given mechanism of immune suppression is tissue- and context-dependent. Indeed, to date, deletion of any single mechanism of Treg cell-mediated immune suppression has not recapitulated the phenotypes observed in Treg cell-deficient mice, indicating that Treg cells use multiple inhibitory mechanisms that are at least partially redundant.

The immunosuppressive mechanisms ascribed to Treg cells thus far can broadly be divided into those that inhibit the activation and function of antigen-presenting cells, the production of inhibitory cytokines that act directly on T cells, disruption of effector T cell responses through deprivation of key cytokines or metabolites, and even direct cytolysis of target cells. Although these mechanisms have been reviewed extensively elsewhere (120, 121), we will briefly touch on some of these as they relate to tissue- and inflammation-specific Treg cell functions.

That Treg cells function differently in different tissue sites is best exemplified by the fact that Treg cells in lymphoid and non-lymphoid organs seem to use distinct regulatory mechanisms that can differentially inhibit T cell priming or effector function. For example, deletion of IL-10 specifically in Treg cells results in development of spontaneous colitis, as well as exaggerated immune responses in skin and lung (122). In contrast, Treg-specific deletion of CTLA-4 results in systemic autoimmunity associated with dysregulated activation of T cells in secondary lymphoid tissues and lymhoproliferation (123). Indeed, one key mechanism by which Treg cells blunt T cell responses is by regulating DC abundance (124, 125), and by maintaining DCs in a less stimulatory state by CTLA-4-mediated stripping of the co-stimulatory ligands, CD80 and CD86 (123, 126). Analysis of Treg cell behavior in secondary lymphoid tissues showed that they serially interact with DCs, and that this in turn inhibited stable contacts between DCs and naïve $CD4^+$ T cells, preventing their activation and priming (127, 128). It is therefore intriguing to speculate that Treg production of IL-10 is a major mechanism by which these cells regulate inflammation at environmental interfaces, whereas CTLA-4-dependent regulation of DC function is a regulatory mechanism that predominates in secondary lymphoid tissues where it controls the initial activation and expansion of naïve autoreactive T cells. Accordingly, although CTLA-4 is expressed by most Treg cells, production of IL-10 is limited to effector Treg cells that upregulate expression of the transcription factor Blimp-1 upon activation (70). That IL-10 production is dramatically enriched in human Treg cells that phenotypically resemble Th1 and Th17 cells further suggests that IL-10 is particularly important for regulation of these types of inflammatory responses (68).

In addition to inhibition of DC function and production of immunoregulatory cytokines such as IL-10, TGF-β, and IL-35, Treg cells can function by limiting the availability of key metabolites and cytokines to effector T cells. This can occur indirectly, as Treg cells promote expression of indolamine 2,3-dioxygenase (IDO) by DCs. IDO is a potent regulatory molecule, which catabolizes tryptophan, reducing the availability of this important amino acid and in the process producing kynurenine, an endogenous ligand for the aryl hydrocarbon receptor that can dampen effector T cell differentiation (129, 130). Additionally, production of adenosine by Treg cells due to their expression of the ectoenzymes CD39/CD73 contributes to their suppressive function *in vitro* and *in vivo* (131). Finally, due to their constitutive expression of the high-affinity IL-2 receptor, Treg cells have been thought to function in part by sequestering IL-2 from responding $CD4^+$ and $CD8^+$ T cells. However, by controlling the concentration of available IL-2, Treg cells can actually promote the generation of certain types of pro-inflammatory effector cells. For instance, IL-2 signaling via Stat5 potently inhibits Th17 cell development (132), and therefore by limiting IL-2 availability Treg cells can actually promote Th17 cell differentiation and immune responses to infection with the fungal pathogen *Candida albicans* (133, 134). Similarly, IL-2 signaling limits Tfh differentiation, and Treg cells are required for efficient Tfh development and germinal center responses during influenza infection (135). Thus, rather than being strictly immunosuppressive, by influencing the immune environment Treg cells can contribute to efficient pathogen clearance and memory formation.

A hallmark of the adaptive immune system is its ability in healthy individuals to mount robust responses to invading pathogens and dangerous toxins without causing excessive tissue damage or development of autoimmunity. Despite the insights into the various immunosuppressive mechanisms employed by Treg cells, a key unresolved question is how Treg cells suppress responses in such an antigen-specific way and are capable of discriminating between beneficial and harmful immune responses. Several lines of evidence indicate that a sizable population of functionally competent T cells capable of causing autoimmunity is actively suppressed by Treg cells. For instance, transfer of Treg cell-depleted naïve T cells into lymphopenic mice rapidly causes colitis and wasting disease (136). Additionally, depletion of Treg cells in adult mice results in the rapid activation of $CD4^+$ effector T cells and development of severe autoinflammatory disease within ~10 days (124). Together, these data demonstrate that potentially harmful cells are present in the normal T cell repertoire, and that Treg cells do not permanently inactivate all autoreactive cells. Suppression of these cells must be maintained in the face of various infections, tissue damage, and sterile inflammatory responses that require the immune system's attention, raising the question of how these cells are kept in check during induction of strong immune responses to foreign antigens.

As discussed above, suppressing DC activity is an effective strategy for preventing the priming of autoreactive T cells in steady-state conditions. However, Treg cell-mediated suppression

of DCs is quickly overcome during infection as a result of direct pathogen recognition via various pathogen sensing systems such as TLRs (39, 137), through activation by pro-inflammatory cytokines (138), or by "licensing" of DCs via CD40 stimulation from activated T cells (139). Additionally, pro-inflammatory cytokines made during infection such as IL-1, IL-6, IL-12, and type-1 IFNs can subvert Treg cell function either directly (94, 140), or by rendering effector T cells "resistant" to Treg cell-mediated suppression (58, 141), and this is required to generate appropriate anti-pathogen responses. Combined with the extensive tissue damage and release of autoantigens that can accompany infection, this would appear to provide ample opportunity for functionally competent autoreactive T cells to escape Treg cell-mediated suppression and undergo activation/functional differentiation in parallel with pathogen-specific cells. However, despite the fact that infection is believed to trigger autoimmune disease in certain susceptible individuals and animal models, in most cases infections are resolved without development of corresponding autoimmune sequela. This concept is well-illustrated by the demyelinating disease that develops following infection with a neurotropic strain of murine hepatitis virus (MHV). Depletion of Treg cells in this context has little or no effect on the magnitude of the anti-viral immune response or viral clearance, but greatly exacerbates neurological pathology and the activation of myelin-specific T cells (142), indicating that at least in this case Treg cells are selectively modulating the activation and functional differentiation of self-reactive T cells.

The mechanisms by which Treg cells restrict the activation of self-reactive cells while allowing anti-pathogen responses to occur remain poorly understood. The fact that these cells would be expected to encounter either self- or foreign antigen presented by the same populations of APCs and in the same cytokine environment indicates that suppression in this case must be exquisitely antigen-specific. However, most functional mechanisms ascribed to Treg cells (inhibition of DC function, production of immunosuppressive cytokines, IL-2 deprivation, metabolic disruption of effector T cells, etc.) would be expected to operate non-specifically on most T cells in the local area. One possibility that must be considered is that due to their self-reactivity, Treg cells directly compete with other autoreactive cells for access to the limited amount of any given self-antigen presented by DCs in secondary lymphoid tissues (**Figure 1**). In such a competition, Treg cells may have a distinct advantage due to their selection in the thymus based on high-affinity interaction with self-antigen, and their increased expression of adhesion and co-stimulatory receptors such as LFA-1 that promote stable T cell:DC interactions (11). Consistent with this notion, Treg cells could outcompete naïve T cells of the same specificity for access to DCs when co-cultured *in vitro* (143). Although the limited understanding of Treg cell specificity has precluded a comprehensive test of this possibility *in vivo*, it is interesting to note that only Treg cells from male mice can effectively ameliorate autoimmune prostatitis caused by Treg cell depletion due to neonatal thymectomy (144). Conversely, autoimmune oophoritis is most effectively controlled by Treg cells from female mice, particularly those isolated from the tissue-draining lymph nodes (145). Thus, despite the fact that both male and female Treg cells presumably contain specificities

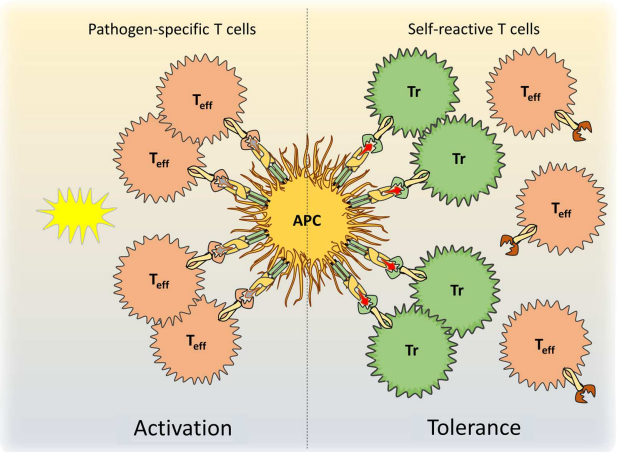

FIGURE 1 | A model for antigen-specific function of Tregs during infection is shown. During pathogen encounter, activated antigen-presenting cells present both self- and pathogen-derived antigens to CD4+ T cells. Whereas pathogen-specific effector T cells are activated (left), competition for access to antigen with Treg cells prevents the activation of autoreactive effector T cells and maintains self-tolerance (right).

for shared and ubiquitously expressed self-antigens present in the prostate and ovaries, these were not sufficient to prevent disease development, indicating a tremendous degree of antigen specificity in these regulatory responses. Additionally, Treg cells with a limited TCR repertoire were unable to ameliorate experimental graft-versus-host-disease as well as those with a more diverse repertoire, and this may reflect a decreased ability to compete with effector cells for access to alloantigen. Finally, anti-CD3 therapy for autoimmunity may work in part by allowing small populations of Treg cells that are "constrained" to specific TCR-dependent niches to expand non-specifically, potentially allowing them to better compete for autoantigen with effector T cells (146).

TISSUE-SUPPORT FUNCTIONS OF Treg CELLS

Activated tissue-resident Treg have been found in multiple tissues such as skin, gut, lung, liver, solid tumors, muscle, and visceral adipose tissue (VAT). As discussed, these tissue Treg cells have altered phenotypes, distinct TCR repertoires, and function differently than Treg cells from lymphoid organs (44, 55, 147, 148). Additionally, some of these Treg cell populations may fulfill tissue-specific functions that are not directly related to their immune functions, and this was recently reviewed by some of the driving researchers in this field (149). For example, VAT Treg cells are a well-characterized population, which were found to specifically express peroxisome proliferator-activated receptor (PPAR)-γ. PPAR-γ, which is considered to be a master regulator of adipocyte differentiation, was recently reported to be a crucial molecule in VAT Treg cell accumulation, phenotype, and function. Mice lacking PPAR-γ specifically in Treg cells showed reduced Treg cell numbers specifically in VAT and PPAR-γ expression by VAT Treg cells was necessary for complete restoration of insulin sensitivity in obese mice (148). Similarly, muscle Treg cells express the growth factor amphiregulin, which acts directly on muscle satellite cells *in vitro* and improves

muscle repair *in vivo* (147). However, in the existing models of tissue homeostasis, it has been difficult to test whether expression of a tissue-specific factor such as PPAR-γ or amphiregulin constitutes a specialized state of the Treg to meet the specific needs of the tissue, or whether it is required for the maintenance of Treg in the tissue. A reduction in Treg numbers in the tissue would likely result in prolonged inflammation, which itself could impair the elaboration of normal tissue-repair mechanisms. The concept of specific tissue-support roles of Treg cells that operate independent of their anti-inflammatory functions can definitively be tested by deletion of the respective genes in Treg cells, or by uncoupling Treg removal from inflammation. One way to achieve the latter would be to perform tissue-repair assays in RAG-deficient mice in which Treg deficiency would not cause inflammation due to the absence of effector T cells. This approach would conclusively answer how much of the observed tissue-support functions of Treg cells are due to their ability to control inflammatory responses that impair normal tissue homeostasis.

REGULATORY MEMORY

The concept of regulatory memory has emerged in recent years, as multiple studies have demonstrated that the regulatory arm of the immune system can provide immunoprotection to transiently encountered antigens (55, 150). Following expression of a neo-tissue-antigen, antigen-specific Tregs become activated and recruited to the target tissue. After preventing or resolving the primary inflammation, these activated Treg reside in the tissue even in the absence of antigen and upon re-encounter of the same antigen they suppress a secondary inflammatory response. Importantly, they do this more efficiently than during the primary encounter displaying similarity to typical tissue-resident effector memory T cells. These Treg cells that reside in the tissues have been termed memory Treg (mTreg) cells. mTreg cells have been described in murine skin where they control autoimmunity in response to inducible antigen expression (55), and in following allogeneic pregnancy (150). Indeed, successful pregnancy requires the activity of maternal Treg cells specific for fetal allo-antigens. These fetal-specific maternal Treg cells develop as pTreg cells during pregnancy and persist at elevated levels after delivery. These persistent Treg cells maintain tolerance to pre-encountered fetal antigen and rapidly re-accumulate during subsequent pregnancy rendering the secondary pregnancy more resilient to inflammatory insults. However, it is important to point out that although in the transgenic system (in which antigen expression could be turned on and off pharmacologically) it is clear that mTreg cell maintenance was antigen-independent (55), in the case of allo-specific fetal tolerance one cannot exclude the possibility that persistent antigen [e.g., microchimerism (151)] is responsible for the maintenance of Treg rather than true antigen-independent memory. Clarification of this point will be crucial before fetal-specific mTreg can be exploited therapeutically.

The discovery of memory Treg raises some obvious questions: what are the evolutionary target-antigens of mTreg cells? In other words, which antigens are expressed/present intermittently and thus require regulatory memory to last between exposures? Although most self-antigens are likely persistently expressed, some, such as proteins in female breast milk, pregnancy-related antigens, and fetal antigens are encountered intermittently. In these cases, the initial expression of the neo-self-antigen or fetal antigens could recruit antigen-specific Treg cells to the respective tissue (i.e., breast, uterus) to then dampen any inflammation upon re-encounter of the antigen in the tissue. This mechanism would increase the success of subsequent pregnancies. Thus, one could speculate that mTreg-specific for intermittently expressed antigens are a mechanism devised to face the challenges and changes that accompany sexual reproduction in mammals. In this way, mTreg are similar to pTreg, which seem to have evolved to mitigate the maternal–fetal conflict (152). Subsequently, both regulatory cell types may evolutionarily have been adapted to mediate microbiota-specific tolerance (33). Microbial antigens present at body surfaces may also be antigens we are only exposed to intermittently depending on changes in the respective flora and on breaches of the epithelial surface of skin or gut that result in increased release of microbial antigens. Thus, mTreg cells may be a useful mechanism to avoid inflammation in response to spikes in exposure to normal microbial flora at body surfaces. Other examples of intermittent antigen-exposure that may require regulatory memory are food antigens and allergens that the gut and skin are exposed to. The existence of allergen-specific mTreg in healthy individuals has not been formally shown but the success of allergen-specific immunotherapy relies on the induction of specific Treg cells that persist over long periods of time (153).

Which tissues/situations are amenable to the induction and maintenance of mTreg? Organs with environmental surfaces such as the skin, gut, and lungs have the highest likelihood of a barrier breach, and therefore, one might hypothesize that these organs have a battalion of self-reactive (and/or microbiota-specific?) mTreg cells positioned to prevent excessive inflammation and tissue damage in case of barrier breach. Indeed, this may have driven the ability of certain epithelial tissues to support mTreg cell maintenance (discussed further below). Additionally, it is possible that the regenerative capacity of a tissue is crucial for the development of mTreg cells. mTreg cells only make sense in tissues that can recover after inflammatory damage. Relatively, non-regenerative tissues such as the pancreas are perhaps less likely to harbor mTreg cells since the pancreatic islets are destroyed in the inflammatory response in type-1 diabetes and regulatory memory would not serve subsequent organ protection. In this context, it is possible that tissue stem cells instruct regulatory memory formation to allow faster regeneration in future inflammatory settings. Indeed, the immunomodulatory potential of stem cells, and in particular mesenchymal stem cells (MSCs), has been studied extensively in recent years. MSCs are pluripotent cells that are present in multiple tissues, including bone marrow, adipose tissue, skin, muscle, blood, and placenta (154). MSCs were shown to induce Treg cells *in vitro* via their production of prostaglandin E(2) and TGF-β (155). Additionally, they modulate their environment by secretion of mediators such as IDO and IL-10. Due to their immunomodulatory functions, numerous clinical studies using MSCs are currently underway to treat inflammatory diseases such as graft-versus-host disease and autoimmunity (156). Stem cells have potentially evolved their ability to induce Treg cells because they seem to require them for their maintenance. For instance, Treg cells are attracted to the bone marrow by the stem cell chemoattractant

CXCL12 (SDF-1) (157). This localization to the niche was crucial for the preservation of the hematopoietic stem-cell niche in the bone marrow as Treg cell depletion resulted in a loss of allohematopoietic stem cells (158). Thus, Treg cells (and specifically mTreg cells) are potentially involved in preserving stem-cell niches from immune attacks and this may be one way in which they provide critical tissue-support functions.

CONTROL OF Treg CELL MAINTENANCE

Due to their potent immunosuppressive function, manipulation of Treg cell abundance is an attractive therapeutic strategy to either boost or inhibit immune responses in a variety of clinical settings (159). However, competition for growth and survival factors acts to limit the size of the Treg cell pool *in vivo*, and as a result clinical trials of adoptive Treg cell therapy have failed to achieve long-term cell engraftment or substantial clinical benefit (79). Although work over the last 10 years has defined several factors that help regulate Treg cell homeostasis, an integrated model of how Treg cell abundance, function, and distribution is controlled during normal and pathological immune responses is still lacking. A better understanding of the mechanisms regulating the abundance of different Treg cell populations is crucial for developing therapies to boost their activity to treat autoimmunity and prevent graft rejection, or to inhibit Treg cells in the contexts of cancer and chronic infection.

In conventional $CD8^+$ and $CD4^+Foxp3^+$ effector T cells, it has become clear that different populations of naïve, effector, and memory T cells have distinct homeostatic requirements, and that this helps preserve the functional diversity of effector and memory T cells while ensuring that an adequate pool of naïve T cells is maintained in order to respond to new threats (160). This is in large part due to changes in the requirements that these cells have for different cytokines that signal through receptors utilizing the γ_c receptor subunit such as IL-2, IL-7, and IL-15. That Treg cells occupied their own homeostatic niches was apparent from early experiments in which Treg cells underwent robust population expansion and ameliorated autoimmune disease development when transferred into Foxp3 mutant mice lacking endogenous Treg cells (9). A similar niche-filling capacity of Treg cells is observed when Treg cells are acutely depleted (161). That Treg cells could be sub-divided into populations with different homeostatic behaviors (and therefore likely subject to distinct sets of proliferative and survival signals) has been appreciated for some time (82). However, the precise nature of these homeostatic niches remains poorly understood.

Consistent with their constitutive expression of the high-affinity IL-2 receptor component CD25, it has become clear that IL-2 plays a central role in Treg cell function and homeostasis. Accordingly, defects in IL-2 or various components of the IL-2 receptor lead to development of autoimmune/inflammatory diseases associated with Treg cell dysfunction. Treg cells themselves do not produce IL-2, and instead are stimulated in a paracrine fashion by IL-2 produced by activated conventional T cells (162). Through regulation of anti-apoptotic proteins such as Bcl-2 and Mcl-1, IL-2 can deliver potent survival signals to Treg cells (161, 163). Additionally, IL-2 can potently drive Treg cell proliferation, especially when present in excess during niche-filling or when administered as super-agonistic IL-2/α-IL-2 immune complexes (161, 164). Paradoxically, after the identification of Foxp3 as a molecular marker of Treg cells it became apparent that the numerical deficiency in peripheral Treg cells in peripheral tissues in the absence of IL-2 signaling is relatively mild (165, 166), and correcting these deficiencies by knocking out the pro-apoptotic factor Bim failed to restore full Treg cell function in IL-2-deficient mice (167). Taken together, these data indicate that maintenance of at least some Treg cell populations is IL-2-independent, and that the effects of IL-2 on Treg cell function *in vivo* are more qualitative rather than quantitative. Indeed, a recent analysis of IL-2 signaling in Treg cells demonstrated that rather than acting as a trophic factor for all Treg cells, IL-2 signaling *in vivo* is largely restricted to "central" Treg cells that access sites of paracrine IL-2 production in the secondary lymphoid tissues via expression of the chemokine receptor CCR7 (25). This quiescent population of Treg cells was particularly sensitive to genetic or antibody-mediated blockade of IL-2 signaling, whereas rapidly proliferating "effector" Treg cells were effectively maintained in the absence of IL-2. IL-2 also promotes specific effector functions in Treg cells such as expression of CTLA-4 (167). As CTLA-4 expression by Treg cells in secondary lymphoid organs help prevent the initial activation and differentiation of autoreactive cells, selective loss of these central Treg cells helps explain why autoimmunity develops in the absence of IL-2 or CD25 despite the presence of effector Treg cells with at least some functional capacity.

In contrast to the IL-2-dependent central Treg cells, the abundance of $CCR7^-$ effector Treg cells is most profoundly influenced by signals through the TCR and associated co-stimulatory receptors such as CD28 and ICOS. That effector Treg cells compete for access to these signals is indicated by the fact that abundance of these cells is intimately linked to the number of antigen-presenting DCs (125). Moreover, the fact that DC-mediated Treg cell population expansion occurred even when IL-2 signaling was blocked indicates that signals through either the TCR or IL-2 act in separate pathways to control Treg cell abundance (25, 168). Consistent with this, although IL-2 signaling was not associated with Treg cell proliferation in central Treg cells, the high rate of homeostatic proliferation of effector Treg cells was completely dependent on continued TCR signaling (168). Moreover, effector Treg cells have a $CD25^{lo}Bcl\text{-}2^{lo}Mcl\text{-}1^{lo}$ phenotype indicative of IL-2 deprivation, and accordingly are highly apoptotic. Thus, after losing access to IL-2-dependent survival signals in secondary lymphoid tissues, effector Treg cells in non-lymphoid organs appear to balance rapid TCR-dependent cell proliferation with a high-rate of apoptotic cell death to maintain their steady-state abundance. In tissues such as the intestines, this creates a largely self-renewing Treg cell pool specific for local antigens that are effectively maintained despite low levels of cellular immigration (25).

Among the co-stimulatory receptors, loss of CD28 has the most dramatic impact of Treg cell abundance (169). However, this may be largely due to defective Treg cell development in the thymus as deleting CD28 specifically in Treg cells after their development did not recapitulate this phenotype, although the CD28-deficient Treg cells were functionally impaired (170). However, blockade of ICOS signaling causes a rapid decline in the abundance of effector Treg cells *in vivo* (25), and this can accelerate development of organ-specific autoimmune disease (171). Interestingly, this was

not associated with defects in effector Treg cell proliferation, indicating that ICOS signaling may regulate effector Treg cell survival, perhaps through engagement of the PI3K/Akt signaling pathway.

The dependence of effector Treg cells on TCR and co-stimulatory signals, and their competition for access to DCs raises the possibility that effector Treg cells exist in multiple TCR-dependent "micro-niches" as was recently described for conventional $CD4^+$ T cells (92). In this scenario, Treg cells specific for any given autoantigen must compete with one another for access to antigen-bearing DCs, thereby linking the abundance of any given Treg cell specificity to the amount of autoantigen presented, and ensuring that a diverse TCR repertoire is maintained in effector Treg cells. Indeed, this is consistent with the data demonstrating that particular TCR specificities are enriched in specific tissue sites (33, 53).

Unlike effector Treg cells that appear to depend on continued TCR and co-stimulatory signals for their maintenance, memory Treg cells can reside in non-lymphoid tissues such as the skin for extended periods in the absence of continued antigen-receptor signaling, raising the question of how these populations are maintained (44, 59). Additionally, memory Treg cells displayed a high-rate of homeostatic proliferation even after antigen withdrawal (59). The continued proliferation and thus maintenance of memory Treg cells may be a consequence of their not requiring many of the signals thought to be essential for the responses of effector T cells, such as Akt and mTOR and becoming relatively independent of TCR-signals after initial activation (172). Additionally, in the absence of the continued TCR and co-stimulatory receptor signals that maintain effector Treg cells, it is likely that memory Treg cells rely instead on specific cytokine signals for their homeostatic maintenance. Surprisingly, although IL-2 was required for the development of memory Treg cells from naïve precursors in the secondary lymphoid tissues, memory Treg cells in the skin showed decreased CD25 expression and maintenance of these cutaneous cells was IL-2-independent. However, IL-7 receptor expression was dramatically upregulated on these cells, and blockade of IL-7R signaling resulted in the loss of memory Treg cells in the skin but not the skin-draining lymph node (49).

That maintenance of mTreg cells in the skin is IL-7-dependent raises the important question of how expression of IL-7R is regulated in these cells in such a tissue-specific manner. Expression of IL-7R in conventional T cells is controlled in large part by the transcription factor, Foxo1, which in T cells is inactivated and removed from the nucleus after phosphorylation by activated Akt following TCR stimulation (173). However, despite the fact that Treg cells rely on continued Foxo1 activity for their suppressive function, most Treg cells in secondary lymphoid organs express low levels of IL-7R (49, 174). This prevents Treg cells from competing with conventional naïve and memory T cells for access to IL-7 produced by stromal cells in these tissues, and implies that IL-7R expression is differentially regulated in conventional T cells and Treg cells. By contrast, antigen-specific Treg cells in murine skin uniformly expressed high levels of IL-7R both in the presence or absence of antigen expression (Iris K. Gratz, unpublished observations). Although the tissue-specific signals directing IL-7R expression by Treg cells in the skin have not been identified, this results in the maintenance of a stable population of tissue-resident mTreg cells even in the absence of continued antigen or IL-2. However, the importance of IL-7 in the maintenance of memory Treg cells in tissues other than the skin has not been examined. For instance, due to their expression of CD122 and the γc chain, Treg cells are equipped to respond to IL-15 trans-presented on the surface of IL-15/IL-15Rα expressing cells, and rather than IL-7, this may help maintain mTreg cells in tissues rich in IL-15 such as the intestine.

Collectively, these recent data support the concept that rather than occupying a single homeostatic niche, multiple pathways of homeostatic maintenance exist for distinct populations of Treg cells in different tissue sites. These include IL-2-dependent maintenance of central Treg cells in secondary lymphoid organs, TCR/ICOS-dependent maintenance of effector Treg cells in inflamed non-lymphoid tissues, and IL-7-dependent maintenance of memory Treg cells in the skin (**Figure 2**).

CLINICAL IMPLICATIONS

Therapies to prevent allograft rejection or treat autoimmune diseases have long relied on general immunosuppression using broadly acting and non-specific medications. Treg cells represent a promising new avenue with the possibility of long-lived and antigen-specific tolerance to self- or foreign-antigens. Reported clinical trials have focused on the expansion of Treg cells *in vivo* and *ex vivo* (175). Applications of both polyclonally expanded Treg-populations and antigen-specific Treg cells are currently moving into the clinic, and results have thus far shown acceptable safety and promising efficacy of the treatment (176). Conversely, inhibition of Treg cell function may enhance immunotherapy to cancer, and help promote resolution of chronic infection.

Resolution of the particular issues of Treg cell biology raised in the above will certainly help in the targeted development of Treg cell-based therapies. For instance, defining Treg cell specificity would allow for a more precise targeting of Treg cell-based therapies to the most appropriate antigens. Many antigens that are targeted by effector cells in autoimmune inflammatory diseases have been defined [e.g., BP180 in bullous pemphigoid (177), desmoglein 3 in pemphigus vulgaris (178), and insulin and other antigens in type-1 diabetes (179)]. Additionally, there is considerable hope that allo-antigen-specific Treg cells will show superior suppressive function compared to polyclonal Treg cells in preventing transplant rejection and graft-versus-host disease. By identifying appropriate antigens, antigen-specific Treg cells could be expanded *ex vivo* and adoptively transferred. Upon interaction with tissue DCs *in vivo*, these Treg cells would likely acquire a tissue-tropic chemokine receptor phenotype and migrate to the same target tissues as their effector T cell counterparts. However, clinical trials with antigen-specific *ex vivo* expanded Treg cells are just starting (180) and it will be crucial to analyze migratory patterns, maintenance in tissues, and suppressive function of these Treg cells.

In addition to controlling their specificity, the identification of tissue- and inflammation-specific Treg cells subsets implies that targeting the "correct" Treg cell population will be critical for effective Treg cell-based immunotherapy. One can envision exposing Treg cells *in vitro* to defined cytokine- and co-stimulatory conditions to induce the expression of specific homing receptors and functional modules with the goal to guide them to the

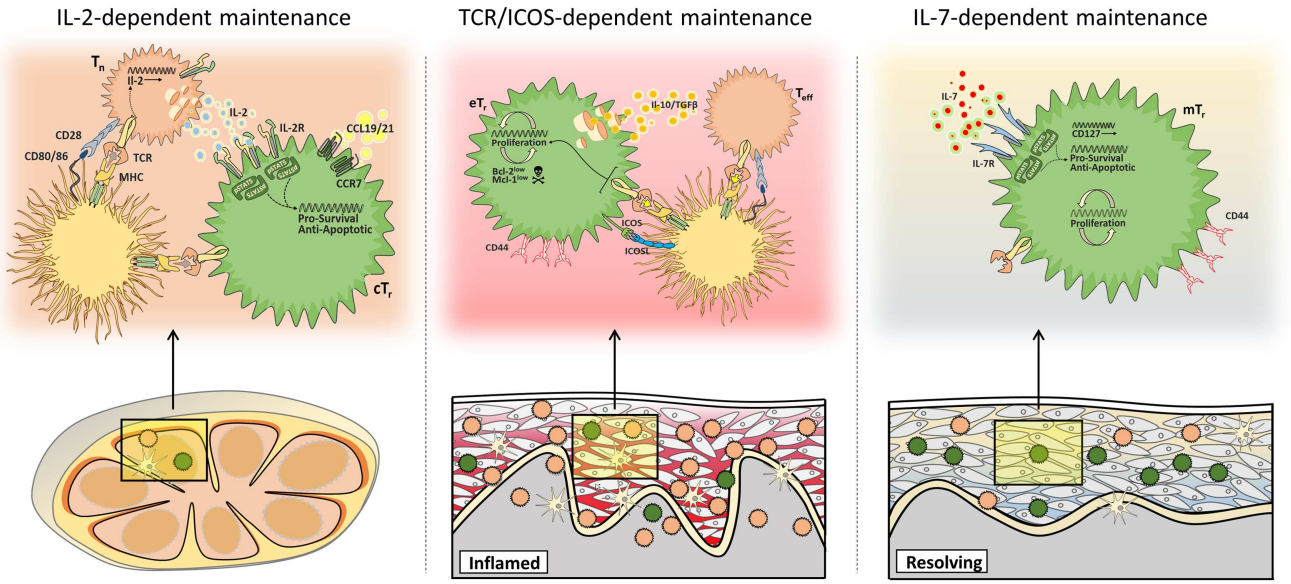

FIGURE 2 | Multiple mechanisms of Treg cell maintenance. Different populations of Treg cells are subject to distinct homeostatic constraints. Central Treg cells (cT_r) access paracrine IL-2 in secondary lymphoid tissues (left), whereas maintenance of effector Treg cells (eT_r) in non-lymphoid tissues depends on continued TCR/ICOS signals (middle), and memory Treg cells (mT_r) in the skin are supported by IL-7/IL-7R-mediated survival signals (right).

appropriate target tissue and hone their suppressive mechanisms. These applications of *ex vivo* expanded Treg cells will benefit tremendously from a better understanding of the development of tissue- and inflammation-specific Treg cell populations, and the control of the immunosuppressive mechanisms they employ. The end results of these efforts to better target Treg cells would include not only increased therapeutic efficacy but also a simultaneous decrease in unwanted off-target effects that could be envisioned upon Treg cell transfer (e.g., generalized immunosuppression or increased risk of tumor development).

A major advantage of adoptive Treg cell therapy is its potential for long-lasting effects without the need to persistently treat with immunosuppressive drugs. However, current applications have struggled with instability and loss of Treg cells after transfer. Therefore, identifying the factors that govern Treg maintenance will not only allow for better survival of transferred Treg cells but will also open the door to new therapies aimed at manipulating (both positively and negatively) the abundance of endogenous Treg cells in different tissue sites for treating autoimmunity, promoting transplantation tolerance, enhancing cancer immunotherapy, or resolving chronic infection. Additionally, the identification of key functional mechanisms and molecules that support Treg cell maintenance and function in specific tissue sites will have a tremendous impact on development of immunotherapies. In this regard, a recent study indicating that the surface molecule neuropilin-1 is essential for Treg cell maintenance and function in tumor environments, but not in other tissue sites, is particularly promising for efforts to inhibit Treg cells as an adjunct cancer immunotherapy (181).

The various developmental stages of Treg cells have only just begun to be defined and understood. For most clinically relevant inflammatory settings, it is not known which stage, naïve, central, effector, or memory (or subtypes of these), is most suitable for therapeutic interventions. However, due to their presumed stability and antigen-independent maintenance, memory Treg cells seem ideally suited to mediate long-term immunoregulatory benefits. However, memory Treg cells have only been described in the skin and the uterus and it is an open question whether they can be found in other target organs and whether their requirements for maintenance differ in different tissue sites. Additionally, better defining the developmental relationship between these different Treg cell populations could provide new insights into how to best promote the generation of immunoprotective Treg cells.

The manipulation of Treg cells to alter the outcome of inflammatory responses is the most obvious translational application of our increasing knowledge of Treg cell biology. However, the recent studies indicating that Treg cells can have specialized tissue-support functions that may lead to a broader range of Treg cell applications. Defining tissue-support functions could yield better therapies for wound healing/tissue-regeneration, metabolic regulation, and potentially other tissue-specific functions. However, more studies on how tissue-resident Treg cells differ from each other and their counterparts found in secondary lymphoid organs are required before we can attempt to therapeutically use and manipulate these populations. Importantly, basic research in this and other key areas of Treg cell biology highlighted in this review will continue in an iterative process with clinical trials, each informing the other as the therapeutic potential of Treg cells is fully realized.

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Impact of inflammatory cytokines on effector and memory CD8+ T cells

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Inflammatory cytokines have long been recognized to produce potent APCs to elicit robust T cell responses for protective immunity. The impact of inflammatory cytokine signaling directly on T cells, however, has only recently been appreciated. Although much remains to be learned, the CD8 T cell field has made considerable strides in understanding the effects of inflammatory cytokines throughout the CD8 T cell response. Key findings first identified IL-12 and type I interferons as "signal 3" cytokines, emphasizing their importance in generating optimal CD8 T cell responses. Separate investigations revealed another inflammatory cytokine, IL-15, to play a critical role in memory CD8 T cell maintenance. These early studies highlighted potential regulators of CD8 T cells, but were unable to provide mechanistic insight into how these inflammatory cytokines enhanced CD8 T cell-mediated immunity. Here, we describe the mechanistic advances that have been made in our lab regarding the role of "signal 3" cytokines and IL-15 in optimizing effector and memory CD8 T cell number and function. Furthermore, we assess initial progress on the role of cytokines, such as TGF-β, in generation of recently described resident memory CD8 T cell populations.

Keywords: signal 3, cytokines, effector, resident memory, memory, CD8 T cells

INTRODUCTION

Naïve CD8 T cells undergo activation when presented with their cognate antigen following a three-signal model. Professional antigen-presenting cells (APCs) provide the crucial first and second signals through the T cell receptor (TCR) and costimulatory molecules, while innate immune cells contribute inflammatory cytokines to promote optimal accumulation and differentiation of effector CD8 T cells (1). Although the role of inflammatory cytokines in maturing professional APCs to stimulate robust T cell responses has been well described (2), investigation of their direct effect on T cells is ongoing. In the following review, we outline mechanistic studies identified for inflammatory cytokine regulation of various stages of the CD8 T cell response and discuss cutting edge research on the cytokine requirements for generation of the novel resident memory T cell (T_{RM}) population.

SIGNAL 3 CYTOKINES AND THE MAGNITUDE OF THE EFFECTOR CD8 T CELL RESPONSE

Initial studies suggesting that cytokines, particularly IL-12, may be important for signaling directly to T cells were made in *in vitro* cultures of T cells and artificial APCs more than a decade ago. Since then, the importance of IL-12 and type I interferon signaling directly to CD8 T cells for optimal effector cell accumulation has been demonstrated both *in vitro* and *in vivo* (3–7). Despite the clear impact of IL-12 and IFNα/β on effector CD8 T cell numbers, it remained unclear how inflammatory cytokines regulated the magnitude of effector CD8 T cell responses. Although several other cytokines have been discussed in the literature recently for T cell differentiation (8, 9); here, we will focus on signal 3 cytokines as originally defined for their role in T cell accumulation (3–7). After their classification as signal 3 cytokines, IL-12 and type I interferons were proposed to enhance accumulation of CD8 T cells following one of two models: via greater survival (2, 10) or by conferring an early proliferative advantage (11, 12). The model for enhanced survival stemmed from 3 days culture experiments, which demonstrated accumulations of cells in cultures containing IL-12 with no detectable changes in cell division. The latter model was supported also by *in vitro* studies, where IL-12 transiently increased expression of CD25, the high affinity IL-2 receptor, peaking at day 2 (11). Hence, previous reports addressing the mechanism by which signal 3 cytokines allow optimal accumulation of effector CD8 T cells were limited to short-term *in vitro* experiments with no clear answer to the question of whether survival or early proliferation, or both, contribute to the magnitude of the CD8 T cell response. Furthermore, the temporal disconnect between signal 3 cytokine-driven CD25 expression and optimal accumulation of effector CD8 T cells many days later has not been assessed (13). Here, we describe a recent study from our lab addressing these knowledge gaps concerning the mechanism by which signal 3 cytokines allow optimal accumulation of effector CD8 T cells *in vivo*.

Utilizing an OT-I T cell adoptive transfer system followed by DC-OVA priming with or without the TLR9 agonist, CpG, to induce signal 3 cytokines, Starbeck-Miller et al. compared CD8 T cells activated *in vivo* in the presence or absence of signal 3 cytokines (14). Gene expression profiling of T cells from these groups at D7 post immunization clearly showed that signal 3 cytokines enhanced transcription of proliferation, but not

anti-apoptosis-associated genes (14). Additionally, analysis of CD8 T cells primed by DC with or without signal 3 showed no differences in proliferation or total cells numbers as late as day 5 post immunization. Thus, the *in vivo* data do not support either of the proposed models for signal 3 activity. Interestingly, both DC and DC + CpG OT-I cells isolated on D4 and moved into *in vitro* cultures failed to divide, although transfer of the same populations to an *in vivo* host revealed more robust proliferation from the CD8 T cells that had been exposed to signal 3 cytokines. This suggested that signal 3 cytokines established a proliferation program, but sustained proliferation required an additional component that was present in a naïve host. Since IL-2 is an important driver of T cell accumulation, Starbeck-Miller et al. monitored expression of the high affinity IL-2 receptor, CD25, on DC versus DC + CpG CD8 T cells. Indeed, IL-12 and type I interferon sustained CD25 expression, allowing for greater IL-2-induced proliferation via activation of the PI3K pathway and expression of FoxM1, a positive cell cycle gene regulator. Importantly, administering the IL-2 neutralizing antibody JES6 from D4-6 removed the proliferative advantage conferred by signal 3 cytokines. Thus, these studies verify, and add mechanistic insight to the model, indicating that signal 3 cytokines neither enhance survival not provide and early proliferative advantage, but rather sustain expression of the high affinity IL-2 receptor, which extends the duration of proliferation after immunization and permits optimal generation of effector CD8 T cells *in vivo.* Interestingly, the effects of IL-12 and type I interferons are not limited to promoting optimal CD8 T cell accumulation, but offer functional advantages to effector CD8 T cells, such as antigen sensitivity, which will be discussed next.

DYNAMIC REGULATION OF ANTIGEN SENSITIVITY BY INFLAMMATORY CYTOKINES

The protective capacity of CD8 T cells depends on their quantity, functional properties, and anatomical distribution (15). High antigen sensitivity, otherwise referred to as functional avidity, strongly correlates with protective immunity against intracellular pathogens (16). Although T cells cannot directly alter the binding affinity of their TCR through processes like somatic hypermutation, it has been shown that monoclonal TCR-transgenic CD8 T cells can increase their functional avidity from early to late effector time points (17). This study suggested that the functional avidity maturation was a fixed property of CD8 T cells. Here, we describe a mechanistic study demonstrating that inflammatory cytokines directly enhance antigen sensitivity of effector and memory CD8 T cells, however this enhanced sensitivity is not hardwired, but rather tuned by the pathogen-specific milieu.

Using a similar DC immunization protocol as indicated previously, Richer et al. activated OT-I CD8 T cells in the presence or absence of signal 3 cytokines (18). Distinct inflammatory milieu were then initiated by co-infection of DC primed mice with *Listeria monocytogenes* (Lm) or lymphocytic choriomeningitis virus (LCMV) and antigen sensitivity was assessed at day 5 after priming. Strikingly, DC-OVA with LCMV infection substantially enhanced antigen sensitivity by more than 10-fold whereas co-infection with Lm enhanced antigen-sensitivity four to six-fold. To determine whether inflammation increased functional avidity via enhanced TCR signaling, Richer et al. isolated OT-I T cells from DC and DC + LCMV mice on D4 and analyzed phosphorylation of downstream TCR signals after TCR ligation (18). Indeed, inflammatory cytokines dramatically enhanced phosphorylation of ZAP-70, PLCgamma, and ERK1/2 in response to TCR stimulation. Importantly, greater ERK1/2 phosphorylation was not observed with PMA stimulation, which bypasses proximal TCR signals, suggesting that inflammatory cytokines increased the antigen sensitivity of the TCR by enhancing proximal TCR signaling. Consistent with the data from effector CD8 T cells, inflammatory cytokines also increased the antigen sensitivity of memory CD8 T cells by enhancing TCR proximal signaling, albeit to a lesser degree than observed with effector CD8 T cells. This study demonstrated how the pathogen-specific inflammatory milieu affects antigen-sensitivity, an essential functional aspect of both effector and memory CD8 T cells. In addition to signal 3 cytokine effects on memory CD8 T cells, we next review a novel role for IL-15 in memory CD8 T cell trafficking.

IL-15-DEPENDENT SYNTHESIS OF SELECTIN LIGANDS

Numerous studies have described the functional differences between memory and naïve CD8 T cells (13, 19). Among such reports, it was demonstrated that memory, but not naïve, CD8 T cells can be rapidly recruited to inflamed lungs in an antigen-independent manner (20). Importantly, this large influx of memory CD8 T cells was shown to provide immediate cytolytic killing against pathogens expressing cognate antigen (21). Although this non-specific recruitment of memory CD8 T cells was shown to depend on CCR5 expression, the molecular mechanisms initiating early "tethering and rolling" events before chemokine recognition by memory CD8 T cells detection remained undefined.

Immune cell homing is a highly regulated process that begins with selectin family proteins. Leukocytes extravasate into inflamed tissue by constructing ligands to P- and E-selectin, which are expressed on activated endothelium. In contrast, L-selectin mediates homeostatic trafficking of naïve and central memory CD8 T cells through lymph nodes. Previous reports concerning the synthesis of P- and E-selectin ligands had been limited to *in vitro* models, which suggested TCR activation was essential to express appropriate selectin ligands. Herein, we describe studies from Nolz et al. that show P- and E-selectin ligand synthesis occurs on memory, but not naïve, CD8 T cells following inflammation *in vivo* (22). Utilizing the model pathogen, LCMV, Nolz et al. observed uniform expression of functional P- and E-selectin ligands on effector populations, but that most memory CD8 T cells did not express functional P or E-selectin ligands. After detecting high selectin ligand expression on non-specifically recruited memory P14 CD8 T cells following several irrelevant pathogen infections, it was demonstrated, through use of blocking antibodies to P- and E-selectin or P-selectin glycoprotein ligand-1, that non-specific recruitment of memory CD8 T cells to inflamed sites was dependent on selectin binding. To investigate the mechanism regulating inflammation-induced selectin ligand expression on memory CD8 T cells, Nolz et al. analyzed expression of the *Gcnt1* gene, which prompts their formation on naïve, effector, and memory CD8 T cells. Although effector CD8 T

cells expressed high levels of *Gcnt1*, naïve, and memory CD8 T cells had minimal expression of this protein. Interestingly, recombinant IL-15 substantially enhanced P- and E-selectin ligand synthesis on memory, but not naive CD8 T cells *in vitro* and Nolz et al. revealed a similar induction of the Gcnt1 protein via immunoblot. *In vivo*, IL-15-deficiency significantly reduced expression of selectin ligands, and subsequent memory CD8 T cell trafficking to inflamed sites, suggesting that P- and E-selectin ligand expression occurs in an IL-15/STAT5-dependent, but TCR-independent manner. Importantly, IL-15-driven P- and E-selectin ligand expression was shown to occur in human memory CD8 T cells, demonstrating conserved trafficking pathways between mouse and human T cells that can be manipulated for therapeutic purposes.

Until now, IL-15 has been referred to, principally, as a maintenance cytokine for memory CD8 T cells. This study investigating the role of IL-15 in the regulation of core 2 O-glycan synthesis on memory CD8 T cells suggests the possibility of other unexplored functions of this important inflammatory cytokine.

TGF-β, IL-33, AND TNF REQUIRED FOR RESIDENT MEMORY CD8 T CELLS

Although the CD8 T cell field has established a paradigm of IL-15-driven homeostatic proliferation as the model of memory CD8 T cell maintenance for circulating T cells, localized CD8 T cell populations in the lung (23), gut (24), and skin (25), among other tissues, have been shown to sustain a sizable pool of memory CD8 T cells despite the absence of IL-15 signaling. Most recently, the T_{RM} population has garnered immense interest for their distinct surface phenotype, local protective capacity, and long-term maintenance in the absence of traditional cytokines. Skin and gut infection models to generate transgenic CD8+ T_{RM} populations are well established (26, 27); hence, we describe recent advances in determining the cytokine signals involved for T_{RM} development and maintenance following either immunization or infection.

Resident memory T cell cells represent a novel, non-circulating class of T cells that persist within extralymphoid tissue and demonstrate superior regional immunity (28). The best-characterized T_{RM} cells express the alpha chain of the αEβ7 integrin (CD103), as well as the sphingosine 1 phosphate receptor ($S1PR_1$) inhibitor CD69, in multiple tissue compartments. Relevantly, both molecules are required for the optimal formation and maintenance of T_{RM} cells in the skin (26). Since *in vitro* and some *in vivo* studies have long since shown that transforming growth factor-β (TGF-β) signaling promotes CD103 expression on immune cells (29–31) and that TGF-β is expressed in the skin epithelium, Mackay et al. investigated whether signaling through the TGF-β receptor was required to upregulate CD103 and establish T_{RM} cells *in vivo* (26, 32). Utilizing one to one adoptive transfer models of WT and *Tgfbr2*$^{f/f}$.dLck-Cre (Tgfbr2−/−) OT-I T cells into C57BL/6 mice followed by infection with OVA-expressing HSV, Mackay et al. indeed demonstrated that Tgfbr2−/− OT-I cells failed to upregulate CD103 and had a dramatically reduced ability to form T_{RM}.

By utilizing acute and chronic infections with LCMV, Zhang et al. delved further into the mechanism behind TGF-β signaling for generation and maintenance of T_{RM} cells (27). Creating equal ratio mixtures of WT and Tgfbr2−/− P14 T cells followed by either LCMV-Armstrong (acute) or Clone 13 (Cl13, chronic) infections, Zhang et al. notices defective maintenance of Tgfbr2−/− cells in Armstrong, but not Cl13-infected hosts. While monitoring integrin expression in secondary lymphoid organs, which are the major source for T_{RM} cells, Zhang et al. detected enhanced expression of α4β7 on Tgfbr2−/− cells in Cl13, compared to Armstrong-infected mice (27). As α4β7 aids in the migration to the gut (33, 34), it was concluded that, although Tgfbr2−/− T_{RM} cells are locally declining in both Armstrong and Cl13-infected mice, the more prominent, enhanced expression of α4β7 on splenic Tgfbr2−/− T cells of Cl13-infected hosts allowed for continual replacement and stabilization of T_{RM} numbers. Thus, TGF-β acts as a negative regulator to T_{RM} formation through α4β7 downregulation, but is required for the maintenance of established T_{RM} cells in the gut through induction of CD103 expression.

The above findings clearly identify the relationship between TGF-β and CD103 expression for persistence of T_{RM} cells; however, CD103 is not required in all T_{RM} niches (35–37). Thus, we outline a complementary study, defining the transcriptional regulation of a ubiquitous T_{RM} marker, CD69, to establish T_{RM} cells, where CD103 may be dispensable. The antagonistic relationship between CD69 and $S1PR_1$ are well established (38). The zinc-finger transcription factor KLF2 catalyzes the expression of $S1PR_1$, known to promote lymph node egress (39). Hence, Skon et al. initially uses adoptive transfer models of KLF2-GFP P14 T cells followed by LCMV-Armstrong infection to monitor KLF2 expression in circulating, compared to resident memory CD8 T cells (40). As expected, T_{RM} cells expressed low levels of both KLF2 and $S1PR_1$, while CD69 expression was increased. Interestingly, *in vitro* cytokine screening revealed that a combination of TGF-β, IL-33, and TNF were capable of inducing a modest downregulation of KLF2 expression. To analyze the effect of $S1PR_1$ expression on T_{RM} formation, Skon et al. overexpressed $S1PR_1$ through retroviral transduction of P14 cells, and demonstrated that failure to downregulate $S1PR_1$ prevented the establishment of T_{RM} cells in the salivary gland, kidney, lamina propria, and intestinal epithelium (40). Hence, these studies propose that migration to non-lymphoid tissue enhances exposure of CD8 T cells to TGF-β, IL-33, and TNF, which triggers some loss of KLF2 expression, subsequently decreasing $S1PR_1$, and allowing CD69 upregulation. Although the upregulation of CD69 may be controlled by multiple factors, increasing the complexity of this process, these studies, among others, clearly demonstrate that the non-migratory T_{RM} population has novel cytokine requirements for their generation and maintenance (**Figure 1**) and that this list of cytokines may continue to expand.

SYNOPSIS

In this review, we outline recent studies uncovering the mechanisms by which inflammatory cytokines regulate various attributes of circulating and resident memory CD8 T cell populations. Although investigation of the role of inflammatory cytokines on T_{RM} cells, and T cells in general, remain far from complete, the field

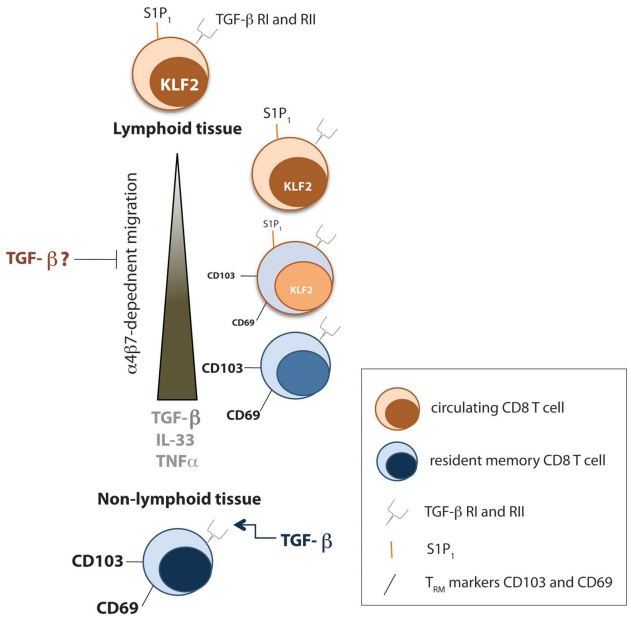

FIGURE 1 | Cytokines involved in TRM formation and maintenance. Early after infection, local TGF-β signals prevent migration of effector CD8 T cells from the spleen to non-lymphoid tissue by downregulating the αEb7 integrin. However, tissue-specific programming during priming of CD8 T cells causes homing to appropriate resident tissue. In addition, the transcription factor KLF2 gets downregulated as effector CD8 T cells travel to non-lymphoid tissue toward a combination of TGF-β, IL-33, and TNFα signals, which causes a decrease in S1P1, allowing upregulation of CD69. Within resident tissue, TGF-β signals sustain TRM numbers.

has made remarkable progress in understanding how the inflammatory environment can directly modulate the number, function, migration, and maintenance of T cells.

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Blowing on embers: commensal microbiota and our immune system

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Vertebrates have co-evolved with microorganisms resulting in a symbiotic relationship, which plays an important role in health and disease. Skin and mucosal surfaces are colonized with a diverse population of commensal microbiota, over 1000 species, outnumbering the host cells by 10-fold. In the past 40 years, studies have built on the idea that commensal microbiota is in constant contact with the host immune system and thus influence immune function. Recent studies, focusing on mutualism in the gut, have shown that commensal microbiota seems to play a critical role in the development and homeostasis of the host immune system. In particular, the gut microbiota appears to direct the organization and maturation of lymphoid tissues and acts both locally and systemically to regulate the recruitment, differentiation, and function of innate and adaptive immune cells. While the pace of research in the area of the mucosal–immune interface has certainly intensified over the last 10 years, we are still in the early days of this field. Illuminating the mechanisms of how gut microbes shape host immunity will enhance our understanding of the causes of immune-mediated pathologies and improve the design of next-generation vaccines. This review discusses the recent advances in this field, focusing on the close relationship between the adaptive immune system and commensal microbiota, a constant and abundant source of foreign antigens.

Keywords: commensal, microbiota, immune system, T cells, regulation

INTRODUCTION

Microorganisms represent the oldest and most ubiquitous forms of life on our planet. These microbes evolved alongside other organisms, like metazoans, with a portion of them colonizing and establishing life-long relationships with larger species. Such mutualistic relationships have been described in plants (1), insects (2), worms (3, 4), fish (5, 6), birds (6), and mammals (7). Shortly after birth, the mammalian mucosal tissues are exposed to the environment and colonized by viruses, bacteria, protozoa, and fungi, resulting in the formation of complex populations of microbes, collectively called the commensal microbiota. The mammalian commensal microbiota constitutes of over 1000 different species of microbes and outnumbers the host cells by 10-fold (8, 9). The intestinal mucosa harbors the largest amount of microbes in the human body; many are beneficial while some, termed pathobionts, are generally harmless, but can turn pathogenic with an imbalance of the microbial community. The commensal microbiota has long been appreciated for its essential contributions to host development and health. Such benefits include metabolism of indigestible food byproducts, generation of nutrients, defense against pathobionts, promotion of angiogenesis and enteric nerve function, maintenance of intestinal structure, and contribution to the development and regulation of the mammalian immune system (10–15).

For better and for worse, the mammalian immune system is one aspect of host physiology that is heavily influenced by commensal microbiota. Both human and animal model research supports the hypothesis that immune-related disorders like inflammatory bowel disease (IBD) (16), cancer (17), diabetes (18, 19), allergies (20), and even obesity (21, 22) may result from dysbiosis of the commensal microbial communities. To better understand how microbiota contributes to the onset and/or exacerbation of such disorders (23, 24), it is important to understand how signals from intestinal microbiota influence the immune system under normal and diseased conditions. This review discusses the effect of gut microbiota on the mammalian immune system, with a particular focus on T cell differentiation, responses, and homeostasis. First, we discuss how the host senses microbes. Then, we review the ability of dendritic cells (DCs) to induce immunogenic or tolerogenic responses to microbial signals. Finally, we examine the signals that microbes provide and their role in modulating T cell differentiation and function.

HOST SENSING OF MICROBES

Commensal microbiota may be largely harmless but it is nevertheless crucial to maintain barriers to prevent opportunistic invasions and, occasionally, an immune response is required to keep potential pathobionts in check. In mucosal tissues, such a response is

promoted by both epithelial and innate immune cells via ligation of various receptors expressed differentially in these cells. The recognition of microbial particles such as DNA, cell wall components, and metabolites occurs in the context of innate toll-like receptors (TLRs), NOD-like receptors (NLRs), G protein-coupled receptors (GPCRs), other pattern-recognition receptors (C-type lectin receptors, RIG-I-like receptors, and AIM-2-like receptors), and yet to be identified receptors expressed by both hematopoietic and non-hematopoietic lineage cells in mucosal tissues. The following section will focus on the use of TLRs and NLRs by the host to sense microbes.

TOLL-LIKE RECEPTORS

Toll-like receptors are pattern-recognition receptors, which bind to evolutionarily conserved molecules expressed by bacteria, viruses, and other microorganisms (25). Some of them (TLRs 2, 3, 4, and 5) are expressed on the cell surface, whereas others (TLRs 3, 7, and 9) are expressed in endosomal compartments of cells (26, 27). TLRs are expressed in high levels on epithelial cells and innate immune cells and can sense various microbial molecules such as double- and single-stranded RNA, LPS, flagellin, CpG (28), ensuring detection of all microbes.

Toll-like receptor ligands have been implicated in the onset and management of various diseases. For instance, TLR signaling has been shown to affect Crohn's disease and psoriatic arthritis in humans (29, 30). Studies from the non-obese diabetic (NOD) murine model of type-1 diabetes have also revealed that disruption of TLR signaling protects these mice from developing diabetes (31). These data indicate that microbial recognition via TLRs has a significant implication in the onset of autoimmune and inflammatory diseases.

Toll-like receptor ligands have also been suggested to modulate mucosal cell function and tissue maintenance. One mechanism is by inducing the synthesis of antimicrobial peptides and tissue-repair factors by intestinal epithelial cells (IECs) (32). Moreover, LPS-induced TLR signaling has been linked to NFκB activation of IECs, which results in promoting either a tolerogenic or a pro-inflammatory environment depending on the microbial context (33). LPS also acts directly on mucosal DCs, affecting their activation and migration to various anatomical sites where they can induce adaptive responses (33). Similar to IECs, TLR signals can induce either activation or tolerance programs in DCs (discussed below).

NOD-LIKE RECEPTORS

NOD-like receptors contain multiple subfamilies and are expressed by many organisms from plants to mammals. Mammalian NLRs play a crucial role in sensing microbes and inducing various pro-inflammatory programs in response to microbial products (34). NLRs are not only essential in responses to pathogens but they are also necessary for the development of intestinal lymphoid tissues, maintenance of commensal communities, and mounting antigen-specific immune responses (35). This section will discuss only the NLRC and NLRP subfamilies of NLR signaling. For a more comprehensive discussion of NLRs, see the recent review by Chen et al. (36).

The NLRC subfamily contains NOD1, which recognizes peptidoglycan-containing mesodiaminopimelic acid (iE-DAP), and NOD2, which recognizes muramyl dipeptide (MDP) (37). One of the major functions of NOD1/2 is the activation of NFκB signaling. Pathogen induction of NOD2 results in NFκB activation and secretion of pro-inflammatory cytokines, especially IL-1 (34). Mutations in NOD2 are linked to Crohn's disease; here, the inability to induce NFκB signaling on IECs and immune cells is thought to impair intestinal mucosal barrier function, resulting in pathologies from invasion of the intestine by commensal bacteria (38). Support for this notion was obtained from work in the mouse. Hence, increased intestinal permeability to commensal bacteria was reported in NOD2 deficient mice, indicating that NOD2 signaling is important for maintaining intestinal epithelial integrity and barrier function (39). Similarly, NOD1 also has a role in maintaining intestinal homeostasis but in other related ways. Thus, NOD1 deficient mice possess a decreased number of intestinal lymphoid follicles and lower expression of CCL20 as well as a change in microbiota composition (35). NOD1 and 2 signaling has been implicated in both pro-inflammatory and immunoregulatory roles as it can induce secretion of IFN-γ, IL-12, IL-6 but also of IL-10 in various models of disease, demonstrating thus suggesting that a fine balance of NLRC signaling must be maintained (40–43). It is clear that NOD1 and NOD2 contribute to regulatory and inflammatory responses to microbes but it is the integration of these signals and those from other receptors, such as NLRP, that likely result in determining whether a regulatory or inflammatory response is appropriate.

NLRP and NLRC signaling are closely associated with the immune system as they affect inflammasome function. The inflammasome is a multiprotein complex that is required for the enzymatic processing of pro-forms of IL-1β and IL-18. Multiple NLRs can activate the inflammasome but they all recognize bacterial flagellin or type III secretion system (44). It has been suggested that NLRP and NLRC signaling is more specific than NOD1/2 signaling since it is triggered not only by these two types of bacterial molecules but it also has regulatory molecules in place that are recruited only by certain species of bacteria that merit an inflammatory response (44, 45). A recent model proposes that inflammasome activation to various microbes is mediated commonly by NLRC4, but the specificity comes from differential NLRP recognition of products from specific bacterial species (46). NLRP4, NLRP3, and NLRP1 have been associated with induction of the inflammasome in response to bacterial, fungal, and viral products; mutations in these genes are associated with higher susceptibility to bacterial and viral infections as well as autoimmunity (47–49).

Even though NLRP activation of the inflammasome was believed to be largely in response to infection and severe cellular stress, it was recently demonstrated that NLRP6 plays a crucial role in regulating interactions with commensal microbiota during steady-state conditions. NLRP6 knockout animals were highly susceptible to DSS-induced colitis and had decreased intestinal IL-18 levels as well as altered microbiota composition (50). It is unclear exactly what triggers NLRP6-mediated activation of the inflammasome but it likely occurs in response to a collection

of environmental signals. Because NLRP activation can occur in the context of microbiota, stress, or infection, the discrimination between pathogenic and commensal microbes likely involves the integration of multiple signals from the inflammasome, NFκB signaling, and other pathways by innate immune cells.

INNATE LYMPHOID CELLS

Innate lymphoid cells (ILCs) comprise a recently described population of innate immune cells, which have been shown to regulate immunity, inflammation, and tissue repair in various anatomical sites (51–53). Like B and T cells, they differentiate from a common lymphoid progenitor but, unlike B and T cells, they lack a rearranged antigen-specific receptor (51–53). Nonetheless, ILCs closely resemble CD4 helper T cells and express many of their transcription factors and molecules. Multiple subsets of ILCs exist, and they are classified into three groups – Groups 1, 2, and 3 – according to the expression of distinct transcription factors and effector molecules (51–53). Group 1 ILCs, such as NK cells, are characterized by the expression of the transcription factors T-bet and/or Eomesodermin and the production of the cytokine IFN-γ in response to stimulation with IL-12 and IL-18 (51–53). Group 2 ILCs' canonical features are the expression of the transcription factors RORα and GATA3 and the expression of IL-5 and IL-13 in response to stimulation with IL-25, IL-33, and TLSP (51–53). Finally, group 3 ILCs, such as lymphoid tissue inducer (LTI) cells, NKp46$^+$, and NKp46$^-$ cells, are characterized by the expression of RORγt and/or T-bet and/or the aryl hydrocarbon receptor (AHR) and upon stimulation with IL-23 and IL-1β, they produce IL-22 and/or IL-17 and/or IFN-γ (51–53).

Innate lymphoid cells inhabit various tissues where they have been shown to direct inflammation at mucosal surfaces, especially in a response to infection, and confer resistance to pathogens (54). Group 1 ILCs include NK cells and a population of human mucosal ILCs, which lack expression of NK surface markers but produce high levels of IFN-γ in response to IL-18 and IL-12 stimulation (55). They are present in high levels in patients with Crohn's disease and contribute to increased inflammation in the intestine (56). A new NKp44$^+$CD103$^+$ population of ILC1s, which produces large amounts of IFN-γ, CCL4, and TNFα, has been recently described in the human intestine (57), and it has been suggested that it is the innate counterpart of tissue-resident memory CD8 T cells. Group 2 ILCs participate in protection against helminth infection in the intestine by producing IL-25 (58–60) as well as in wound healing in the airways via the production of amphiregulin, which induces epithelial cell proliferation (61, 62). Group 3 ILCs have been shown to regulate CD4 T cell responses to commensal bacteria in the intestine of mice as well as to prevent bacterial dispersal to peripheral tissues (63, 64). Recently, it has also been revealed that intestinal macrophages act through ILC3 to induce the production of tolerogenic molecules (IL-10 and retinoic acid) by intestinal DCs or macrophages and thus promote the induction and homeostasis of regulatory T cells in the intestine and the mesenteric lymph nodes (65). Even though ILCs are a novel population of innate immune cells, it has been well demonstrated that they play a key role in mucosal defense from pathogens as well as in maintenance of mucosal tissues.

DENDRITIC CELL FUNCTION DEPENDS ON LOCATION AND ENVIRONMENT

Mucosal DCs are in constant contact with commensal microbiota via various pattern-recognition receptors. Because DCs have the unique ability to elicit a robust T cell response (66), they are differentially conditioned based on anatomical location and local antigenic load to be either tolerogenic or pro-inflammatory in response to the same signal. In the lungs, TLR4 signaling on DCs results in antigen-specific CD4 T cell mediated inflammation (67). On the contrary, TLR4 signaling in intestinal DCs is shown to be tolerogenic (68), suggesting that the environmental cues and location determine how a DC integrates such signals. In the context of other TLR ligands, administration of CpG in TLR4 deficient mice reduces their susceptibility to systemic allergy development, arguing for a tolerogenic role of TLR9 signaling (69). In contrast, when CpG is administered to germ-free mice, it causes increased IFN-γ and IL-17 production in the intestine of these mice, conferring protection against intestinal parasites, indicating a more activating role for TLR9 (69). Based on these data, it is widely accepted that the environmental context in which DCs integrate the same signal determines whether an inflammatory immune response occurs.

At mucosal sites, unlike in other lymphoid tissues, DCs perform unique roles and occupy distinct niches (70). In addition to specifying the inflammatory or anti-inflammatory nature of the T cell response, DCs also direct the homing of effector cells. In the mesenteric lymph node, they can promote upregulation of CCR9 and α4β7 on B and T cells (71, 72), thereby facilitating their migration back to the intestine (73, 74). In the intestine, DCs translocate to and reside in the lamina propria (LP), Peyer's patches, isolated lymphoid follicles, and mesenteric lymph node, where they sample and present luminal and self antigens to T cells (75). In the Peyer's patches, DCs are divided into three groups: CXCR1$^+$ DCs, CCR6$^+$ DCs, and CCR7$^+$ DCs. Each group has distinct functions and characteristics. CXCR1$^+$ DCs are found in the Peyer's patch in close proximity to M cells, positioned there with the purpose to sample luminal antigens in a TLR-dependent manner (76). CCR6$^+$ DCs are migratory and are found in the dome of the Peyer's patches, from where they readily translocate to the follicle-associated epithelium in response to microbial stimulation. Finally, CCR7$^+$ DCs are found in the T cell areas of the Peyer's patches, where they can induce T cell activation (77, 78) and migration (73, 74), and IgA production by B cells (79, 80) in response to microbial signals (81).

As Peyer's patches are rare along the intestinal tract, the majority of gut-associated DCs are more frequently found in the LP of the small intestine. LP DCs, often differentiated from circulating precursors (82), express tight junction proteins that allow them to reach their dendrites between IECs and sample the luminal contents directly (83). This phenomenon depends on CX3C chemokines and TLR ligation (84, 85). CX3CR1 deficient mice exhibit impaired luminal sampling by DCs and are thus more susceptible to *Salmonela typhimurium* infection (85). This finding suggests that luminal sampling by DCs may be critical for protective immune responses against intestinal pathogens.

As mentioned above, intestinal DCs are thought to be more tolerogenic than systemic DCs (86, 87). It has been shown that

stimulation of intestinal DCs with LPS results in elevated IL-10 secretion, whereas the same stimulation of circulating DCs results in the production of a variety of pro-inflammatory cytokines (88, 89). The mechanisms that condition intestinal DCs to produce IL-10 in response to LPS stimulation, but not systemic DCs, are still largely unknown. It has been suggested that reduced TLR expression (88, 89) and hyporesponsiveness to TLR ligation (68) as well as negative regulation of NFκB pathways via NOD2 signaling play a role in the desensitization of intestinal DCs to microbial antigens (40–43).

Dendritic cells play a key role in promoting T cell differentiation and responses to microbiota in the gut as well as systemically. Some studies have shown that intestinal DCs can transport self and microbial antigens to the mesenteric lymph nodes (90) where they orchestrate T cell activation and differentiation into effector cells. Intestinal DCs are not exclusively tolerogenic and can induce pro-inflammatory T cell responses by secreting IL-12 and IL-23. IL-12 is a master inducer of T helper 1 (Th1) responses (91) and thus plays an important role in Th1-associated autoimmune diseases, such as IBD (92–94). IL-23 has been implicated in inflammatory T helper 17 (Th17) responses (95) in murine models of joint (96) and intestinal inflammation (97–100) as well as psoriasis (101, 102). Conversely, in addition to Th1 and Th17 responses, intestinal DCs can induce T regulatory cells in the context of retinoic acid, TGF-β, and IL-10 in the gut, suggesting that intestinal DCs can serve to direct T cell differentiation so as to induce the appropriate response based on different contexts (75, 86, 87). DCs can sense microbial antigens from commensal and pathogenic microbes and ideally are able to differentiate between the two in order to induce appropriate T cell responses, protecting the host from infection but at the same time creating a tolerogenic environment for the commensal microbiota to thrive.

COMMENSAL MICROBIOTA MAINTAINS T CELLS' POISED STATE

Many dramatic effects of commensal microbiota on host T cells have been documented. Following microbial colonization, immune cells are recruited, induced to differentiate and to reside in the gut (13). At steady state, the intestine houses a large number of T cells, which produce IL-17, IL-22, IFN-γ, and IL-10 (69, 103). In the absence of microbiota, there are deficiencies in the production of these cytokines (15, 103), indicating that commensal microbes control their constitutive production in the gut. CD4 T cell numbers have been shown to be decreased in germ-free mice, affecting T helper 1 (Th1) and T helper 17 (Th17) cells, although regulatory T cell (Treg) frequencies remain the same (69, 103). When germ-free mice are conventionalized, a broad Th1, Th17, and Treg expansion occurs and cytokine production is recovered in the gut, indicating that the accumulation of functional CD4 T cells in the gut is microbiota dependent (104). Furthermore, upon infection with a mucosal pathogen, CD4 T cells in the intestine respond to both the pathogen as well as to the commensal microbiota that permeate the intestinal epithelial layer, demonstrating the ability of the adaptive immune system to overcome its tolerance to commensals and its ability to contain pathobionts and opportunistic pathogens (105). Among the CD8 T cell populations in the gut, intraepithelial lymphocytes (IELs) are significantly decreased in germ-free mice, and they are restored upon colonization (106). Because the host needs metabolites and essential nutrients from microbes, yet at the same time it must protect itself from infection, maintaining a balance between pro- and anti-inflammatory T cell populations in the gut is essential.

In addition to affecting the local populations, there is evidence supporting the notion that commensal microbes also influence the generation and function of the host immune system more broadly. Peripheral lymphoid organ structure and function are disrupted in the absence of commensal microbiota (107). Moreover, T cell responses to systemic antigens are also altered in the absence of commensal microbiota. Recent studies have suggested that commensal microbiota keeps the immune system primed and ready to respond during the steady state and conventional mice respond more robustly to infection than germ-free mice (108). This section will discuss the effect of commensal microbiota on T cell responses both at mucosal sites and systemically.

TH17 CELLS

IL-17 producing Th17 cells are represented in high numbers in the LP of the small intestine, where they play a role in protection against extracellular pathogens (103). The differentiation of Th17 cells is dependent on their expression of the transcription factor RORγt, and it is driven by signals from TGF-β and IL-21 or IL-6 (109). Additionally, Th17 cells require IL-23 for maturation and survival (110). As the number of Th17 cells is dramatically decreased in the small intestine of germ-free animals, there is a widely accepted notion that commensal bacteria are required to cue Th17 differentiation (103). A TLR-independent mechanism for the promotion of Th17 differentiation in the gut has been suggested, as signals through MyD88 are not necessary for the induction of Th17 cells (103). Furthermore, data from mice mono-associated with segmented filamentous bacteria (SFB) indicate that even a single species of commensal bacteria is enough to direct T cell differentiation toward a Th17 bias (104, 111).

Not only do microbiota-derived signals stimulate the differentiation and accumulation of Th17 cells in the intestinal LP, but they also maintain Th17 cell homeostasis and survival. Studies have indicated that treatment with the antibiotic vancomycin decreases Th17 populations in the small intestine of conventional mice (103, 112), whereas treatment with a complex cocktail of antibiotics, results in a decreased Th17 frequency in the mesenteric lymph node (113). In other mucosal sites, such as the skin, resident commensal microbiota have been shown to induce Th17 and Th1 differentiation to protect the host from pathogens and possible opportunistic microbes (114).

REGULATORY T CELLS

Maintaining tolerance to resident bacteria is the key to preventing inflammatory diseases in mucosal tissues. Before the importance of tolerance was widely accepted, it was commonly believed that an unknown pathogen was the trigger for IBD. However, successful treatment of the disease with immunosuppressive drugs has lead to the hypothesis that IBD may result from defects in tolerance to otherwise non-pathogenic gut commensals (115–118).

The idea that T cells must be tolerized to commensal microbiota was suggested decades ago, when in an adoptive transfer

model, naïve CD4 T cells caused colitis (119) but were held in check by another population of CD4 T cells (118), now known as regulatory T cells (Tregs). For the purposes of this review, Tregs are T cells that express the master regulator transcription factor, Foxp3, and display anti-inflammatory activity, including secretion of TGF-β and/or IL-10 (120). Extrapolation from early studies led to the proposal that regulatory T cells are required to prevent aberrant T cell responses to resident microbes. Scientists were thus surprised when data from the small intestine of germ-free mice emerged to show that commensal bacteria are not necessary for the development of Tregs in that organ (121–123). Furthermore, when germ-free Tregs were assayed, they were able to suppress colitis-like symptoms albeit not as well as conventional Tregs in the adoptive transfer model of the disease (122, 124), indicating that functionally and developmentally, small intestinal Tregs are independent of commensal microbiota. Even though the small intestine houses a large amount of T cells, it is the colon that houses the largest load of microbiota and is the site of colitis observed in these experiments.

Recent studies on the effect of gut microbiota on Treg development and function have elucidated that colonic Tregs are significantly decreased in germ-free mice, indicating that, in the colon, commensal microbiota is the major inducer of colonic Tregs (125, 126). The exact mechanism of how the induction of colonic Tregs occurs remains unknown. It has been suggested that certain species of microbiota, such as Clostridium clusters IV and XIVa, can induce Foxp3$^+$ Treg generation in the colon (125). In order to determine whether commensal microbiota is required for the differentiation of naïve peripheral T cells into colonic Tregs, several groups looked at the expression of an Ikaros-family transcription factor, Helios, which is thought to signify thymic origin of Tregs. They showed that in the colons of germ-free mice, most of the Tregs were Helios^{hi}, suggesting that these T cells became Tregs in the thymus (so-called thymic or tTreg), whereas their counterparts in conventional mice were mostly Helios^{lo}, indicating that these CD4 T cells converted to Tregs in the peripheral tissues (so-called peripheral or pTreg) (125, 127). These results suggested that in conventional hosts, colonic Tregs are differentiated outside of the thymus (presumably in the gut) in response to foreign antigens. Because the use of Helios in the field is still quite controversial (128, 129), other experimental approaches were utilized to confirm the importance of commensal microbiota on colonic Treg differentiation and function. Subsequent work using Neuropilin-1 (Nrp-1) as a marker to detect tTregs confirmed that induction of pTregs in the colon was mediated by commensal microbiota (130). Another study using a fixed TCRβ showed that colonic Tregs utilize different TCRs from systemic circulating Tregs, reaffirming the notion that they recognize distinct gut antigens (127). Additionally, TCRs from colonic Tregs, expressed as transgenes, were unable to induce generation of tTregs, again supporting the notion that colonic Tregs were peripherally induced in response to commensal antigens (127).

As the mechanism is still unknown, multiple models have been offered to explain how microbiota supports an abundance of pTregs in the gut. It has been proposed that preferential expansion of pTregs occurs in the intestine either by microbial components influencing pTregs directly or indirectly through the products of bacterial metabolism, such as short-chain fatty acids (131–133), and from presentation of bacterial peptides (such as PSA) by innate immune cells (134–136) and/or from effector T cells (129, 137, 138). Another hypothesis offers that the foreign antigen load in the gut is so large that, given a limited capacity for antigen presentation, self-peptides are displaced and the increased prevalence of pTregs simply reflects the increased density of their targets (i.e., foreign-peptide/MHC-II) and decreased availability of tTreg targets (i.e., self-peptide/MHC-II) (127). The question of the specificity of small intestinal pTregs still remains open. It is possible that these Tregs are generated in response to self or dietary antigens, as the abundance of these antigens is greater in the small intestine than in the colon.

Fostering a balance between tolerating commensal microbiota and maintaining the ability to mount an immune response to microbial pathogens is crucial for the survival of the host. Despite the seeming bias toward induction of tolerogenic responses in the gut, immune responses still occur readily against pathogens to protect the host from infections. The mechanisms utilized by the host to distinguish between the commensal and pathogenic bacteria are still poorly understood and are under intense investigation.

INTRAEPITHELIAL LYMPHOCYTES

Because the intestinal mucosa harbors various opportunistic bacteria, the host has evolved the ability to house cytotoxic killer T cells in close proximity to potential sites of pathogen entry, like the intraepithelial layer of the intestine. The lymphocytes that reside there, also known as IELs, are composed of CD8 T cells that are recruited to and remain in that compartment for the duration of the host's life. There are three types of IELs, each bearing different characteristics and functions: αβ TCR CD8αα, αβ TCR CD8αβ T cells, and γδ TCR T cells. Whereas αβ TCR IELs respond mainly to pathogenic challenge of the epithelial mucosa, γδ TCR IELs participate in would healing and tissue repair by producing pro-inflammatory cytokines and chemokines and recruiting neutrophils, eosinophils, and T cells.

αβ TCR IEL differentiation and maintenance depend on TLR recognition of bacterial signals as MyD88 deficient and germ-free mice exhibit diminished numbers of these cells (139–141). Functionally, in germ-free and antibiotic-treated mice, the cytotoxic activity of αβ TCR IELs is significantly decreased when compared to conventional mice (140), a phenotype that can be rescued by supplying exogenous endotoxin. This suggests that αβ TCR IEL function is both induced and maintained by ubiquitous bacterial components (139, 142).

Similarly, recent data have elucidated the dynamic relationship between commensal bacteria and γδ IELs during mucosal injury. γδ TCR IELs restrict the spread of bacteria to the mesenteric lymph node following intestinal injury as shown by the lack of such a response in γδ TCR IEL-deficient mice (143). γδ TCR IELs exhibit their functions by producing keratinocyte growth factor, which causes epithelial cell proliferation and restoration of barrier functions in the gut (144). Germ-free animals, even though they have a similar numbers of γδ IELs as their conventional counterparts, have significantly decreased ability to promote mucosal injury repair and prevention of invasion by opportunistic pathogens (143). This problem is evidenced by their decreased

ability to produce antimicrobiotal peptides, such as RegIIIγ, as well as pro-inflammatory chemokines and cytokines, such as IL-1β, KC, and MIP2α (143), indicating that commensal microbes are required to promote γδ IEL function.

Continued research will reveal the crosstalk between microbiota and these two populations of IELs as they directly relate to intestinal homeostasis. IELs are able to produce both anti- and pro-inflammatory cytokines and promote both strong cytotoxic and tissue-repair responses.

SYSTEMIC T CELL RESPONSES TO INFECTION

Effects of gut microbiota on innate immune responses to systemic viruses and bacteria have been well demonstrated in the literature (108, 145–147). However, the mechanisms whereby microbiota affects non-mucosal T cell responses have been difficult to comprehend as peripheral T cells are not in direct contact with commensal bacteria. Because T cells confer long-term protection and memory against pathogens, understanding the role of microbiota on T cell responses to infection is crucial. Studies from the 1970s indicated that germ-free and conventional animals exhibit similar immune responses to systemic infection by *Salmonela paratyphi* or lymphocytic choriomeningitis virus (LCMV) (146).

A few groups infected germ-free mice with systemic pathogens in the 1990s. Even though they compared mainly antibody production to assess the immune responses in these mice, a speculation can be made that antibody production is directly connected to T cell responses as T cell help is required for class switching and somatic hypermutation. One group, which infected mice with MCMV and subsequently with *Klebsiella pneumoniae,* concluded that germ-free mice were significantly more susceptible to both pathogens and did not clear the infection in various organs at the same rate as SPF counterparts (148). Moreover, they showed increased mortality by bacterial infection (148). Histologically, the spleens and livers of germ-free animals were more severely affected by the infection and recovered more slowly (148). Another group, which infected germ-free mice with *Salmonella typhimurium,* showed that they were more susceptible to systemic infection than conventional controls (149). Furthermore, they noted a decrease in IgG and IgM responses in the germ-free mice. It could be inferred that the inability to clear a viral and intracellular bacterial infections as well as the decreased antibody production is a result of an impaired T cell response in germ-free mice. Indeed, with improved techniques, more recent work has suggested that systemic immune responses are dampened in the absence of commensal microbes.

Germ-free animals were found to have a higher susceptibility to a prolonged and non-limiting *Listeria monocytogenes* infection compared to conventional animals, a phenomenon supposedly due to the inability of germ-free mice to accumulate T cells at inflammation sites (145, 150). Although *Listeria monocytogenes* is naturally an intestinal pathogen, the method utilized in these studies resulted primarily in infection of the host spleen. Likewise, adaptive immune responses to viral infection of non-mucosal sites are also shaped by the presence of commensal microbes. Antibiotic-treated mice generate significantly fewer virus-specific effector CD4 and CD8 T cells when compared to untreated controls during influenza infection (151). Functionally, CD4 and CD8 T cells in antibiotic-treated mice produce lower amounts of pro-inflammatory cytokines, which correlates with increased virus titers in these mice (151). Further investigation by Abt et al. indicated that antibiotic-treated mice more readily succumbed to influenza infection when compared to conventional counterparts (108). T cell responses in antibiotic-treated mice indicated a decrease in CD8 virus-specific T cells, further confirming that commensal microbiota modulates T cell responses to systemic viral infection (108). The authors concluded that the higher susceptibility of antibiotic-treated mice to infection was due to an inability of macrophages to respond to type-1 interferon and limit viral replication. However, they did not expound upon the relationship between the macrophage defect and the CD8 T cell phenotype they observed. It could be inferred that commensal-derived signals provide tonic signaling to innate immune cells, which in turn, influence the ability of these cells to effectively activate naïve T cells and convert them to fully functional effectors. A detailed understanding on the effect of commensal microbiota on systemic T cell responses is yet to be provided.

CONCLUSION

Commensal microbiota plays a crucial role in the development, homeostasis, and regulation of the immune system. With the current rise of autoimmune and inflammatory diseases, the importance of inducing and maintaining tolerance to commensal bacteria is increasingly appreciated. As a constant source of foreign antigens, microbiota plays a pivotal role in inducing tolerance to beneficial bacteria as well as in maintaining the immune system poised to defend the host against pathogens. Since the adaptive immune system is often implicated in microbiota-associated inflammatory and autoimmune conditions, understanding its relationship with commensals is crucial. Metaphorically speaking, if the host immune system is a house, the commensal microbes may be likened to the embers in a fireplace. At homeostasis, they remain glowing, providing constant minimal heating for the house. However, when the house so requires, i.e., when an immune response is necessary, they help create a powerful flame.

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Resident-memory CD8 T cells and mTOR: generation, protection, and clinical importance

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Tissues such as the lung, skin, intestinal epithelium, and reproductive tract serve as a barricade against pathogen exposure for the entire body. Specifically within the skin and mucosal tissues, a population of resident CD8 T cells plays a salient role in the protection against infection. Resident-memory CD8 T cells (T_{RM}) are a long-lived subset of memory CD8 T cells that do not re-circulate after taking up residence in the tissues. Traditionally memory CD8 T cells were conceptually divided into two subsets central (T_{CM}) and effector (T_{EM}) memory where T_{CM} preferentially localized within secondary lymphoid tissues (SLO) and T_{EM} circulated throughout the peripheral tissues. While the concept of T_{CM} and T_{EM} has been considerably explored, memory CD8 T cells found within barrier tissues do not totally fit within the T_{CM}/T_{EM} paradigm. Through the study of circulating CD8 T cells, our understanding of memory CD8 T cells has grown tremendously in the last 25 years. We now understand that it is not sufficient to simply generate large numbers of circulating memory CD8 T cells in order to enhance protection against localized infection. Developing clinical strategies that can enhance protection against mucosal pathogens will require a clear understanding of how memory CD8 T cells are generated and maintained within barrier tissues at the sites of initial pathogen exposure. We will outline our current understanding of T_{RM} in respect to their generation, functional importance, and how future studies must shed light on how we can exploit T_{RM} to develop the next generation of effective vaccines.

GENERATION OF TISSUE-RESIDENT-MEMORY CD8 T CELLS

CD8 T cells are primed within tissue draining lymph nodes and lymphoid tissues. Once primed, CD8 T cells receive tissue-specific signals that allow them entry into tissues, which at steady-state are normally non-permissive to T cell migration (1). Upon entry into mucosal or skin tissues CD8 T cells take up residence and do not re-circulate (2, 3). For T cells to enter the small intestine the integrin α4β7 and chemokine receptor CCR9 are important (4, 5). α4β7 and CCR9 expression is induced by dendritic cell-derived signals like the vitamin A metabolite retinoic acid (6). α4β7 is transiently expressed and the timing of its expression correlates with the window of opportunity for T cell migration into the small intestine (2, 4, 5). Primed T cells that enter mucosal tissues differentiate into T_{RM} in response to tissue-derived signals, not limited to but including TGF-β and IL-15 (5, 7). Tissue-derived signals like TGF-β and IL-15 are not uniquely confined to barrier tissues, as their availability is also important for circulating CD8 T cells. T_{RM} originate from KLRG1$^-$ effector cells, are not terminally differentiated, however, express lower levels of CD127 and CD122 than circulating memory CD8 T cells (7, 8). While T_{RM} share a common naïve precursor with blood and SLO memory cells they are inherently different from their circulating counterparts (9). Unlike circulating memory CD8 T cells, T_{RM} maintain expression of CD69 and elevated levels of Granzyme B, attributes akin to effector cells (2, 10, 11). Generation of T_{RM} is dependent on CD69 as overexpression *S1pr1* or deletion of CD69 in CD8 T cells limits T_{RM} formation (7, 12). T_{RM} also upregulate the integrin subunit CD103 whereas circulating memory CD8 T cells remain CD103$^-$ (2, 8). A qualitative feature that distinguishes circulating T_{CM} and T_{EM} from T_{RM} is that regardless of infection or anatomical location T_{RM} share a signature of core gene transcripts (7). Within this signature are genes involved in chemotaxis, adhesion, and co-stimulation. Expression of some genes associated with circulating memory cells are decreased, e.g., *Eomes*, *S1pr1*, and *Ly6C*. The signaling events that regulate the transcriptional programing of T_{RM} is unknown.

mTOR AND T_{RM} FORMATION

The mammalian target of rapamycin (mTOR) kinase is a central regulator of many cell processes, including survival, differentiation, and proliferation. It orchestrates cellular responses to external and internal environmental cues. Signaling through the mTOR pathway is an important event for memory formation (13). During each stage of T cell priming, mTOR is activated i.e., TCR/CD28/IL-12. However, inhibiting mTOR in effector CD8 T cells increases the number of memory CD8 T cells in the circulation by increasing the number of effector CD8 T cells that are CD127^{hi}KLRG1$^-$. This suggests that while mTOR is needed early during activation for effector formation, at some point during the effector phase mTOR could be attenuated or turned off in order for effector cells to progress toward memory differentiation.

We recently reported that accumulation of CD8 T cells in the small intestine and

FRT is critically dependent on prolonged mTOR signaling (14). We showed that blocking mTOR signaling during the effector phase inhibits the accumulation of effector CD8 T cells in the mucosa thereby limiting T_{RM} formation. This suggests that mTOR controls signals that either direct the migration of CD8 T cells into these barrier tissues or influences their survival. More importantly, because one naive CD8 T cell can give rise to memory in both the SLO and mucosal, our observations indicate that mTOR may regulate the divergence between circulating and resident-memory CD8 T cells. It is also possible that mTOR controls the context in which memory precursors receive signals from cytokines and growth factors. Circulating memory CD8 T cells interact with different stromal and hematopoietic cells than tissue-resident-memory CD8 T cells and this could influence their overall function and survival. For example, IL-15 is an important homeostatic cytokine for memory CD8 T cells, received through a contact-dependent mechanism known as trans-presentation, and for T_{RM} formation (5, 15). Within the memory CD8 T cell populations both IL-15-dependent and IL-15 independent populations have been described. mTOR can be activated by IL-15 and inhibition of mTOR leads to a predominantly IL-15 independent memory population (16, 17).

Many cell types within different tissues produce IL-15 and as a result T_{RM} may receive IL-15 signals more frequently than circulating T_{EM} that may only receive transient IL-15 signals when they circulate through the tissues. While TGF-β induces apoptosis in circulating effector CD8 T cells, T_{RM} generated from effector CD8 T cells rely on TGF-β signals (7, 18). Since mTOR has broad affects on cell physiology and is activated in effector CD8 T cells, mTOR may be central in regulating the unique transcriptional program in T_{RM}. mTOR signaling is mediated by two distinct complexes, mTORC1 and mTORC2 that control responses to environmental cytokine milieus (13). In the absence of mTOR, T cells are unable to respond to cytokines that direct their differentiation (19). The differentiation of naïve CD4 T cells into Th1 and Th2 subsets, requires mTORC1 and mTORC2 signaling, respectively. Loss of mTORC1 signaling in CD4 T cells blocks their ability to upregulate T-bet in the presence of IL-12 (20). IL-12 mediated mTORC1 signaling in CD8 T cells sustains the expression of T-bet and promotes differentiation into effector cells (21). Moreover, inhibiting mTORC1 increases the expression of Eomes and skews memory CD8 T cells toward IL-15 dependence (17). CD4 T cells that lack mTORC2 signaling have diminished responses to IL-4 and IL-13. Less is known about the function of mTORC2 in CD8 T cells. However, given CD4 and CD8 T cells' shared dependence on the same cytokines or cytokine signaling pathways for their differentiation, the role of mTORC2 in CD8 T cell function will be an important direction for future study.

ARE T_{RM} REQUIRED FOR PROTECTION?

A major question is whether T_{RM} positioned at sites of exposure are critical to protect against local re-exposure. The presence of T_{RM} can provide local protection (3). However, circulating memory CD8 T cells can migrate into barrier tissues upon re-challenge and provide protection. However, antigen must first make its way to mucosal draining lymph nodes in order to prime circulating memory CD8 T cells and recruit them to infected tissues. Due to their proximity to infected tissue T_{RM} may be able to respond a pathogen exposure more rapidly than circulating memory CD8 T cells (22). The mucosa is the major route of entry for HIV infection. Within a few hours to days after exposure, HIV can breach the mucosal barrier, infect resting CD4 T cells, and presumably establish latent virus reservoirs (23). In non-human primate models of infection, the latent reservoir is seeded rapidly sometime during the first 3 days of infection creating a big challenge for the immune system (24). The time required for circulating memory CD8 T cells to mount a response may not be quick enough to prevent the establishment of HIV infection. It is now evident that eliminating infected founder cell populations in the area of initial HIV entry is a critical requirement for the immune system to provide protection. Robust T_{RM} responses at the portal of HIV entry may be critical component. Whether T_{RM} can directly kill local infected cells is not explicitly known. The surface area of the mucosa barrier is immense and it is estimated that for every T_{RM} cell there could be tasked to survey as many as $\sim 10^3$ cells (25). Therefore, it is important to know the concentration of T_{RM} that would constitute a critical mass to provide protection. However, recent work has shown that T_{RM} may do more than kill infected cells. T_{RM} can rapidly respond to local challenge and recruit new antigen-specific memory CD8 T cells from the circulation as well as bystander cell populations (26). Moreover, T_{RM} can activate neighboring NK cells and B cells by providing a local source of IL-2, IFN-γ, and TNF-α (27). Collectively, these recent findings suggest that T_{RM} could provide protection not only by killing of infected cells in barrier tissues, but also by taking part in pathogen sensing and initiation of the immune response. Thus it is important to know whether vaccine prime-boost regimens, which can generate large numbers of T_{RM}, will ultimately lead to protection against mucosa-acquired infections such as HIV and HSV.

MANIPULATING T_{RM} FOR CLINICAL BENEFIT

The observation that upon reactivation T_{RM} are capable of inducing activation of innate cells and even protecting against an antigenically unrelated pathogen may have broader implications for vaccine design (27). It is understood that local priming of CD8 T cells or priming within mucosa draining lymph nodes can generate effector CD8 T cells that can become T_{RM}. Building vaccines that favor priming at these tissue sites and the establishment of long-lived T_{RM}, while challenging, is important, and may include the following strategies. Orally administered vaccines can induce CD8 T cells that accumulate within tissues such as the female reproductive tract and small intestine of the gastrointestinal tract, while attractive the longevity and functional attributes of the memory cells generated from this approach need to be further elucidated (28). Prime-boost strategies using local challenge potentially can increase the number of T_{RM} to a level that can provide protection. Identifying key regulators of T_{RM} formation, as candidate adjuvants to more traditional vaccine strategies, in order to re-direct primed effectors to mucosal tissues to generate increased numbers of T_{RM}. However, the

ability of these re-positioned T_{RM} to persist like their counterparts that are generated *in situ* is still an area of research that warrants further investigation. On the other hand, CD8 T_{RM} cells may also play a central role in tissue destruction in organ-specific autoimmune disorders. The same factors that are important for the generation of T_{RM} may be potential targets for blocking pathogenic CD8 T cell responses in the small intestine of patients with celiac disease. Our findings that low-doses of rapamycin, a pharmacologic mTOR inhibitor, blocked the accumulation of CD8 T cells within the intestinal mucosa suggest the mTOR pathway as a candidate for therapeutic intervention (14). Moreover, we used a model of T cell mediated enterocyte destruction that may have some similarities to celiac disease pathogenesis. Using this model, we demonstrated that rapamycin was capable of blocking the accumulation of CD8 T cells specific for antigen expressed exclusively within the small intestine. In this model, the autoimmunity becomes fatal upon addition of systemic inflammation and the administration of low-dose rapamycin reversed intestinal destruction and enhanced survival. For tumor immunity one hurdle is positioning enough functional CD8 T cells within the tumor milieu. Interestingly, a small population of memory CD8 T cells has been identified in the spleen and lymph nodes that share characteristics of T_{RM} in that they do not re-circulate, and express CD69 (29). It is of significant interest to determine if an equivalent population of T_{RM} populates the tumor and if so how to manipulate these cells to the point where they have a negative impact on tumor growth.

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