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Persistence in Temporary Lung Niches: A Survival Strategy of Lung-Resident Memory CD8$^+$ T Cells

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Abstract

Respiratory virus infections, such as those mediated by influenza virus, parainfluenza virus, respiratory syncytial virus (RSV), severe acute respiratory syndrome coronavirus (SARS-CoV), rhinovirus, and adenovirus, are responsible for substantial morbidity and mortality, especially in children and older adults. Furthermore, the potential emergence of highly pathogenic strains of influenza virus poses a significant public health threat. Thus, the development of vaccines capable of eliciting long-lasting protective immunity to those pathogens is a major public health priority. CD8$^+$ Tissue-resident memory T (T_{RM}) cells are a newly defined population that resides permanently in the nonlymphoid tissues including the lung. These cells are capable of providing local protection immediately after infection, thereby promoting rapid host recovery. Recent studies have offered new insights into the anatomical niches that harbor lung CD8$^+$ T_{RM} cells, and also identified the requirement and limitations of T_{RM} maintenance. However, it remains controversial whether lung CD8$^+$ T_{RM} cells are continuously replenished by new cells from the circulation or permanently lodged in this site. A better understanding of how lung CD8$^+$ T_{RM} cells are generated and maintained and the tissue-specific factors that drive local T_{RM} formation is required for optimal vaccine development. This review focuses on recent advance in our understanding of CD8$^+$ T_{RM} cell establishment and maintenance in the lung, and describes how those processes are uniquely regulated in this tissue.

Keywords: lung, memory CD8$^+$ T cells, tissue-resident memory

Introduction

Memory T cells have been divided into two distinct subsets based on their distinct migratory properties (107). Central memory T (T_{CM}) cells express lymph node-homing receptors L-selectin (CD62L) and CC-chemokine receptor 7 (CCR7), and preferentially circulate between lymph nodes and blood. Effector memory T (T_{EM}) cells lack the expressions of these receptors and circulate between the blood and nonlymphoid barrier tissues such as the skin, lung, intestines, and female reproductive tract. Upon secondary infection, T_{EM} cells exhibit immediate effector functions at the site of infection, while T_{CM} cells undergo extensive expansion in the draining lymph nodes before migrating to the site of infection and eliminating virus-infected cells (106). It has recently emerged that memory T cells in the nonlymphoid tissues, which had previously been classified as a circulating T_{EM} population, include a noncirculating cell population that resides permanently within the peripheral tissues. These cells have been termed tissue-resident memory T (T_{RM}) cells (34) and comprise the majority of memory T cells in the nonlymphoid tissues that confer immediate protection against peripheral infection (119). Low levels of T_{EM} cells also transit the peripheral tissues and contribute to local protection (83). More recent studies have revealed that T_{RM} cells are present in wide variety of lymphoid and nonlymphoid tissues, including brain, salivary glands, thymus, spleen, lymph nodes, liver, kidneys, pancreas, heart, and dorsal root ganglia (96). The generation and maintenance of T_{RM} cells in each of these tissues differ significantly, indicating a major role for tissue-specific instruction (53). Therefore, there is a need to identify the unique signals underlying each tissue microenvironment and the molecular mechanisms that instruct T_{RM} formation.

During primary respiratory virus infections, antigen-specific CD8$^+$ T cells are crucial to the elimination of virus-infected cells and in the case of influenza viruses, cross reactive CD8$^+$ T cell-mediated immunity can provide

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protection against different viral strains (heterosubtypic immunity) (21). Thus, understanding the mechanisms by which CD8$^+$ T_{RM} cells are established in the lung has important implications for vaccine development. Following resolution of respiratory virus infections, CD8$^+$ T_{RM} cells persist in at least two distinct compartments of the lung: the lung interstitium/parenchyma and the lung airways (44). CD8$^+$ T_{RM} cells in the lung interstitium/parenchyma are mainly found as confluent peribronchiolar cell infiltrates in the interstitium and alveolar spaces (123). Note that the term ''lung parenchyma'' indicates a part of lung involved in gas exchange, such as the alveoli, alveolar ducts, and respiratory bronchioles, but does not include the lung interstitium. In contrast, CD8$^+$ T_{RM} cells in the lung airways are localized primarily in the epithelial layers of the upper respiratory tract and can be easily isolated by bronchoalveolar lavage (BAL) (25,47,48). Both T_{RM} populations confer rapid protection against secondary infection (37,91,135), and the number of antigen-specific CD8$^+$ T_{RM} cells in those tissues correlates with the efficacy of protection. Importantly, however, the molecular and cellular mechanisms underlying their recruitment, differentiation, maintenance, and recall differ significantly (44). Thus, the precise discrimination of those populations is necessary to comprehensively understand CD8$^+$ T cell-mediated antiviral immunity in the lung.

This review will focus primarily on influenza and parainfluenza virus infections and discuss recent insights into the course of CD8$^+$ T_{RM} cell establishment in the lung interstitium/parenchyma and airways, from initial priming, to tissue migration, local differentiation, and maintenance.

Priming of CD8$^+$ T Cells Following Respiratory Virus Infection

CD8$^+$ T cell priming following a respiratory virus infection occurs in the lung-draining mediastinal lymph nodes (MLN) after lung-resident antigen-presenting cells (APCs) have transported viral antigens to that site. Lymph node-resident CD8α^+ conventional dendritic cells (DCs) that acquire antigens from migrant respiratory DCs were initially thought to be pivotal in the initiation of antiviral CD8$^+$ T cell responses (10). It has recently become apparent, however, that two subsets of respiratory DCs transport viral antigens from the lung and prime naive CD8$^+$ T cells in the MLN (60). Moreover, those two DC subsets exhibit distinct T cell stimulatory functions and also regulate the tempo of migration to the MLN upon infection, resulting in the generation of distinct memory CD8$^+$ T cell subsets in both lymphoid and nonlymphoid tissues (11,35). In brief, CD103$^+$ respiratory DCs that reside between/below epithelial cells possess the specialized ability to uptake apoptotic cell-associated antigens (e.g., virus-infected cells) and cross-present them on the MHC class I molecules (20,43,46). Upon infection, migration of antigen-laden CD103$^+$ respiratory DCs to the MLN dominates the early stage of infection (2–4 days) (36,60). These CD103$^+$ respiratory DCs express high levels of the costimulatory molecule CD24, which has been decorated with high mobility group box 1 (HMGB1), a damage-associated molecular pattern (DAMP) released from dying cells, thereby providing strong stimulatory signals to CD8$^+$ T cells through its ligand, receptor for advanced glycan end-products (RAGE) (61). CD8$^+$ T cells activated by these CD103$^+$ respiratory DCs proliferate vigorously and become potent effector cells that preferentially home back to the lung to eliminate virally infected cells (36,52). Since entry into the peripheral tissues is necessary for subsequent differentiation into CD8$^+$ T_{RM} cells, priming with CD103$^+$ respiratory DCs is potentially the primary factor controlling the development of T_{RM} cells. In contrast, CD11b^{hi} respiratory DCs transport and cross-present viral antigens in the MLN at later time points during the infection (5–7 days) (7,60,94). Those include cells that originally reside in the lung interstitium and new emigrants to the lung in response to inflammation (e.g., monocyte-derived DCs) (35). CD11b^{hi} respiratory DCs uniquely upregulate CD70, the ligand for CD27, in response, in part, to thymic stromal lymphopoietin (TSLP) secreted by virus-infected lung epithelial cells (136), and are thus capable of providing costimulatory signals to CD8$^+$ T cells (7). Several lines of evidence support the notion that CD11b^{hi} respiratory DCs contribute less to antiviral CD8$^+$ T cell immunity than CD103$^+$ respiratory DCs because only the selective loss of CD103$^+$ respiratory DCs leads to a severe reduction in the antigen-specific CD8$^+$ T cell responses (36,46,60,100). The weaker stimulatory potential of CD11b^{hi} respiratory DCs presumably explains their characteristics: preferential generation of the memory CD8$^+$ T cell population that persists in the secondary lymphoid organs, rather than generation of fully differentiated effector CD8$^+$ T cells (61). Thus, the distinct functionality of respiratory DC subsets critically impacts the memory CD8$^+$ T cell heterogeneity.

A study by Mikhak *et al.* has suggested that lung DCs are capable of imprinting CD4$^+$ T cell homing to the lung through selective upregulation of CCR4 (93). However, since lung DCs from naive animals that are expanded by injection of fms-like tyrosine kinase 3 ligand (Flt3L) have been demonstrated in this study, it is unclear which DC populations contributed to the lung imprinting. Considering the migratory property of activated T cells to the lung, it is likely that DCs purified from Flt3L-treated lung tissues largely contain lung-resident CD103$^+$ DCs, but not CD11b^{hi} DCs. It is interesting to speculate whether lung imprinting signals could also affect CD8$^+$ T cells.

Recruitment of CD8$^+$ T Cells to the Lung

New definitions provided by intravascular staining

Intravascular (i.v.) staining of cells in the bloodstream by i.v. injection with specific antibodies before harvesting the cells has revolutionized the analysis of lung T_{RM} (5,6). Since the lung is a highly vascularized organ, it became apparent that a majority of CD8$^+$ T cells purified from the lung tissues in earlier studies were contaminants from the blood. In fact, >95% of total CD8$^+$ T cells in naive animals and $\sim$50% of antigen-specific memory CD8$^+$ T cells in mice that had recovered from an influenza virus infection were found to be derived from the lung vasculature (6,126). Note that because the lung airways are segregated from the blood vessels, i.v. staining has essentially no impact on the cells in these tissues. However, a careful reinterpretation of previously published data regarding cells in the lung interstitium/parenchyma analyzed without i.v. staining is required. For instance, it has been reported that antigen-specific CD8$^+$ T cells generated by intraperitoneal infections were efficiently recruited to the interstitium/parenchyma, but not to the lung airways when the

effector T cell numbers peak (9–11 days) even in the absence of progressive infection or inflammation in the lung (122). Furthermore, parabiosis experiments in which pairs of mice are surgically joined revealed that significant numbers of memory $CD8^+$ T cells could also be recruited to the lung as up to half of the cells in this organ were replaced by circulatory $CD8^+$ T cell populations (63,84). By using i.v. staining, those conclusions have been revised. First, although effector $CD8^+$ T cells migrate into the interstitium of normal lungs more efficiently than naive $CD8^+$ T cells (33), the extent is much less than that observed in the presence of infection/inflammation in the lung (123). Second, the migration of circulating memory $CD8^+$ T cells to the lung under steady-state conditions is also relatively limited, as the ratio of new immigrants to resident cells never exceeds 20% (123). On the basis of these new findings, we propose that the migration of effector as well as naive /memory $CD8^+$ T cells into the noninflamed lung be termed as ''basal recruitment'' and be distinguished from ''active recruitment'': migration of antigen-specific effector $CD8^+$ T cells to the lung in response to inflammation in the tissues (Fig. 1). Strict discrimination between basal and active recruitment is important because tissue-derived instructive factors (e.g., antigen and inflammatory stimuli) that dictate T_{RM} differentiation differs significantly depending on how the cells were recruited.

Influence of chemokines on the active and basal recruitment of $CD8^+$ T cells to the lung

Unlike skin and intestines, where specialized adhesion molecules and chemokine receptors regulate selective migration of T cells to those tissues (e.g., integrin $\alpha 4\beta 7$ and

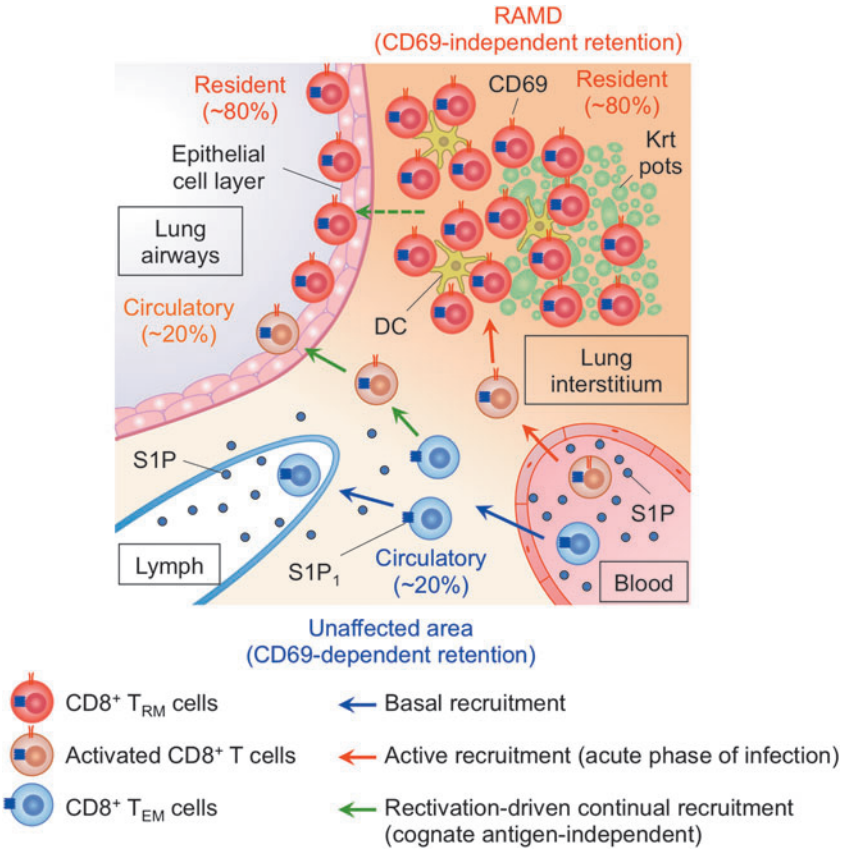

FIG. 1. Compartmentalization of $CD8^+$ T_{RM} cells and $CD8^+$ T_{EM} cells in the lung. Memory $CD8^+$ T cells in the lung consists of a major ($\sim$80%) population of T_{RM} cells and a minor ($\sim$20%) population of T_{EM} cells. During the acute phase of a respiratory virus infection, effector $CD8^+$ T cells are recruited to the lung (active recruitment) and acquire tissue-derived instructions necessary for differentiation into terminal effector cells. $CD8^+$ T_{RM} precursors are recruited to the site of tissue damage during later stages of the infection and receive instructive signals from several factors (such as local antigen and TGF-β) before differentiating into T_{RM} cells. $CD8^+$ T_{RM} niches (RAMDs) are created as a consequence of tissue remodeling and provide temporal spaces for the maintenance of $CD8^+$ T_{RM} cells. $CD8^+$ T_{RM} cells in the RAMDs are maintained in a CD69-independent manner due to spatial separation from the lymphatics. Because $CD8^+$ T_{RM} cells in the lung airways are short-lived, this population may be maintained by the continual recruitment of cells from the $CD8^+$ T_{RM} pool in the lung interstitium/parenchyma. $CD8^+$ T_{EM} cells are recruited to the uninfected lung interstitium during steady state (basal recruitment). Those cells are segregated from the $CD8^+$ T_{RM} niches and residual antigen-presenting cells in the lung, thereby causing them to exit from this tissue through the lymph in response to S1P-induced chemotactic signal. Some cells are activated by antigen-independent stimulus in the interstitium and transiently express CD69. CD69-mediated inhibition of $S1P_1$ leads to temporal retention of $CD8^+$ T_{EM} cells in the interstitium, which potentially enable subsequent recruitment of cells to the lung airways. RAMDs, repair-associated memory depots; S1P, sphingosine 1-phosphate; T_{EM}, effector memory T; TGF-β, transforming growth factor-β; T_{RM}, tissue-resident memory T.

CCR9 for the intestines, and cutaneous lymphocyte-associated antigen [CLA], CCR4, and CCR10 for the skin, respectively), molecules that specifically regulate T cell trafficking to the lung have not yet been reported. Instead, general factors such as lymphocyte function-associated antigen-1 (LFA-1) (125) and inflammatory chemokine receptors CCR5 and CXC chemokine receptor 3 (CXCR3) have been shown to be involved (134).

CCR5 ligands are constitutively expressed in the normal lung and regulate the basal recruitment of CCR5$^+$ effector CD8$^+$ T cells to the interstitium (33). Upon respiratory virus infection, the expression of CCR5 binding chemokines as well as CXCR3 binding chemokines is upregulated in the lung (64,134), and various cell types are involved in the secretion of these chemokines, including epithelial cells, DCs, macrophages, endothelial cells, and mast cells (22,99,124). CCR5 is also transiently expressed on the surface of antigen-experienced CD8$^+$ T cells in the circulation shortly after respiratory virus infection (peaking at day 2 postinfection), and this upregulation is probably induced by proinflammatory cytokines (64). Since only limited numbers of virally primed antigen-specific CD8$^+$ T cells exist at this time point, CCR5-mediated active recruitment of cells to the lung airways is antigen independent (23). This influx (3–5 days) is a part of acute response during respiratory virus infections (44), and antigen-nonspecific memory CD8$^+$ T cells recruited to the lung airways provide ''innate'' protection (64). Nevertheless, the lack of CCR5 alone has essentially no impact on the active recruitment of expanded antigen-specific effector CD8$^+$ T cells to the lung (5–10 days) (30,66), suggesting the redundancy of signals through various inflammatory chemokine receptors in this process.

In contrast to CCR5, CXCR3 is expressed in a large fraction of antigen-specific effector as well as memory CD8$^+$ T cells, and plays a major role in the active recruitment of those cells to the inflamed lung (30,77,111). After entry into the lung, CXCR3 ligands guide effector CD8$^+$ T cells to the sites of infection/inflammation in the lung and accelerate effector maturation (66). Some cells maintain the expression of CXCR3 and are preferentially recruited to the vicinity of virus-infected epithelial cells and airway lumen, while cells that receive signals from IL-12 and IL-15 downregulate CXCR3 and are retained in the peribronchioarterial area where they provide a ''second wave'' of protection (1). Interestingly, Lim *et al.* have demonstrated that neutrophils that infiltrated the lung during the early phase of infection (around day 4) leave long-lasting trails of CXCL12 that guide and accelerate the migration of effector CD8$^+$ T cells to the lung airways in a CXCR4-dependent manner (76). Thus, CXCR3 and CXCR4 cooperatively regulate the active recruitment of cells into the inflamed lung. CXCR3 also contributes to the basal recruitment of memory CD8$^+$ T cells to the lung airways in the absence of any infection in the lung (116). This feature may reflect, in part, the superior functionality of CXCR3^{hi} memory CD8$^+$ T cells in mounting recall responses against respiratory virus infection (45).

It has also been reported that CXCR6$^+$ memory CD8$^+$ T cells accumulate in the lung following intranasal, but not intradermal delivery of antigen (72,73). In fact, the expression of CXCL16 is strongly enhanced in response to inflammatory stimuli, thereby accelerating the active recruitment of effector T cells into inflamed tissues (2,38,85). Interestingly, this chemokine is constitutively expressed in the lung (18,127). These observations strongly suggested that CXCR6 contributes to both active and basal recruitment of CD8$^+$ T cells to the lung.

Temporal Retention and Positioning of CD8$^+$ T Cells in the Lung

Inhibition of tissue egress

It is well known that sphingosine 1-phosphate (S1P) plays a key role in the egress of lymphocytes from the lymph nodes and thymus (4,86), and recent studies indicate that this is also the case for the nonlymphoid tissues (71,78,115,123). S1P is present at high levels in the blood and lymph, which results in the continuous downregulation of its receptor S1P receptor-1 ($S1P_1$) on circulating T cells (102,110). Upon tissue entry, CD8$^+$ T cells instantly recover surface expression of $S1P_1$ due to the relatively low concentration of S1P in the parenchymal tissues (15) and are thereby subjected to opposite chemotaxis toward the lymphatics (78,115). Hence, a balance between the S1P-mediated exit signal and the chemokine-mediated migratory signals controls the direction of T cell migration, which potentially reflects the differential dynamics between T_{EM} and T_{RM} cells within the peripheral tissues.

The T cell expression of $S1P_1$ is regulated by at least two distinct mechanisms: local cytokine-induced transcriptional downregulation of *S1pr1*, which encodes $S1P_1$ (115), and the activation maker CD69-mediated posttranscriptional antagonism (8,114). Under the basal recruitment (in the absence of strong chemokine signaling in the tissues), forced expression of $S1P_1$ or genetic deletion of CD69 results in the significant loss of tissue-circulating CD8$^+$ T cells as well as CD8$^+$ T_{RM} precursors in the nonlymphoid tissues, including the lung (78,115,123), which is likely due to the accelerated tissue egress mediated by $S1P_1$. Importantly, although pharmacological inhibition of $S1P_1$ by the agonist FTY720 leads to transient accumulation of CD69$^-$ CD8$^+$ T cells in the lung, this was reversed when FTY720 treatment was discontinued (123). These findings suggest that T cells are committed to leave tissues during steady-state conditions, unless $S1P_1$ expression is inhibited. Since recruitment of CD8$^+$ T cells into the lung does not lead to the downregulation of *Klf2* or its downstream target *S1pr1* (123), local reactivation and subsequent upregulation of CD69 are likely crucial for the temporal retention of tissue-circulating CD8$^+$ T cells in the lung under the basal recruitment conditions (Fig. 1). In contrast, we have demonstrated that this is not the case during the acute phase of infection where the lack of CD69 has a little impact on the active recruitment of effector CD8$^+$ T cells to the lung unless a CD69-intact wild-type competitor T cell is present (123). Thus, during the acute phase of infection, inflammation-induced chemotactic signaling overrides the S1P-mediated exit signal, which may explain the appearance of CD8$^+$ T cells specific for unrelated antigens in the lung (23,123).

CCR7 is also known to regulate the egress of effector CD8$^+$ cells from the lung interstitium through the lymph (12,19,56). This is consistent with the fact that activated respiratory DCs migrate to the MLN in a CCR7-dependent manner (39,42). As is the case with $S1P_1$, antigen recognition in the lung leads to downregulation of CCR7 on effector

$CD8^+$ T cells, suggesting that $S1P_1$ and CCR7 cooperatively accelerate the egress of antigen-specific $CD8^+$ T cells from the lung. It is also noteworthy that antigen-dependent selective retention of effector $CD8^+$ T cells may prevent overt pathogenesis by decreasing the number of bystander T cells at the site of infection (56). However, as most CCR7 should be already downregulated when expanded effector $CD8^+$ T cells leave the MLN and all of previous data were analyzed in the presence of large proportions of cell contaminants in the blood, analysis using i.v. staining would be required to precisely understand the role of this chemokine receptor.

Integrin-mediated retention in the specific microenvironment of the lung

In addition to CD69, high levels of integrin $\alpha 1\beta 1$ (VLA-1: very late antigen-1) and $\alpha E\beta 7$ (detected by CD49a and CD103, respectively) expression are unique hallmarks of $CD8^+$ T_{RM} cells in the lung compared to circulatory memory $CD8^+$ T cell populations in the lymphoid and nonlymphoid tissues, including T_{EM} cells in the lung (123). VLA-1 preferentially binds to type IV and type I collagen (40,58,128). Type IV collagen constitutes the major structural component of basement membranes of the vascular endothelium and airway epithelium, while type I collagen is widely distributed in the lung interstitium (104). In contrast, integrin $\alpha E\beta 7$ binds to E-cadherin, which forms adherens junctions between lung airway epithelial cells (98). Analyses using blockade antibodies or knockout mice have revealed that both VLA-1 and integrin $\alpha E\beta 7$ are not required for the active recruitment of $CD8^+$ T cells to the lung (74,103). Instead, these integrins fine-tune the distribution of migrant $CD8^+$ T cells within the lung. For example, VLA-1 promotes distribution of cells in close proximity to the basement membranes of the blood vessels as well as the airways (103,104). The binding of VLA-1 to type IV collagen, in combination with signaling through tumor necrotic factor (TNF) receptor II, also protects effector $CD8^+$ T cells in the airways from apoptosis during the acute phase of infection (105). Interestingly, compared to effector $CD8^+$ T cells, effector $CD4^+$ T cells recruited to the lung exhibit lower VLA-1, but higher VLA-2 (integrin $\alpha 2\beta 1$ detected by CD49b), which prefer type I, but not type IV collagen, allowing $CD4^+$ T cells to localize preferentially in the lung interstitium, but not in the airways (104). Integrin $\alpha E\beta 7$ also promotes retention of effector $CD8^+$ T cells in the lung airways (51,74). It is important to note, however, that such integrin-mediated retention signals are likely redundant or supportive as the lack of one of those integrins does not lead to the significant loss of effector $CD8^+$ T cells in the lung airways (74,103).

It is currently unclear what signals are responsible for the upregulation of VLA-1 on $CD8^+$ T cells. Antigen-specific $CD8^+$ T cells in nonlymphoid tissues are mostly VLA-1^+, indicating the universal role of this integrin in the retention of cells that are ''basally'' recruited to the nonlymphoid tissues (103). Interestingly, large numbers of VLA-1^+ $CD8^+$ T cells accumulate in the lung following respiratory, but not systemic infections/vaccinations (108,123). Furthermore, local inflammation in the presence of cognate antigen, but not inflammation alone, enables the conversion of circulating $CD8^+$ T cells into VLA-1^+ $CD8^+$ T_{RM} cells in the lung (123), suggesting the impact of local reactivation on the upregulation or maintenance of VLA-1 expression on actively recruited $CD8^+$ T cells to the inflamed nonlymphoid tissues. In support of this, CD49a expression defines a marker of $CD8^+$ T_{RM} cells having a highly cytolytic potential (13). However, additional experiments are required to precisely resolve the question where and when upregulation of VLA-1 occurs. On the other hand, transforming growth factor-β (TGF-β) signaling is known to be necessary for the upregulation of integrin $\alpha E\beta 7$ as lack of this signal results in the complete loss of $CD103^+$ $CD8^+$ T cells in the lung (51,133). $CD4^+$ T cell-derived interferon-γ (IFN-γ) potentially contributes to the secretion of TGF-β in the lung, thereby helping $CD8^+$ T cell retention in the airways by inducing the expression of CD103 (70). Based on these data, the upregulation of integrins in the lung and the consequential regulation of tissue distribution of the effector T cells could also be considered a consequence of local tissue-derived instruction that promotes T_{RM} differentiation.

Differentiation of Lung $CD8^+$ T_{RM} Cells

Second hit with cognate antigen in the lung

It is well established that effector $CD8^+$ T cells recruited to the lung subsequently encounter respiratory DC subsets that present cognate antigen. This ''second hit'' with cognate antigen at the site of infection improves the cell capacity to secrete IFN-γ (88) and induces additional rounds of proliferation (87). In addition to stimulating the $CD8^+$ T cells, respiratory DCs trans-present IL-15 to prevent rapid apoptosis of the cells (89). While these antigen stimulation processes regulate terminal effector differentiation, several studies have indicated that local antigen recognition also serves as a primary tissue-derived instructive factor requisite for effective T_{RM} differentiation (9,59,90,97,123,133).

In the case of surface and mucosal tissues, such as skin and vagina, $CD8^+$ T_{RM} cells can be generated independent of cognate antigen. For example, forced recruitment of cells to the epithelial tissues by antigen-independent local inflammation or topical chemokine administration results in the establishment of $CD8^+$ T_{RM} cells (81,113), a method known as ''prime-pull.'' Furthermore, basal levels of $CD8^+$ T_{RM} cells can be deposited in multiple nonlymphoid tissues even after systemic infection in the absence of any local antigen presentation or inflammation (115,119). It should be noted that such bystander deposition of $CD8^+$ T_{RM} cells does not occur in the lung. As we have recently demonstrated, $CD8^+$ T cells actively recruited to the lung by antigen-independent inflammation completely disappear after resolution of inflammation in the lung (123). In contrast, the combination of local inflammation and cognate antigen successfully promotes lung $CD8^+$ T_{RM} cell development (123). Thus, the lung is a unique tissue where local antigen is required for the establishment of $CD8^+$ T_{RM} cells. This appears to be based on the structural differences between lung and other surface/mucosal tissues, which will be discussed in detail later. Despite the fact that $CD8^+$ T_{RM} cells can be established in the skin independent of cognate antigen, there is a significant increase in the T_{RM} formation when antigen is present (59,97), indicating that although local antigen recognition is not necessary for the establishment of T_{RM} in all tissues, it nevertheless enhances T_{RM} deposition. Hence, a second hit with cognate antigen in the

peripheral tissues plays a key role not only for terminal effector differentiation but also function as pivotal tissue instruction for T_{RM} differentiation.

It is not understood how signals elicited by the local antigen can induce either terminal effector differentiation or T_{RM} development. How is this decision checkpoint regulated? There are at least four potential explanations. First, the decision to become a terminal effector or T_{RM} cell following local reactivation may be cell-intrinsically precommitted before recruitment, perhaps reflecting whether the cells originated from short-lived effector cells (SLECs) or memory precursor effector cells (MPECs) (57,80). Second, as previously described, a division of labor between respiratory DC subsets may also take place even within the lung, as CD103$^+$DCs, but not CD11b^{hi} DCs, preferentially drive CD103 expression upon CD8$^+$ T cell activation (133). Third, fate decisions between terminal effector and T_{RM} may be determined by temporal deviation of reactivation (16). For example, in the case of CD4$^+$ T cells, McKinstry *et al.* have shown that late antigen recognition, which is necessary for memory formation, occurred at days 5–8 postinfection, and have termed this time window as the ''memory check point'' (90). Whereas a second hit for terminal effector differentiation may occur a little earlier, as cognate antigen-presenting respiratory DCs were transferred intranasally on day 3 and analyzed by day 5 (88). Actually, this third hypothesis is mainly attributed to the fourth hypothesis in which effector versus memory fate decision may be regulated by the strength of CD8$^+$ T cell activation. Both, the levels of antigen presentation (17) as well as the levels of the ''third signal'' induced by inflammatory cytokines (14) influence the activation status. Such antigenic as well as inflammatory signals may be redundant at early phases of infection, which bias CD8$^+$ T cell differentiation toward terminal effector cells, while weak signaling at later time points preferentially promotes memory differentiation (16). Distinct microdistribution of CD8$^+$ T cells also influences differential acquisition of the activation signals. For example, CXCR3^{hi} cells receive stronger stimulatory signals and preferentially become terminal effectors, while lack of this receptor puts cells away from the core of inflammatory microenvironment, and ultimately promotes memory differentiation (50,66,69). All those factors must cooperatively regulate CD8$^+$ T cell fate following late antigen recognition.

There is strong evidence that CD8$^+$ T_{RM} precursors receive bona fide TCR signaling in the peripheral tissues since the cells exhibit high levels of Nur77 expression (9,59). However, the molecular mechanisms by which TCR signaling-induced events elicit T_{RM} formation remain unclear. CD4$^+$ T cells produce IL-2 in response to late antigen recognition, and autocrine IL-2 signaling at the memory check point improves memory CD4 T cell survival in the spleen, MLN, and lung, (90) suggesting it as a potential mechanism. Another observation is that inhibition of the mammalian target of rapamycin (mTOR) during the early phase of infection selectively impairs the formation of CD8$^+$ T_{RM} cells in the small intestine, while simultaneously enhancing memory generation in the spleen (118), suggesting that mTOR expression in response to local reactivation may play a role in the T_{RM} differentiation by modulating the metabolic status. Clearly, a great deal more study is required to understand how local antigen restimulation optimizes CD8$^+$ T_{RM} formation.

Local cytokine-mediated instruction

In addition to late antigen recognition, local cytokine signaling is also crucial for the formation of CD8$^+$ T_{RM} cells in the lung. TGF-β is produced by a wide variety of cell types in the lung, including alveolar macrophages, neutrophils, activated alveolar epithelial cells and endothelial cells (31). Although TGF-β is known as a profibrotic cytokine and its overproduction is critically associated with pulmonary fibrosis (31), influenza virus-infected animals recover without the acquisition of lung fibrosis (68,123), suggesting that TGF-β production as well as its activation is rather stable during the course of infection. This is true despite the fact that some strains of influenza virus surface protein can activate latent TGF-β in the lung (109). Local TGF-β signaling does not require Sma- and Mad-related protein 4 (Smad4) (51), and plays a key role in the downregulation of T-box transcription factors Eomes and T-bet in effector CD8$^+$ T cells, both of which are required for effector to T_{RM} transition (82). Upon skin CD8$^+$ T_{RM} differentiation, Eomes expression is fully extinguished, while T-box expression remains at a low level, which sustains CD122 expression (IL-15 receptor β-chain) and enables to receive IL-15-medaited survival signal (82). As T-bet imposes repression of CD103, the downregulation of T-bet reciprocally leads to the expression of integrin $\alpha E\beta 7$.

Although the requirement of TGF-β in T_{RM} differentiation seems universal for all nonlymphoid tissues, IL-15 may not be essential for the establishment of CD8$^+$ T_{RM} cells in the lung. In fact, IL-15 production is increased especially in cells purified from the airways and also the lung tissues following influenza virus infection, and lack of IL-15 results in transient reduction in the number of effector CD8$^+$ T cells in the airways at the early phase of infection (7–12 days) (130). However, such reduction is no longer observed at the memory phase of infection without any treatment (131), indicating that the lack of IL-15 essentially has no impact on the ultimate generation of CD8$^+$ T_{RM} cells in the lung.

At later time points during an acute virus infection (around day 7), effector CD8$^+$ T cells recruited to the lung produce a significant amount of IL-10 in response to CD4$^+$ T cell-derived IL-2 and innate lymphoid cell-derived IL-27 (101,120,121). CD8$^+$ T cell production of IL-10 is correlated with its terminal maturation and is vital in preventing excess inflammation in the lung (121). Since IL-10 induces activation of the signal transducer and activator of transcription 3 (STAT3) that promotes memory CD8$^+$ T cell differentiation, it is tempting to speculate that IL-10 produced by terminal effector CD8$^+$ T cells at the memory check point may impact neighbor as well as late-comer CD8$^+$ T_{RM} precursors, and promote memory maturation in the lung.

Niche-dependent establishment of CD8$^+$ T_{RM} cells in the lung

In the case of skin, CD8$^+$ T_{RM} precursors recruited to the skin persist in an epidermal niche that is originally occupied by dendritic epidermal $\gamma\delta$ T cells (DETCs). This results in their lifelong persistence (137). Because normal lung tissues do not exhibit such preformed niches to displace, additional ''space'' is required for the cells to inhabit. It has long been believed that lung CD8$^+$ T_{RM} cells are maintained in the ectopic lymphoid tissues developed in response to respiratory virus infections, such as inducible bronchus-associated

lymphoid tissues (iBALT) (95). However, our group has demonstrated that such structures are primarily populated with $CD4^+$ T cells as well as B cells, but relatively few numbers of $CD8^+$ T cells (123). Rather, $CD8^+$ T_{RM} cells are enriched specifically in niches created at the site of tissue regeneration after injury, which are termed as repair-associated memory depots (RAMDs) (123). Histologically, RAMDs represent confluent foci of peribronchiolar lymphocytic infiltrates with diffuse thickening of alveolar walls in surrounding area. Thus, the niches exist primarily in the lung interstitium with partial extension to the lung parenchyma. The appearance of cytokeratin-expressing cell aggregates, known as Krt pots, is a unique hallmark of RAMDs. Krt pots comprise distal airway stem cells that begin to emerge in the lung around day 7 postinfection, proliferate vigorously, and subsequently differentiate and reconstruct the damaged lung tissues (68,129,140). However, it is unclear whether those cells directly impact the differentiation of $CD8^+$ T_{RM} cells. $CD8^+$ T_{RM} cells in the RAMDs do not form a specific organized structure and are simply sequestered in this site, while $CD4^+$ T_{RM} cells in the iBALT typically form clusters and surround B cell follicles (123). Such distinct distributions between $CD8^+$ and $CD4^+$ T_{RM} cells in the lung clearly reflect their division of labor upon recall, in which $CD8^+$ T_{RM} cells exert their function as cytotoxic T lymphocytes (CTLs) at the damaged site, while $CD4^+$ T_{RM} cells and B cells need to interact with each other in the iBALT for sustained germinal center formation (3). There is also rigid compartmentalization between lung $CD8^+$ T_{RM} cells and $CD8^+$ T_{EM} cells that circulate between the lung and blood. For instance, $CD8^+$ T_{EM} cells in the lung are widely, but sparsely distributed in the unaffected lung interstitium, and never involved in the RAMDs unless *de novo* niches are newly created (123). As described previously, $CD8^+$ T_{EM} cells exit lung tissues mainly through S1P-induced chemotaxis to the lymph. In contrast, inhibition of $S1P_1$ is no longer required for the retention of $CD8^+$ T_{RM} cells in the RAMDs due, in part, to limited access to S1P gradient in this microenvironment (123). Importantly, not only tissue-circulating $CD8^+$ T_{EM} cells but also effector $CD8^+$ T cells are incapable of being involved in the RAMDs later than the peak of $CD8^+$ T cell response in the lung (around day 10, which also reflects the peak of tissue damage) (123). Because administration of cognate antigen in combination with the prime-pull strategy enables *de novo* creation of the RAMD and subsequent establishment of $CD8^+$ T_{RM} cells in the lung (123), the availability of cognate APCs in the RAMDs likely restricts the numbers of $CD8^+$ T cells deposited. Indeed, there is a competition among antigen-specific effector $CD8^+$ T cells to interact with cognate APCs in the inflamed microenvironment (97). Interestingly, this competition occurs even between effector $CD8^+$ T cells with distinct specificities when epitopes specific for those effectors are presented on the same DCs, thereby shaping the local repertoire (97). Such conventional- as well as cross-competition of local antigens may explain the selective deposition of $CD8^+$ T_{RM} cells expressing high-affinity TCRs (32).

Maintenance of Lung $CD8^+$ T_{RM} Cells

A revised theory on continual recruitment and permanent deposition

A primary definition of T_{RM} cells is that they are maintained in the lymphoid and nonlymphoid tissues without recirculation. This definition clearly applies to lung airway memory $CD8^+$ T cells because, once recruited, those cells do not return to the circulation or the lung interstitium (47). However, maintenance of this population differs significantly from that of $CD8^+$ T_{RM} cells in other mucosal tissues. For example, although lung airway memory $CD8^+$ T cells are not highly apoptotic, it has demonstrated that the half-life of this population is $\sim$2 weeks (24). Such a short lifespan is likely due to the biophysical effects of the harsh airway environment: cells are being cleared by phagocytic cells or removed through mucociliary clearance. Furthermore, airway memory $CD8^+$ T cells do not proliferate (47). Based on these findings, a concept emerged that memory $CD8^+$ T cells in the airways are continuously replaced by new cells recruited from the circulation as a process of memory T cell maintenance. This was confirmed by the continuous appearance of LFA-1^+ cells in the airways even in the situation that memory $CD8^+$ T cells downregulate LFA-1 within 48 h after entry into the airways (24). Importantly, however, our recent findings have confirmed that only a limited number of cells in the lung airways are continually replaced by cells from the circulation. In brief, ratios of memory $CD8^+$ T cells recruited from the circulation to the airways peaked at only $\sim$20% by 2 weeks after parabiotic surgery, and this ratio was maintained for up to 7 weeks (123). Thus, while $\sim$20% of memory $CD8^+$ T cells in the airways are new recruits, $\sim$80% are obviously segregated from blood-born memory $CD8^+$ T cells, just like T_{RM} cells in other mucosal tissues. This raised a question whether the latter cell population can survive for long in the harsh airway environment. We speculate that continual recruitment is also essential for $\sim$80% of memory $CD8^+$ T cells in the airways. If this is the case, their source may be $CD8^+$ T_{RM} pools in the lung interstitium/parenchyma, but not memory $CD8^+$ T cells in the circulation (Fig. 1).

As is the case with cells in the lung airways, memory $CD8^+$ T cells in the lung interstitium/parenchyma consist of at least two distinct memory T cell subpopulations: $\sim$80% bona fide T_{RM} cells present in the RAMDs and $\sim$20% T_{EM} cells present the lung interstitium (123) (Fig. 1). In the steady state in the lung, there is a balance between basal recruitment-mediated influx and S1P-mediated efflux through the lymph that maintains the flat ratio of $CD8^+$ T_{EM} cells (Fig. 1). We hypothesize that a small fraction of cells are activated by antigen-independent inflammatory stimuli, potentially due to exposure with airborne contaminants. These cells upregulate CD69 expression, causing them to transiently persist in the interstitium, and some of them are then recruited to the airways through a process of basal recruitment (Fig. 1). The mechanisms by which $CD8^+$ T_{RM} cells in the RAMDs are maintained remain unclear with possibilities, including homeostatic proliferation or prolonged longevity. There is evidence that $CD8^+$ T_{RM} cells in the lung interstitium/parenchyma and airways are maintained independent of homeostatic cytokine IL-15 (131). This is consistent with the relatively lower level of expression of IL-15 receptor β on memory $CD8^+$ T cells in the lung compared to those in the spleen (112). Nevertheless, these findings do not exclude a possibility that other factors may drive homeostatic turnover of $CD8^+$ T_{RM} cells in the lung RAMDs, such as residual antigen-induced reactivation. Although numerous questions remain in this field, the discovery of specific niches in the lung interstitium/parenchyma has substantial implications in

understanding the factors regulating the maintenance of CD8$^+$ T_{RM} cells in the lung.

Niches and residual antigen: factors that potentially restrict the numbers of CD8$^+$ T_{RM} cells maintained in the lung

Following a respiratory virus infection, the number of antigen-specific CD8$^+$ T cells in the lung peaks on day 10 and then declines dramatically as infection subsides. This is followed by the establishment of CD8$^+$ T_{RM} cells at around 1 month postinfection. The absolute number of CD8$^+$ T_{RM} cells in the lung wanes over time, which results in a decrease in the protective efficacy of these cells against secondary infection with a homologous virus (67). For example, CD8$^+$ T_{RM} cell-mediated protective immunity is essentially lost at 4–6 months postinfection (135). This contrasts with the situation in the skin where CD8$^+$ T_{RM} cells can persist up to 1 year (137). The shorter lifespan of lung CD8$^+$ T_{RM} cells could be explained by our recent findings regarding the niche-dependent maintenance of CD8$^+$ T_{RM} cells in the lung (123). As described above, CD8$^+$ T_{RM} cells are predominantly accumulated in the RAMDs: disorganized peribronchiolar foci that are temporarily created at the site of tissue damage. In fact, peribronchiolar foci still remain in the lung at a month postinfection, despite the fact that inflammatory responses have largely abated at this time point. As tissue regeneration proceeds, the size of the RAMDs shrinks over time and tends to disappear several months postinfection. Thus, we suggest that the decrease in numbers of CD8$^+$ T_{RM} cells in the lung for the first couple of months simply depends on the size of the RAMDs. We also suggest that an organized lymphoid structure like iBALT persists for longer periods and low numbers of CD8$^+$ T_{RM} cells persist in the iBALT following disappearance of RAMDs. This hypothesis is based on the idea that lung tissues do not initially have preexisted niches in which T cells can persist. Thus, the progressive loss of temporarily created ''spaces'' significantly restricts the long-term maintenance of CD8$^+$ T_{RM} cells.

As discussed above, cognate antigen that remains in the RAMDs is also a potential factor regulating the number of CD8$^+$ T_{RM} cells in the lung. In fact, viral antigen can be detected in the peribronchiolar lymphocytic infiltrates (62) as well as bronchial epithelial cells (41) at least a month postinfection. Furthermore, CD8$^+$ T_{RM} cells, but not T_{EM} cells, express CD69 as well as PD-1, indicative of recent activation (123,135). Those observations suggest that residual antigen presentation is limited in the RAMDs, but not in the unaffected lung interstitium. Thus, the reduced CD8$^+$ T_{RM} persistence in the RAMDs is also potentially caused by reduction in the level of residual antigen presentation. Importantly, despite the fact that PD-1 impairs the protective efficacy of memory CD8$^+$ T cells in the lung (28,92), accumulating evidence suggests that these cells never succumb to functional exhaustion (25,44). Thus, the level of residual antigen presentation must be lower than that exhibited during a typical chronic infection. In line with this, PD-1 as well as other potential inhibitory molecules may act to prevent excessive immunopathology (26,27,29) by maintaining the cells in a quiescent state (49). Furthermore, reactivation of CD8$^+$ T_{RM} cells in the lung leads to sustained expression of interferon-induced transmembrane protein (IFITM3), which is involved in conferring resistance against subsequent virus infection (132). Hence, in contrast to chronic infection, the repeated acquisition of weak cognate signals may be beneficial rather than harmful for the maintenance of CD8$^+$ T_{RM} cells in the lung. A remaining question is how APCs avoid being eliminated by antigen-specific CD8$^+$ T_{RM} cells. It is tempting to hypothesize that PD-1-mediated partial inhibition may play a role in this escape without inducing the global exhaustion.

It has also demonstrated that residual antigen presentation persists in the MLN for several months after acute respiratory virus infections (54,55,62,75,122,138). Because memory CD8$^+$ T cells in the MLN and the lung airways exhibit similar activated phenotypes, it has been suggested that reactivation by residual antigen in the MLN induces the migration of memory CD8$^+$ T cells from the MLN to the lung airways, resulting in the continual influx of cells to the airways (138). In other words, reactivation in the MLN induces phonotypic changes in memory T cells from lymph node-surveying T_{CM} to peripheral tissue homing T_{EM} cells (122,138). However, it should be noted that the basal levels of continual recruitment of cells to the airways also occur in the absence of residual antigen (65). Furthermore, memory CD8$^+$ T cells in the circulation gradually lose the expressions of Blimp-1 and Hobit (117), key transcription factors regulating tissue retention (79). As a result, the efficacy of continual (basal) recruitment to the airways wanes over time (117). One should also be mindful of the fact that signature markers of T_{RM} cells, such as CD69 and CD103, could be upregulated on lung-circulating blood-born CD8$^+$ T_{EM} cells in certain tissue environments (65) or basal levels of TNF secreted in the lung (117). However, it is still unclear whether T_{EM}-derived CD69$^+$CD103$^+$ cells acquire certain tissue residency. (117,123) Thus, a more precise analysis of migratory property is required to define CD8$^+$ T_{RM} cells in the lung. Nevertheless, lung-circulating CD8$^+$ T_{EM} cell populations should not be neglected as those populations could be majority when bona fide T_{RM} cells disappeared (117), and have an ability to contribute, in part, to the protective immunity upon rechallenge (116).

Concluding Remarks

Ultimately, a better understanding of CD8 memory in the lung is essential for the development of safe and effective vaccines capable of generating long-lasting antigen-specific memory CD8$^+$ T cells. Despite a great deal of progress in understanding CD8$^+$ T cell memory in the lung and recent success in generating lung CD8$^+$ T_{RM} cells by vaccination (37,133,139), our identification of specific niches for CD8$^+$ T_{RM} cells in the lung and other data raises a fundamental possibility that lung CD8$^+$ T_{RM} cells are by necessity short lived (e.g., several months). This is primarily due to the lack of preexisting CD8$^+$ T_{RM} niches in the lung and the short-lived nature of these niches. Furthermore, peribronchiolar foci fill alveolar spaces, thereby reducing the efficacy of gas exchange, indicating that a risk (tissue damage and resultant functional impairment) is unavoidable to create niches for lung CD8$^+$ T_{RM} cells. Thus, long-term maintenance of CD8$^+$ T_{RM} cells in the lung and repeated tissue damage are two sides of the same coin, and future study should be focused on the balance between protective efficacy and immune

pathology when considering the vaccines that target $CD8^+$ T_{RM} cells in the lung.

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