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Memory T Cell Populations in the Lung Airways Are Maintained by Continual Recruitment

Kenneth H. Ely, Tres Cookenheim, Alan D. Roberts, and David L. Woodland

Effector memory T cell populations in the periphery play a key role in cellular immune responses to secondary infections. However, it is unclear how these populations are maintained under steady-state conditions in nonlymphoid peripheral sites, such as the lung airways. In this study, we show that LFA-1 expression is selectively down-regulated following entry of memory T cells into the lung airways. Using Sendai virus as a mouse model of respiratory virus infection, we use LFA-1 expression levels to demonstrate that effector memory T cell populations in the lung airways are maintained by continual recruitment of new cells from the circulation. The rate of memory cell recruitment is surprisingly rapid, resulting in replacement of 90% of the population every 10 days, and is maintained for well over 1 year following viral clearance. These data indicate that peripheral T cell memory is dynamic and depends on a systemic source of T cells. The Journal of Immunology, 2006, 176: 537–543.

The CD8⁺ T cell population plays a central role in the control of acute respiratory virus infections through the secretion of antiviral cytokines and lysis of infected lung epithelial cells (1–3). Following resolution of the infection, populations of long-lived memory CD8⁺ T cells are established in secondary lymphoid organs, such as the spleen and lymph nodes, and in a wide range of peripheral sites, including the lung airways (4–6). In addition, de novo bronchus-associated lymphoid tissues are established in the lung parenchyma that support functional germinal centers and can substitute for conventional lymph nodes (7). Secondary virus infection results in an accelerated recall response due to the increased numbers and enhanced activation state of memory T cells (2, 5). Memory cells in the lung airways are typically of the effector memory phenotype and are able to generate an immediate response to the virus when viral loads are relatively low (8). Memory cells in the secondary lymphoid organs typically display a central memory phenotype and expand during the recall response to generate a supply of new effector cells that are subsequently recruited to the lung airways (9).

The factors affecting the maintenance of memory T cell subpopulations in various anatomical sites are incompletely understood. The numbers of memory cells in the secondary lymphoid organs appear to be stably maintained by a process of IL-15- and IL-7-driven homeostatic proliferation (10–16). In contrast, the numbers of memory cells at peripheral sites can vary with time, but the factors that regulate this are unclear. In the case of lung airway memory CD8⁺ T cells, there is an initial decline in the number of Ag-specific cells that subsequently stabilizes 6 mo postinfection (5, 17). There is a similar decline in the number of memory CD4⁺ T cells in the lung airways (18).

Recently, Klonowski et al. (19) investigated the trafficking of memory CD8⁺ T cell populations to various peripheral tissues using parabiotic mice. These studies suggested that memory cell populations could be established in some sites by a process of recruitment that is independent of inflammation or G-coupled receptors. Moreover, T cell recruitment to the intestinal mucosa and the brain was delayed, suggesting that these compartments may have restricted entry requirements. However, it was not clear from these studies how memory T cells are maintained over the long-term in peripheral sites that lack any form of organized lymphoid tissues, such as the lung airways.

An interesting characteristic of effector memory CD4⁺ and CD8⁺ T cells in the lung airways is that the majority of cells express low levels of LFA-1 (CD11a/CD18). This feature distinguishes lung airway memory cells from other memory T cell populations in the animal. In this study, we demonstrate that down-regulation of LFA-1 expression occurs on memory T cells following migration into the lung airways, and can be used as a marker of recent recruitment into this compartment. We have used this marker to determine the minimal rate of recruitment of memory T cells to the lung airways under steady-state conditions, and demonstrate that both CD4⁺ and CD8⁺ effector memory cells are maintained by continual recruitment of new cells.

Materials and Methods

Viruses, mice, and infection

Sendai virus (Enders strain) was grown, stored, and titered, as previously described (5). Female C57BL/6 (CD45.2) and B6.SJL-Ptprc<sup>−</sup> Pep3/BoyJ (CD45.1) mice were purchased from The Jackson Laboratory. Animals were housed under specific pathogen-free conditions. Mice (6–8 wk) were anesthetized by i.p. injection of 2,2,2-tribromoethanol and intranasally infected with 250 50% egg infectious doses of Sendai virus. All mouse studies were approved by Trudeau Institute Animal Care and Use Committee.

Tissue harvest and staining

Tissues were harvested and stained, as previously described (5). Lung airway and peritoneal cells were recovered by lavage with HBSS (5 × 1 ml for the airways and 1 × 5 ml for the peritoneum), followed by plastic adherence (1 h at 37°C). Lung tissue, mesenteric lymph nodes, and spleen cells were obtained by straining through nylon mesh and enriching by either centrifugation in a 40/80% Percoll gradient (lung tissue), plastic adherence (mesenteric lymph nodes), or panning on anti-mouse IgG, followed by RBC lysis in buffered ammonium chloride (spleen). Cells were
stained with anti-CD11a PE, anti-CD8 PerCP or anti-CD4 PerCP, and Sendai-nucleoprotein324–332 (NP324–332/Kb) allophycocyanin tetramer or Sendai-hemagglutinin-neuraminidase419–433 (HN419–433/Aβ) Alexafluor-647 multimer. All anti-mouse mAbs used were purchased from either BD Biosciences, eBioscience, or R&D Systems (CD11a, clones M17/4, 2D7; CD18, clone M18/2; CD8, clone 53-6-7; CD44, clone IM7; CD49a, clone Ha 31/8; CD69, clone H1.2F3; Ly-6c, clone HK1.4; CXCX3, clone 220/030). Multimers and tetramers were generated by the Trudeau Institute Molecular Biology Core, as described previously (2, 20). Flow cytometry was performed on a FACSCalibur cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Annexin V staining

Lung airway memory cells were isolated by lavage from mice that had previously recovered from an intranasal Sendai virus infection. Isolated cells were stained with Sen-NP324–332/Kb allophycocyanin tetramers for 1 h, followed by staining with mAb specific for CD8 and CD11a for 30 min. Cells were stained for annexin V using a reagent kit from BD Biosciences. Briefly, cells were washed twice with PBS and then resuspended in 100 μl of 1× binding buffer. A total of 5 μl of FITC-labeled annexin V was added to the cells and incubated for 15 min at room temperature in the dark. Following incubation, an additional 100 μl of binding buffer was added, and flow cytometry was performed on a FACSCalibur cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Adoptive transfers and CpG treatment and vaccination

Isolated donor cells were transferred intratracheally (i.t.) into the lung airways, as described previously (21) and for i.p. at 10⁶ donor cells/250 μl of PBS into the peritoneal cavity of recipient mice. In other cases, isolated CD8-enriched donor splenocytes were transferred i.v. at 2×10⁶ donor cells/200 μl of PBS into mice that had previously recovered from intranasal Sendai virus infection 3–4 mo before. In some cases, the recipient mice were administered 12 μg/μl of PBS of CpG-deoxyoligonucleotides (CpG) (Midland Certified Reagent) intranasally 1 day after transfer. Cells were then recovered at various times thereafter, and host and donor cells were distinguished on the basis of CD45 expression.

CFSE labeling of lung airway resident memory cells

Mice that had recovered from an intranasal Sendai virus infection (30–45 days postinfection) were administered 100 μl of 2 mM CFSE via i.t. instillation with a bent and blunted 20-gauge needle. Labeled lung airway cells were isolated by lavage 24 and 96 h after i.t. administration of CFSE. Isolated cells were stained with NP324–332/Kb allophycocyanin tetramers for 1 h, followed by staining with mAb specific for CD8 and CD11a for 30 min. Flow cytometry was performed on a FACSCalibur cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Results

Ag-specific memory T cells in the lung airways express low levels of LFA-1

Previous studies have demonstrated that CD8⁺ and CD4⁺ T cells specific for the immunodominant NP324–332/Kb and HN419–433/Aβ epitopes, respectively, are established in the lung airways and in multiple lymphoid and nonlymphoid tissues following intranasal Sendai virus infection (5, 8, 18, 22–24). Typically, memory cells express high levels of the LFA-1 (CD11a/CD18) integrin that is expressed high levels on NP324–332/Kb-specific memory T cells in the lung airways (23, 24). This led us to hypothesize that the lung airway environment may influence the down-regulation of CD11a expression on memory T cells after they have been recruited into the airways. To test this possibility, we used a dual adoptive transfer system in which splenocytes from mice that had recovered from Sendai virus infection 45 days prior were transferred both i.t. and i.p. into the same CD45.1 congenic mice (the i.p. transferred population was labeled with CFSE to confirm that there was no migration of cells from the peritoneum to the lung airways). At various times posttransfer, cells were recovered from the lung airways and analyzed for CD11a expression on donor NP324–332/Kb-specific memory T cells. As shown in Fig. 2A (top panel), CD11a expression was down-regulated within 48 h after transfer of cells directly into the lung airways. A more detailed kinetic analysis (Fig. 2B) indicated that the loss of CD11a expression in the airways occurred within 40 h. The down-regulation of CD11a expression on these memory cells was specific for the lung airway environment as the same cells introduced into the peritoneum failed to change CD11a expression (Fig. 2A, middle panel). The down-regulation of CD11a does not appear to be mediated at the level of transcription, as sorted CD11alow airway cells and CD11ahigh memory splenocytes showed equivalent levels of CD11a message by real-time PCR (our unpublished observations). Interestingly, the reduction in CD11a expression on the donor memory T cells was complete inasmuch as we did not see a subset of cells that retained a CD11ahigh phenotype. This contrasted with the situation with lung-resident memory T cells in which a small subpopulation of CD11ahigh memory T cells is always observed (Fig. 1). Similar data were also obtained with adoptively transferred CD4⁺ T cells (our unpublished observations).

To determine whether the loss of CD11a expression on lung airway memory T cells was permanent, we asked whether CD11a...
expression could be restored when the cells were removed from the lung airway environment. As shown in Fig. 2A (bottom panel), NP324–332/Kb-specific memory T cells from the lung airways rapidly increased their levels of CD11a expression when transferred into the peritoneum of naïve mice. This was not due to selective survival of a small subset of CD11ahigh cells in the donor population because greater than 50% of the input population was recovered at 48 h (our unpublished observations). Thus, the lung airway environment influences the expression of CD11a on memory T cells.

Down-regulation of CD11a expression on T cells in the lung airways does not correlate with apoptosis

Previous studies have suggested that memory T cells in the lung airway are unable to re-enter the circulation and are ultimately removed from this site by apoptosis (21). Therefore, we speculated that the decrease in CD11a expression on airway memory T cells may be a feature of cells that have become apoptotic. To examine this possibility, lung airway cells from mice that had recovered from a Sendai virus infection were stained with annexin V, which marks early apoptotic cells. As shown in Fig. 3 (upper panels), only low frequencies of CD11ahigh cells stained with annexin V. Furthermore, annexin V staining was similar in both the CD11ahigh and CD11alow subpopulations (Fig. 3, lower panels), indicating that there was no correlation between the time that the cells had been present in the lung airways and the likelihood that the cells were apoptotic. This is consistent with previous studies showing that flow cytometrically sorted CD11alow lung airway cells are fully functional in terms of their capacity to mediate proliferative and migration responses to virus challenge in adoptive transfer studies (27). Thus, down-regulation of CD11a on lung airway memory cells is not directly linked to apoptosis.

The expression of surface markers on memory T cells is differentially regulated by the lung airway environment

To determine whether other integrins or cell surface molecules were down-regulated in the lung airways, we examined the expression of other surface markers on memory splenocytes introduced into the lung airways by i.t. transfer. As shown in Fig. 4, the expression of other surface molecules including CD49a, CD69, and CXCR3 remain unchanged (or slightly increased) following i.t. transfer of these cells into the lung airways. Thus, down-regulation of CD11a is not a general characteristic of all integrins and activation molecules on the cell surface. However, Ly-6c was down-regulated on memory T cells transferred into the lung airways, albeit with slower kinetics than CD11a.
Memory T cells are recruited to the lung airways under steady-state conditions

Although the data indicated that cells transferred directly from an intranasal Sendai virus infection, as described in Materials and Methods. Isolated cells were enriched for CD8 cells on a negative selection column and subsequently transferred i.t. into naive recipient mice. Recipient mice were sacrificed at the indicated times, and bronchoalveolar lavage was collected and stained for congenic marker, NP₃₂₄₋₃₃₂/Kb tetramers, and specific marker. The data are representative of two separate experiments.

FIGURE 4. Expression of surface markers on lung airway memory cells is differentially regulated. A single cell suspension of splenocytes was prepared from mice that had previously recovered from an intranasal Sendai virus infection, as described in Materials and Methods. Isolated cells were enriched for CD8 cells on a negative selection column and subsequently transferred i.t. into naive recipient mice. Recipient mice were sacrificed at the indicated times, and bronchoalveolar lavage was collected and stained for congenic marker, NP₃₂₄₋₃₃₂/Kb tetramers, and specific marker. The data are representative of two separate experiments.

In situ labeled lung airway cells down-regulate CD11a expression

The previous data indicated that memory T cells that enter the lung airways either by direct transfer (i.t. administration) or through the circulation (i.v. administration) down-regulate CD11a expression. To further confirm that CD11a is down-regulated on memory T cells entering the lung airways under steady-state conditions, we directly labeled lung airway cells in situ with CFSE delivered i.t. As shown in Fig. 6, essentially all of the CD44high or NP₃₂₄₋₃₃₂/Kb-specific lung airways cells recovered 24 h after labeling were CFSE positive, and 21% of these cells expressed high levels of CD11a. Seventy-two hours later, the majority of cells in the lung airways were CFSE negative, suggesting significant recruitment of non-CFSE-labeled cells. Furthermore, the frequency of CFSE labeled that expressed high levels of CD11a was substantially reduced at this time point. Because T cells are not thought to proliferate in the

FIGURE 5. Intravenously transferred memory cells are recruited to the lung airways under steady-state conditions. Splenocytes were isolated and CD8 enriched from CD45.2 mice that had recovered from respiratory virus infection 40–50 days prior. Cells were transferred i.v. into CD45.1 recipient mice that had recovered from Sendai virus infection 4 mo prior. On days 12 (CD44) or 14 (NP₃₂₄₋₃₃₂/Kb) posttransfer, single cell suspensions were obtained from the airways, lungs, and spleens of recipient mice, as described in Materials and Methods. Alternatively, recipient mice were given 12 μg of CpG-oligonucleotides/μl of PBS intranasally 1 day posttransfer, and lung airway cells were isolated 65 h post-CpG treatment (Airways-CpG). Either CD8, CD44, or NP₃₂₄₋₃₃₂/Kb-specific T cells were analyzed for CD45.2 or CD45.1 and CD11a expression by flow cytometry. The numbers in the figure represent the relative frequencies of CD11a low and CD11a high cells in the donor population. The data depict two independent experiments.
lung airways (21, 28), the reduction of CD11a expression is consistent with a conversion from a CD11a\textsuperscript{high} cell to a CD11a\textsuperscript{low} phenotype. Although the rate of loss of CD11a expression in the labeled memory T cell population is lower than that seen in the i.t. transfer study, this difference may be explained by the fact that i.t. CFSE labeling also labels some cells in the lung parenchyma (our unpublished data) that may migrate into the airways. Taken together, the in situ labeling studies confirm that memory CD8\textsuperscript{T} cells in the airways lose CD11a expression over time.

**Memory CD8\textsuperscript{T} cells in the lung airways are maintained by continual recruitment**

Together, the data presented above indicate that CD11a expression is down-regulated in the lung airways and high expression marks recently arrived airway memory cells. We next took advantage of our observations regarding CD11a expression to analyze memory cell recruitment to the lung airways under steady-state conditions at different times post-Sendai virus infection in otherwise unperturbed, unmanipulated mice. As shown in Fig. 7, the percentage of NP\textsubscript{324–332/K\textsuperscript{b}}-specific memory T cells in the lung airways that were CD11a\textsuperscript{high} at 1 mo postinfection was \( \approx 30\% \) (in contrast, essentially 100\% of the memory cells in the lung parenchyma and spleen were CD11a\textsuperscript{high}). Because \( \approx 30,000 \) NP\textsubscript{324–332/K\textsuperscript{b}}-specific cells were in the airways at this time point, this corresponds to \( \approx 9,000 \) NP\textsubscript{324–332/K\textsuperscript{b}}-specific memory T cells that had been recently recruited. If one assumes that it takes 40 h for newly recruited cells to fully down-regulate CD11a (based on the i.t. transfer data) and that recruitment is a stochastic process, 90\% of the memory cells in the airways would be replaced every 10 days. Interestingly, an even higher proportion of NP\textsubscript{324–332/K\textsuperscript{b}}-specific memory T cells in the lung airways was CD11a\textsuperscript{high} (50\%) at 13 mo postinfection (Fig. 7). This indicates that even at late times postinfection, the population of memory cells in the airways is highly dynamic and maintained at the population level by the constant recruitment of new cells. However, the absolute rate of recruitment at this time is decreased to \( \approx 1000 \) NP\textsubscript{324–332/K\textsuperscript{b}}-specific T cells every 40 h because the number of NP\textsubscript{324–332/K\textsuperscript{b}}-specific memory cells in the airways at this time was lower (2000 cells).

In contrast to CD8\textsuperscript{T} cells, the absolute numbers of Ag-specific memory CD4\textsuperscript{+} T cells present in the lung airways decline very rapidly within the first 2 mo of infection, and subsequently drop below levels of detection (18). Substantial rates of recruitment were observed for HN\textsubscript{419–433/A\textsuperscript{b}}-specific memory T cells at early time points (Fig. 1), but the subsequent decline in cell numbers suggested that steady-state recruitment was not sustained over the long-term (our unpublished observations).

**Discussion**

We have demonstrated that LFA-1 (CD11a/CDS18) is rapidly down-regulated following recruitment of the cells into the lung airways. Thus, CD11a\textsuperscript{high} cells in the lung airways represent cells that have been recruited into the lung airways within the last 40 h. We have taken advantage of this to investigate the trafficking of memory cells to a peripheral site in a completely unperturbed and unmanipulated situation. This approach has revealed that memory T cells in the airways are maintained by a dynamic process of continual recruitment under steady-state conditions. Although our data specifically address memory T cells in the lung airways, we believe that similar mechanisms may regulate T cell memory at other peripheral sites as well (19). Thus, one of the roles of homeostatic turnover of memory cells may be to produce precursor cells to maintain the peripheral pool of recruitable memory cells. Consistent with this hypothesis, airway memory cells acquire BrdU staining at the same rate as secondary lymphoid organs, despite the fact that the airways are unable to support local proliferation (21, 28). This suggests that airway memory T cells are recruited from populations of effector memory cells undergoing continual recruitment under steady-state conditions. Although our recent experiments suggested that steady-state recruitment was not sustained over the long-term (our unpublished observations).
homeostatic proliferation at secondary lymphoid sites. Interestingly, the slow decline in total memory cell numbers in the airways over the first year postinfection may reflect the progressive loss of effector memory cells in the secondary lymphoid organs (29, 30). Thus, the pool of recruitable cells may decline over time.

Although it is apparent from these data that lung airway memory cell populations are highly dynamic, the underlying mechanisms are unknown. These cells appear to be present in the epithelial layer and are not located in organized lymphoid tissues that could serve as a recruiting site. One possibility is that epithelial cells or other cells in the airways constitutively secrete chemokines that attract resting memory cells into the airways from the circulation. Alternatively, it is possible that the lungs are continually challenged by low levels of environmental Ags and that the memory T cell recruitment is essentially a response to low grade inflammation. Interestingly, the continual recruitment of cells into the lung airways, coupled with the fact that cell numbers remain relatively stable, suggests that cells must also be continually cleared. Because memory cells in the airways are not highly apoptotic and do not appear to re-enter the circulation (our unpublished data) (21, 28, 31), it is likely that cells are being cleared from the lung airways by phagocytic cells or removed through mucociliary clearance. The presence of activated phagocytic cells would be consistent with a low level of persistent inflammation in the lungs.

The role of LFA-1 down-regulation on memory cells in the lung airways is not known. LFA-1 on naive cells is in an inactive state, but undergoes a conformational change following TCR ligation or exposure to specific cytokines or chemokines into an active form (32–34). The activated integrin has increased ligand-binding affinity and lateral mobility, which allows clustering and promotes firm adhesion of migrating effector/memory cells to the endothelial wall before extravasation. Dixon et al. (35) showed that LFA-1 and ICAM were partially responsible for lung adhesion of adoptively transferred allogeneic Th1 cells. More recently, Thatte et al. (36) have demonstrated that blockade of CD11a on transferred cells prevented their retention in the lung parenchyma. Therefore, it is possible that while CD11a may facilitate entry into the lung airways, the subsequent down-regulation of CD11a expression could serve as a trapping mechanism to keep memory cells in the airways. Consistent with this, effector/memory cells that have entered the lung airways cannot re-enter the circulation (28). Additionally, down-regulation of LFA-1 may allow alternate adhesion molecules, such as VLA-1, to enhance localization of memory cells to the airway matrix (37). Indeed, our data show that, in contrast to CD11a, CD49a (the α-chain of the VLA-1 heterodimer) is not down-regulated (Fig. 3) and that VLA-1 expression of airway memory cells is high. Thus, the loss of CD11a on airway cells could ensure that airway memory cells are retained in the lung airways to act as first-line responders upon infection. An alternative possibility is that the down-regulation of LFA-1 allows cells to be released from the epithelial layer so that they can be readily removed through the action of the mucociliary escalator. Thus, this may be a mechanism to prevent excessive accumulation of memory T cells in the lung airways.

Another aspect of LFA-1 expression is its role in maintaining tight interactions between the CTL and target cell during cytotoxic responses. A recent study by Anikeeva et al. (38) clearly demonstrated that LFA-1-mediated target cell firm adherence is necessary for target cell lysis by CTL, but not required for granule release. Previous data from our laboratory have shown that memory cells isolated from the lung airways lack immediate CTL activity, but regain that capacity after in vitro restimulation (5). The loss of LFA-1 expression on these cells could reasonably explain this observation. Furthermore, airway memory cells retain the ability to re-express LFA-1 in vitro (our unpublished observations), and when removed from the airway environment and transferred into another location in vivo (Fig. 2) or transferred i.v. into a naive host and intranasally infected (27).

The mechanism of LFA-1 down-regulation on lung airway cells is unknown. However, recent studies suggest that somatostatin (a neuropeptide that is expressed in the lung) can down-regulate Rap1, which in turn influences LFA-1 mRNA expression in splenocytes (39, 40). Somatostatin treatment of splenocytes also increases the expression of neuraminidase in spleen cells, which can also alter LFA-1 expression (40). However, an earlier study by Leszczynski et al. (41) demonstrated that a somatostatin analog, angiopeptin, decreased rat leukocyte adhesiveness without altering LFA-1 expression, as measured by flow cytometry. Thus, this potential mechanism of LFA-1 down-regulation needs further study.

In summary, the data show that memory T cells undergo phenotypic changes upon entering the lung airway environment, most notably LFA-1 expression. We have characterized the kinetics of LFA-1 down-regulation and have used this marker to identify memory cells newly recruited into the lung airways. We have shown that lung airway memory T cells are maintained by a dynamic process of continual recruitment. Importantly, there appears to be a significant relationship between memory T cell pools in the secondary lymphoid organs and those in peripheral tissues. These findings suggest that vaccines designed to promote T cell memory at peripheral sites must also induce robust systemic T cell memory to maintain peripheral memory populations.

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Disclosures
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