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CUTTING EDGE

Cutting Edge: Effector Memory CD8$^+$ T Cells in the Lung Airways Retain the Potential to Mediate Recall Responses[1]

Kenneth H. Ely, Alan D. Roberts, and David L. Woodland[2]

Previous studies have shown that long-lived memory CD8$^+$ T cells persist in the lung airways following the resolution of a murine Sendai virus infection. These cells are CD11a^{low}, noncytolytic, and do not proliferate in the lung airways raising the possibility that they are "end stage" or terminally differentiated memory cells. In this current report, we investigated the functional characteristics of these cells by analyzing their capacity to respond to secondary viral infection outside of the lung environment. We show that, after transfer into the bloodstream, CD11a^{low} memory T cells from the lung airways can return to the secondary lymphoid tissue and respond to a secondary viral challenge. Furthermore, these cells re-express CD11a, which may contribute to their migratory and proliferative capacity. These data demonstrate that lung airway memory CD8$^+$ T cells are not terminally differentiated cells and retain the capacity to mediate recall responses to infection. The Journal of Immunology,* 2003, 171: 3338–3342.

T cells (CD8$^+$) play 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, a population of long-lived effector memory CD8$^+$ T cells is established in the lung airways (4, 5). These resident lung airway memory CD8$^+$ T cells display a highly activated phenotype characterized by the expression of acute activation markers, such as CD69 and CD25. However, these cells are not in cycle and persist in the airways with a half-life of ~40 days (5). Interestingly, the declining number of resident lung airway memory cells correlates with the declining efficacy of cellular memory against secondary infection, suggesting that these cells play an important role in the control of secondary infections (6). In support of this, lung airway memory T cells have been shown to mediate substantial antiviral activity in a Sendai virus model (7). The underlying mechanism of viral control is not known, but it is likely that early cytokine production may play a key role in reducing viral replication.

Despite the role that lung airway memory cells may play in controlling viral infections, they do not proliferate in the lung airways following secondary Sendai virus infection (8, 9). In addition, the majority of CD8$^+$ memory cells in the lung airways express CD11a at low levels which distinguishes them from memory T cells at other sites (8, 10). These observations raise the question of whether CD11a^{low} memory cells in the lung airways are simply end-stage cells or whether they are maintained in a nonproliferative state by the lung environment. To address this issue, we used an adoptive transfer system that allowed direct comparison between the proliferative ability of intratracheally (i.t.)[3] and i.v. transferred memory T cells from the lung airways during secondary Sendai virus infection. We demonstrate that memory cells from the lung airways include cells that retain the ability to proliferate and migrate in response to homologous infection when transferred systemically. These data indicate that the lung environment normally limits the expansion of these cells in response to cognate Ag despite the fact that they retain the potential to mediate recall responses to infection.

Materials and Methods

Viruses, mice, and infection

Sendai virus (Enders strain) was grown, stored, and titered as previously described (1). Female C57BL/6, B6.Pl-*Thy1*^{a}/Cy (Thy1.1), and B6.SJL-*Ptprc*^{a} *Pep3*/BoyJ (CD45.1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). 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 (EID_{50}) of Sendai virus.

Bronchoalveolar lavage (BAL)

Mice were anesthetized and then bled by cutting the descending aorta. Lung airway cells were collected by five consecutive 1-ml lavages with HBSS. Cells were adhered on plastic for 1 h and nonadherent cells were collected for FACS analysis. Collected cells were either stained with anti-CD11a PE (BD PharMingen, San Diego, CA) for sorting or CFSE-labeled as described below.

CFSE staining and adoptive transfer of BAL cells

In one set of experiments, Thy1.1$^+$ and CD45.1$^+$ Sendai memory mice were euthanized and lung airway cells were collected as described above. BAL cells were stained with 0.5 μM CFSE for 10 min at room temperature and were

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[3] Abbreviations used in this paper: i.t., intratracheal; BAL, bronchoalveolar lavage; EID_{50}, 50% egg infectious dose; MLN, mediastinal lymph node.

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washed extensively. Labeled cells were resuspended in 100 μl/HBSS and transferred into naive C57BL/6 recipient mice by i.t. instillation using a blunted and bent 20-gauge needle at a donor to host ratio of 5:1. Recipient mice were then challenged with an intranasal infection with 250 EID_{50} Sendai virus.

Tissue harvest, preparation, and flow cytometry

BAL were harvested from recipient mice 11 or 12 days postinfection. Harvested cells were stained with allophycocyanin-conjugated tetramer specific for the immunodominant Sendai NP epitope ($NP_{324-332}/K^b$) for 1 h, followed by staining with PerCP-conjugated anti-CD8, and either PE-conjugated anti-Thy1.1 or anti-CD45.1 Abs for 20 min and fixed overnight. Stained samples were analyzed as described below. In other experiments, BAL cells were collected, plastic-adhered, and stained with anti-CD11a PE for 20 min. Stained cells were sorted into $CD11a^{high}$ and $CD11a^{low}$ populations on a FACSVantage cell sorter (BD Immunocytometry Systems, San Jose, CA) with diva enhancement software, CFSE-labeled, and transferred into naive recipients as above or sorted cells were transferred without CFSE and stained as above with the exception of the addition of anti-CD11a FITC in place of CFSE.

Flow cytometry

MHC class I-peptide tetramers were generated by the Molecular Biology Core Facility (Trudeau Institute, Saranac Lake, NY) as described previously (11). Cells were stained with $NP_{324-332}/K^b$ allophycocyanin-specific tetramer for 1 h at room temperature as previously described (5). Tetramer-labeled cells were then stained with anti-CD8 PerCP and FITC-conjugated mAbs specific for CD11a, and PE-conjugated mAbs specific for Thy1.1 (BD PharMingen, San Diego, CA) or CD45.1 (eBioscience, San Diego, CA) on ice for 20 min and fixed overnight with 1% paraformaldehyde in PBS. Samples were run on a BD Biosciences FACSCalibur flow cytometer (San Jose, CA) and data were analyzed using FlowJo software (Treestar, San Carlos, CA). The percentage of tetramer-positive cells among $CD8^+$ T cells was calculated by dividing the number of tetramer-positive $CD8^+$ events by the total number of events in the $CD8^+$ gate. The absolute number of tetramer-positive cells was calculated using the percentage of tetramer-positive cells among the total live cell gate and the number of cells isolated per mouse in each tissue as indicated by trypan blue staining.

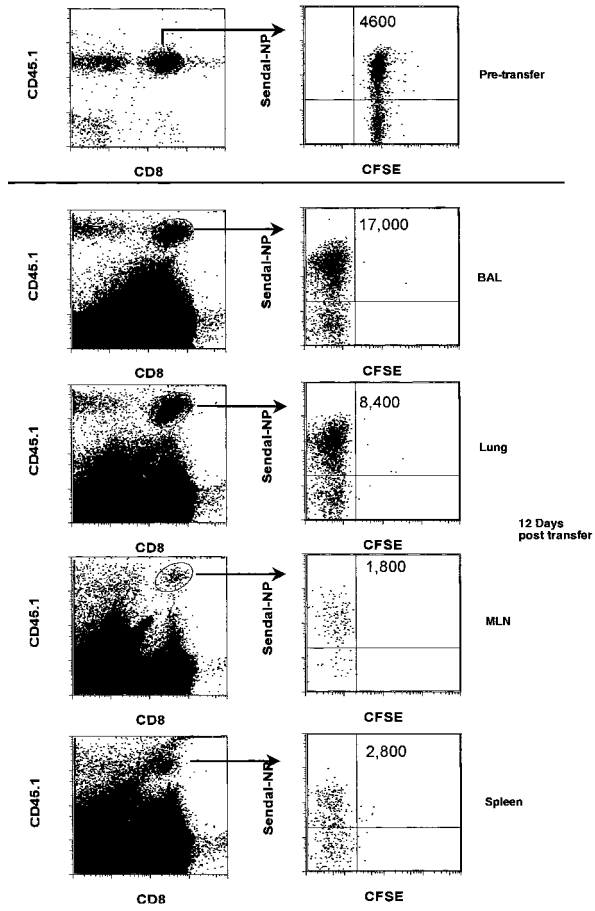

FIGURE 1. CD8 memory T cells from the lung airways proliferate after i.v. transfer. BAL was collected from $CD45.1^+$ 30–35 day Sendai memory mice, CFSE-labeled, and transferred i.v. into naive C57BL/6-recipient mice which were then intranasally infected with 250 EID_{50} Sendai virus on the following day. The profile of CFSE-labeled donor cells is shown in the *upper right panel*. Eleven days postinfection (12 days posttransfer), the recipient mice were euthanized and BAL, lungs, MLN, and spleen were harvested and cells were analyzed by FACS for the expression of CD45.1, CD8, $NP_{324-332}/K^b$ tetramer, and CFSE intensity (*lower panels*). *Lower left panels* are lymphocyte gates and *lower right panels* are the cells represented by the circled gate in the *lower left panels.* The numbers in the *right panels* represent the total number of donor cells transferred or recovered. Data are representative of two separate experiments.

Results and Discussion

Lung airway resident memory $CD8^+$ T cells retain the ability to proliferate in vivo

To analyze the functional capacity of lung airway memory cells, the BAL was collected from $CD45.1^+$ congenic donor mice that had recovered from a Sendai virus infection. The cells were plastic-adhered and nonadherent cells were CFSE-labeled and i.v. transferred into $CD45.2^+$ naive recipient mice. One day later, recipient mice were intranasally infected with a sublethal dose of Sendai virus. Eleven days postinfection (12 days posttransfer), the recipient mice were killed and cells from lung airways and lung parenchyma, draining lymph node, and spleen were isolated and examined for the presence of donor ($CD45.1^+$) $NP_{324-332}/K^b$-specific memory T cells. As shown in Fig. 1, donor memory T cells were found in the lung airways, lung parenchyma, draining lymph node, and the spleen 12 days posttransfer. All of the recovered cells expressed low levels of CFSE indicating that they had proliferated extensively following Sendai virus infection of the recipient. Consistent with this, the number of cells recovered had increased from an input of ~4,600 $NP_{324-332}/K^b$-specific T cells to >30,000 cells in the lungs, mediastinal lymph node (MLN) and spleen combined by 12 days posttransfer. This level of expression was similar to that observed using splenocytes from mice that had recovered from Sendai virus infection as donor cells (an average 20-fold expansion of Ag-specific donor cells with no expansion observed during an irrelevant influenza infection, data not shown). In addition, we also found that i.v.-transferred lung airway memory cells could be detected in the lung airways 36 days posttransfer (data not shown) indicating that these cells develop into lung airway resident memory T cells following resolution of the infection. These results demonstrate that lung airway resident memory $CD8^+$ T cells retain the ability to proliferate and migrate in response to homologous infection following systemic transfer.

In separate experiments, we also compared the ability of lung airway memory T cells to proliferate following simultaneous i.t. and i.v. transfer. Thus, BAL cells from either $Thy1.1^+$ or $CD45.1^+$ donor mice were transferred either i.t. ($Thy1.1^+$) or i.v. ($CD45.1^+$) into the same $CD45.2^+$, $Thy1.2^+$ recipient mice and then intranasally infected with Sendai virus. Eleven days postinfection, the numbers and distribution of i.t.-transferred (Thy1.1) and i.v.-transferred (CD45.1) donor cells were determined. As shown in Fig. 2, despite a strong proliferative and migratory response of the i.v. transferred cells (~20,000 $NP_{324-332}/K^b$-specific cells detected in the lung airways 11 days

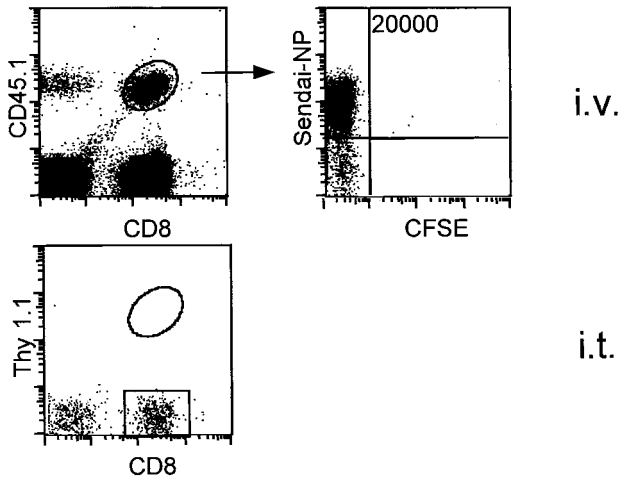

FIGURE 2. CD8 memory T cells present in the lung airways disappear during secondary infection. BAL was collected from both Thy1.1$^+$ and CD45.1$^+$ Sendai memory mice, CFSE-labeled, and transferred either i.t. or i.v., respectively, into the same naive C57BL/6 recipient mice, which were then intranasally infected with 250 EID_{50} Sendai virus on the following day. BAL was harvested 12 days postinfection and nonadherent cells were stained for either Thy1.1 to identify i.t.-transferred cells or CD45.1 to identify i.v.-transferred cells. Gated populations of NP-specific CD8$^+$ T cells and CFSE intensity are shown. *Upper panels*, Intravenously transferred CD45.1$^+$ cells recovered within the lymphocyte gate (*left*) and the $NP_{324-332}/K^b$ and CFSE profiles of cells within the CD45.1, CD8 gate (*right*). *Lower left panel*, There were no i.t.-transferred CD8$^+$, Thy1.1$^+$ cells recovered. Data shown are one of two separate experiments.

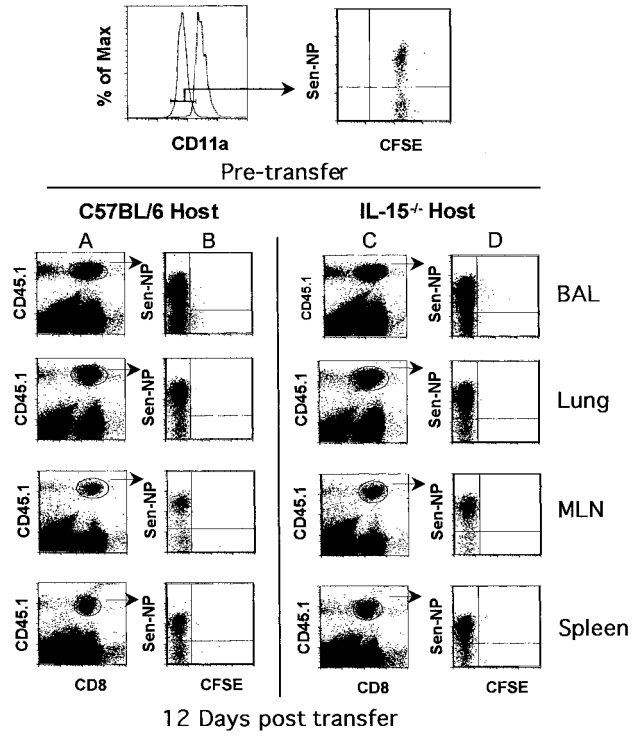

FIGURE 3. CD11a^{low} virus-specific lung airway memory cells proliferate and migrate in an IL-15-independent manner during secondary virus infection after transfer. BAL was collected from CD45.1 memory mice, FACS sorted for CD11a^{low} and CD11a^{high} populations (*upper left*, postsort composite). The CD11a^{low} population was CFSE-labeled (*upper hand panel*) and transferred i.v. into naive C57BL/6 or IL-15$^{-/-}$ (CD45.2) recipient mice. Recipient mice were intranasally infected with 250 EID_{50} Sendai virus the following day. Eleven days postinfection (12 days post transfer), the recipient mice were euthanized and BAL, lungs, MLN, and spleen were harvested and cells were analyzed by FACS for the expression of CD45.1, CD8, $NP_{324-332}/K^b$ tetramer, and CFSE intensity. *Columns A* and *B*, The distribution of $NP_{324-332}/K^b$ tetramer and CFSE-labeled cells from the CD45.1$^+$, CD8$^+$ cells within the lymphocyte gate collected from C57BL/6 recipients. Columns *C* and *D*, The distribution of $NP_{324-332}/K^b$ tetramer and CFSE-labeled cells from the CD45.1$^+$, CD8$^+$ cells within the lymphocyte gate collected from IL-15$^{-/-}$ recipients. Data shown are representative of two separate experiments.

post-Sendai virus infection), we were unable to detect i.t.-transferred cells. These data are consistent with previous studies showing that i.t.-transferred memory cells do not expand in the lung following viral challenge and are deleted during the acute inflammatory response (7–9). Thus, these results suggest the lung environment controls the proliferative capacity and survival of these memory cells.

CD11a^{low} BAL cells migrate and proliferate in response to homologous infection after i.v. transfer

The previous data demonstrate that lung airway memory cells are able to proliferate when removed from the lung environment. However, it is possible that the data in Fig. 1 represent the outgrowth of a small percentage of contaminating cells from the lung parenchyma or recent immigrants that express high levels of CD11a (5). Indeed, we have recently shown that recent arrivals to the lung airway express higher levels of CD11a and then progressively lose this high expression over time (8). To address this issue, we isolated lung airway memory cells from CD45.1$^+$ donor mice that expressed low levels of CD11a by FACS. Sorted CD11a^{low} cells were then i.v. transferred into C57BL/6 (CD45.2$^+$) recipient mice and intranasally infected with Sendai virus as described above. Donor cells were recovered from the airways and parenchymal tissue of the lung, as well as, draining lymph node and spleen on day 11 post-Sendai virus infection. As demonstrated in Fig. 3, *columns A* and *B*, the transferred CD11a^{low} Sendai memory cells proliferated in response to homologous infection as shown by the decrease in CFSE intensity. This proliferation resulted in a 5-fold increase in the total number of $NP_{324-332}/K^b$-specific donor T cells recovered from recipient mice (Table I). These data confirm that CD11a^{low} memory cells in the lung airways retain the ability to mediate recall responses by proliferating and migrating to the site of infection following Sendai virus challenge. Furthermore, the data indicate that i.v.-transferred CD11a^{low} lung airway memory T cells isolated 11 days after Sendai virus infection upregulated CD11a in all tissues examined (Fig. 4). CD11a is an adhesion and activation marker that is involved in the extravasation of cells out of the circulation. The loss or blockade of this

Table I. *Intravenously transferred CD11a^{low} lung airway memory T cells proliferate and migrate to the lungs in C57BL/6-recipient mice following intranasal Sendai virus infection*[a]

Tissue	Number of $NP_{324-332}/K^b$-Specific CD45.1$^+$/CD8$^+$ Cells Recovered[b]
BAL	42,000
Lung	8,500
MLN	12,500
Spleen	34,000
Liver	7,000

[a] Twenty-three thousand $NP_{324-332}/K^b$-specific donor cells transferred.
[b] Twelve days after transfer (11 days postinfection).

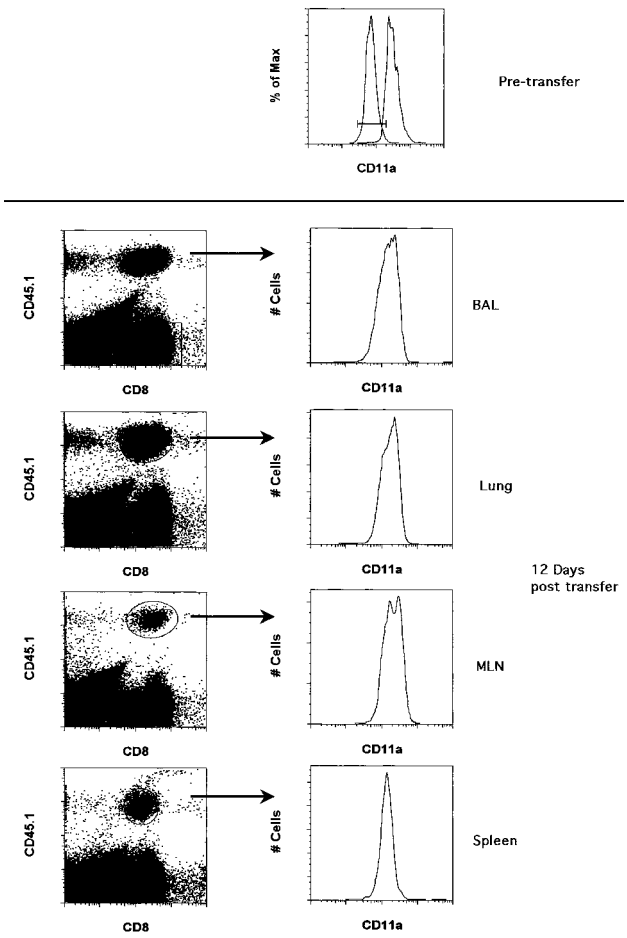

FIGURE 4. Intravenously transferred CD11a^{low} lung airway memory cells re-express higher levels of CD11a during Sendai virus infection of naive hosts. BAL was collected from CD45.1 memory mice, FACS sorted for CD11a^{low} and CD11a^{high} populations (*upper left* composite postsort). The Cd11a^{low} population was and transferred i.v. into naive CD45.2 recipient mice. Recipient mice were intranasally infected with 250 EID_{50} Sendai virus the following day. Eleven days postinfection, the recipient mice were euthanized and BAL, lungs, MLN, and spleen were harvested and cells were analyzed by FACS for the expression of CD45.1, CD8, and CD11a. Panels show the distribution of CD11a on cells within the CD45.1, CD8 gate.

molecule can impair T cell trafficking. For example, pretreatment of transferred effector cells with anti-LFA-1 Ab resulted in decreased endothelial cells adhesion and retention of transferred cells in the lung compared with control treated animals (12). Thus, the re-expression of CD11a on i.v.-transferred BAL cells may account for their ability to migrate throughout the recipient host.

IL-15, along with IL-7, has been identified as a factor necessary for homeostatic maintenance of memory CD8$^+$ T cells (13–15) and also induces chemokines that attract activated or memory CD8$^+$ T cells to the site of inflammation (16). However, the role of IL-15 in the initial expansion of CD8$^+$ cells and the generation of memory is less clear. Some reports cite a requirement for IL-15 (17), while in other systems it is reported that IL-15 is dispensable for the generation of CD8$^+$ memory cells (15, 18). Thus, we investigated whether lung resident CD11a^{low} memory cells required host IL-15 to respond to proliferate and extravasate back into the lung airways following i.v. transfer. As shown in Fig. 3, *columns C* and *D*, lung airway CD11a^{low} memory cells proliferated and migrated back to the lung airways in IL-15$^{-/-}$-recipient mice following Sendai virus infection demonstrating that host IL-15 is not required for this response.

Taken together, the data show that lung airway resident memory T cells retain the potential to proliferate, re-express CD11a, and migrate to the site of infection in the lung airways following i.v. transfer indicating that they are not terminally differentiated cells. However, these same cells failed to proliferate and persist when placed directly into the lung airways suggesting that the lung environment is not permissive for the proliferation of these cells. In this regard, it has been reported that local factors, such as lung surfactants may inhibit the proliferation of T cells in the lung (19–22). It is possible that inhibition of T cell proliferation in nonlymphoid tissues is an important mechanism to avoid inappropriate or dysregulated T cell response. Thus, it may be the case that T cell proliferation is always restricted to lymphoid tissues to ensure appropriate regulation of the response. Furthermore, the failure of T cells proliferate in the lung airways suggests that the size of the memory deposited in the lung airways is a function of the magnitude of recruitment to the airways during resolution of the infection. This hypothesis, along with the observation that lung airway memory cells are able to mediate recall responses, has implications for the development of vaccines designed to promote cellular immunity in the lung.

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