The Journal of Immunology

Long-Term Maintenance of Virus-Specific Effector Memory CD8 $^{+}$ T Cells in the Lung Airways Depends on Proliferation

Robert J. Hogan, Linda S. Cauley, Kenneth H. Ely, Tres Cookenham, Alan D. Roberts, Jean W. Brennan, Simon Monard and David L. Woodland

J Immunol 2002; 169:4976-4981; ;
doi: 10.4049/jimmunol.169.9.4976
http://www.jimmunol.org/content/169/9/4976

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Long-Term Maintenance of Virus-Specific Effector Memory CD8$^+$ T Cells in the Lung Airways Depends on Proliferation[1]

Robert J. Hogan,[2] Linda S. Cauley, Kenneth H. Ely, Tres Cookenham, Alan D. Roberts, Jean W. Brennan, Simon Monard, and David L. Woodland[3]

Recent studies have shown that virus-specific effector memory T cells can be recovered from the lung airways long after clearance of a respiratory virus infection. These cells are thought to play an important role in the recall response to secondary viral infection. It is currently unclear whether these cells actually persist at this site or are maintained by continual proliferation and recruitment. In this study, we have analyzed the mechanisms underlying the persistence of memory CD8$^+$ T cells in the lung airway lumina following recovery from a respiratory virus infection. The data identify two distinct populations of memory cells. First, a large population Ag-specific CD8$^+$ T cells is deposited in the airways during the acute response to the virus. These cells persist in a functional state for several weeks with minimal further division. Second, a smaller population of Ag-specific CD8$^+$ T cells is maintained in the lung airways by homeostatic proliferation and migration to lung airways after viral clearance. This rate of proliferation is identical to that observed in the spleen, suggesting that these cells may be recent immigrants from the lymphoid organs. These data have significant implications for vaccines designed to promote cellular immunity at mucosal sites such as the lung. ***The Journal of Immunology*****, 2002, 169: 4976–4981.**

Memory T cell responses to influenza and parainfluenza infections are characterized by accelerated and enhanced CD8$^+$ T cell accumulation in the lungs (1–4). It was originally thought that memory T cells that persist in secondary lymphoid tissues (such as the spleen and lymph nodes) play a central role in mediating pulmonary recall responses. However, we have recently shown that substantial numbers of Ag-specific CD4$^+$ and CD8$^+$ T cells can be recovered from the lung airways more than 1 year after resolution of either an influenza or Sendai virus infection (5–7). These lung-derived T cells are distinct from lymphoid memory T cells in that they express markers characteristic of highly activated T cells, including CD25 and CD69 (5, 6). They also produce antiviral cytokines and rapidly acquire cytolytic activity in response to Ag similar to the effector memory cells described by Sallusto et al. (8). We speculated that these Ag-specific memory T cells in the lung airways provide a first line of defense against subsequent infection (3). From their location at the site of initial infection, memory T cells in the lung airways may respond immediately to reinfection and may also accelerate the recruitment of new memory cells from secondary lymphoid organs. Although the early production of antiviral cytokines such as IFN-γ may not be sufficient to eliminate the virus, it may help to reduce the viral load encountered by the memory T cells recruited from secondary lymphoid sites later in the response. More recently, it has emerged that pools of memory T cells also persist in other peripheral tissues, such as the kidney, fat pads, salivary glands, and liver (9–12). One study estimated that at least half of all memory CD8$^+$ T cells reside in nonlymphoid peripheral tissues, consistent with the idea that peripheral memory T cells play a major role in combating mucosal infections (12–15).

Although effector memory cells from peripheral tissues have been well characterized, we have very little understanding of the mechanisms that regulate these cell populations. We have previously shown that the absolute numbers of T cells in the lung airways after influenza or Sendai virus infections are initially high and then decline over the course of about 6 mo with a $t_{1/2}$ of ~40 days (5). The cell numbers then stabilize at a relatively low basal level. Interestingly, the decline and subsequent stabilization in the numbers of memory cells in the lung airways correlate with the loss of protective cellular immunity (1). Consistent with this concept, intratracheal transfer studies have confirmed that memory T cells in the lung airways can mediate substantial control of secondary viral infections in the absence of Abs (6).

Since the numbers of peripheral memory T cells show a strong correlation with protective cellular immunity, it is essential that we understand the mechanisms that maintain memory T cells at peripheral sites such as the lung. In the current manuscript, we have analyzed the persistence of memory CD8$^+$ T cells in the lung airways following recovery from a Sendai virus infection. Initially, we assumed that since the lung airways interface with the external environment, memory T cells at this site would be very short-lived. However, the data show that a large bolus of memory CD8$^+$ T cells remains in the lung tissue for several months after the resolution of the acute response. These cells persist in the lung airways without proliferating, although their absolute numbers decline steadily over time. There is also a minor population of memory CD8$^+$ T cells in the lung airways that proliferates after the resolution of the acute response. These cells may account for the stabilization of memory T cell numbers in the lung airways over the longer term. These data represent an important step forward in our understanding of peripheral memory T cell populations and have significant implications for vaccine development.

Trudeau Institute, Saranac Lake, NY 12983

Received for publication June 4, 2002. Accepted for publication August 29, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Public Health Service Grants AI-37597, HL-63925, and HL-69502 (to D.L.W.) and F32 AI-10590 (to R.J.H.) and by funds from the Trudeau Institute.

[2] Current address: Division of Virology, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD 21702.

[3] Address correspondence and reprint requests to Dr. David L. Woodland, Trudeau Institute, P.O. Box 59, Saranac Lake, NY 12983. E-mail address: dwoodland@trudeauinstitute.org

0022-1767/02/$02.00

Materials and Methods

Viruses, animals, and infections

The Enders strain of Sendai virus was grown, stored, and titrated as previously described (4). Female C57BL/6, B6.PL-Thy1a/Cy (Thy1.1$^+$), and B6.SJL-Ptprca Pep3b/BoyJ (Ly5.1$^+$) mice were purchased from the Animal Breeding Facility at the Trudeau Institute (Saranac Lake, NY). Mice (6–12 wk) were anesthetized by i.p. injection with 2,2,2-tribromoethanol and infected intranasally with either 125 or 500 50% egg infectious doses (EID_{50})[4] of Sendai virus.

Peptides

Sendai virus peptides (nucleoprotein (NP) 324–332) and influenza virus ($NP_{366-374}$) peptides were purchased from New England Peptide (Fitchburg, MA). Peptide purity was evaluated using reversed-phase HPLC analysis.

In vivo 5-bromo-2-deoxyuridine (BrdU) labeling

BrdU (Sigma-Aldrich, St. Louis, MO) was administered either in sterile drinking water (0.8 mg/ml) or by i.p. injection of 0.2 ml of PBS/BrdU solution (4 mg/ml) at the indicated times during infection. In experiments where mice were fed water containing BrdU, the solution was replaced daily. For pulse-chase experiments, animals were given normal drinking water at the end of the pulse period.

Tissue preparation

Single-cell suspensions were prepared from spleens and mediastinal lymph nodes (MLN) by passage through cell strainers. Spleen cells were depleted of erythrocytes by treatment with buffered ammonium chloride solution. Bronchoalveolar lavage (BAL) cells were collected by lavage of the lungs three to four times with 1 ml of HBSS. T cells were isolated from the remaining lung tissue by mechanical disruption through cell strainers. The cells were subsequently resuspended in 80% isotonic percoll and layered with 40% isotonic percoll. After centrifugation at 400 $\times$ *g* for 25 min, the cells at the 80%/40% interface were collected, washed, and counted.

Intratracheal cell transfers and in situ labeling of cells in the lung airways

BAL cells were collected from C57BL/6 mice at >30 days after Sendai virus infection. Where indicated, donor cells were labeled with CFSE (Molecular Probes, Eugene, OR) by incubation in HBSS containing 0.5–0.7 μM CSFE for 10 min in the dark. The cells were then washed and resuspended in PBS at a concentration of 0.5–2 $\times$ 10^7 cells/ml before transfer. Thy1.1$^+$ C57BL/6 recipient mice were anesthetized and 100 μl (0.5–2 $\times$ 10^6 cells) of the cell suspension or PBS were instilled into the lungs (via the trachea) using a 1-ml syringe fitted with a blunted 20-gauge needle. In some experiments, CFSE-labeled cells were subsequently isolated from the lung airways and restimulated for 4–5 days in vitro, using either the Sendai (Sen) $NP_{324-332}$ or influenza (Flu)-$NP_{366-374}$ peptides (2 μg/ml final concentration), human rIL-2 (10 U/ml final concentration) (R&D Systems, Minneapolis, MN), and a cell density of 1 $\times$ 10^6/well in 24-well plates. All wells received 1 $\times$ 10^6 gamma-irradiated (4000 rad) Ly5.1$^+$ naive spleen cells as APCs. For in situ labeling of cells in the lung airways, 80 μl of PBS containing CFSE (0.2 mM) was instilled into the trachea using a blunted 20-gauge needle.

MHC tetrameric reagents, staining, and analysis

MHC class I peptide tetramers were generated by the Molecular Biology Core Facility at the Trudeau Institute as described previously (16). The Sen-$NP_{324-332}/K^b$ tetramer is highly specific for CD8$^+$ T cells that recognize the Sendai virus $NP_{324-332}/K^b$ epitope (2, 17). Staining with tetrameric reagents was performed for 1 h at room temperature. After washing, the cells were stained with anti-CD8 Tricolor (Caltag Laboratories, Burlingame, CA) or anti-CD8-CyChrome and biotinylated, FITC-conjugated, or APC-conjugated Abs specific for Ly5.1 (CD45.1) or Thy1.2 (BD PharMingen, San Diego, CA) on ice for 20 min. Stained samples were run on either a BD Biosciences FACScan or FACSCalibur flow cytometer and data were analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA). In some experiments, B cells were depleted before staining by panning on anti-Ig-coated flasks.

[4] Abbreviations used in this paper: EID_{50}, 50% egg infectious dose; NP, nucleoprotein; BrdU, 5-bromo-2-deoxyuridine; MLN, mediastinal lymph node; BAL, bronchoalveolar lavage; Flu, influenza; Sen, Sendai.

BrdU staining was performed as previously described (18). Briefly, 1–2 $\times$ 10^6 cells were surface stained as indicated above and treated with 0.9 ml of FACS Lysing Solution (BD Biosciences) for 15 min at room temperature. The cells were then fixed overnight at 4°C in 1 ml of 1% paraformaldehyde/PBS containing 0.05% Nonidet P-40. After washing, cellular DNA was denatured with 50 Kunitz units of DNase I (bovine pancreas; Sigma-Aldrich) for 30 min at 37°C. The cells were then washed with PBS containing 5% FCS and 0.5% Nonidet P-40 and incubated with 5 μl of anti-BrdU/FITC (BD Biosciences) for 45 min on ice. The cells were washed twice before analysis by flow cytometry.

The percentage of tetramer-positive cells among total live cells was calculated by dividing the number of tetramer$^+$/CD8$^+$ events by the total number of events in a live cell gate.

Cell cycle analysis

T cells were isolated from the lung airways and surface stained as indicated above. After washing, the cells were resuspended in 1 ml of PBS containing 2% FCS, 0.1% NaN_3, 0.5% saponin, and 10 μg of Hoechst 33342 for 30–60 min on ice. Data were acquired using a FACSVantage SE cell sorter fitted with an argon laser emitting at 488 nm and a krypton laser with UV optics. The data were analyzed using FlowJo software (Treestar, San Carlos, CA), and doublets were excluded by width and area analysis.

Results

Two distinct populations of memory CD8$^+$ T cells remain in the lungs following recovery from a Sendai virus infection

Substantial numbers of $NP_{324-332}/K^b$-specific CD8$^+$ T cells persist in the lung airways of C57BL/6 mice following recovery from an intranasal Sendai virus infection (5, 6). These cells are located primarily in the airway epithelium and the lamina propria (data not shown) and express markers that are generally associated with acute activation (CD25$^+$ and CD69$^+$) (3). The absolute numbers of Ag-specific memory CD8$^+$ T cells in the airways decline slowly over the first 6 mo postinfection (with a $t_{1/2}$ of ~40 days) and then stabilize thereafter (5). To identify the mechanisms that control this biphasic decline in numbers of memory T cells in the lung airways, we first asked whether the cells were proliferating. Mice were given BrdU in the drinking water for a period of 8 days during either the acute phase of infection (days 0–8) or after a stable memory population had been established (days 25–32, Sendai virus is cleared by day 10). T cells were then isolated from the lung airways and tissues on day 33 and $NP_{324-332}/K^b$-specific T cells were analyzed for the incorporation of BrdU. As shown in Fig. 1, when BrdU was administered between 0 and 8 days of the infection, ~90% of the $NP_{324-332}/K^b$-specific memory T cells in the lung airways were BrdU positive on day 33. This indicates that virus-specific CD8$^+$ T cells had divided during the acute infection and incorporated BrdU, but the majority had not divided substantially after that time. However, at least 10% of the cells were BrdU negative, indicating that some cells had continued to divide after the virus had been cleared. To confirm this, we analyzed BrdU incorporation later in the response after infectious virus was cleared (days 25–32 postinfection). As shown in Fig. 1, 14% of the $NP_{324-332}/K^b$-specific T cells in the lung airways on day 33 were BrdU positive, indicating that some cell division at this late time point. Similar data were obtained for cells isolated from the lung tissues (Fig. 1). The reciprocal nature of the BrdU profiles in these experiments suggested that there were two populations of $NP_{324-332}/K^b$-specific memory T cells in the lung airways. One was a large static population of nonproliferating cells (90%) that persisted in the lung airways following the acute infection. The other was a smaller population of cells that continued to divide after resolution of the primary infection (10%).

To further investigate this hypothesis, we analyzed the stability of these two BrdU-defined populations over time. As shown in Table I, memory T cells that had been labeled during days 1–8 declined from 90% of the recovered cells at day 33 to 0% by day

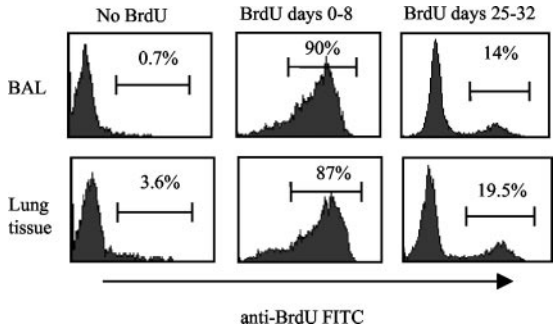

FIGURE 1. The majority of Sendai virus $NP_{324-332}/K^b$-specific memory $CD8^+$ T cells in the lung undergo minimal proliferation following resolution of the primary infection. Three groups of C57BL/6 mice were intranasally infected with 500 EID_{50} Sendai virus. One group was given normal drinking water (No BrdU, $n = 3$), a second group was given BrdU water from days 0 to 8 postinfection (BrdU days 0–8, $n = 3$), and a third group was given BrdU water from days 25 to 32 postinfection (BrdU days 25–32, $n = 3$). At day 33 after infection, BAL cells and cells isolated from the lung tissue (after lavage) were pooled within each group and stained with Sen-$NP_{324-332}/K^b$ tetramer-PE, anti-CD8-Tricolor, and anti-BrdU-FITC Abs. The data are shown as anti-BrdU incorporation among $CD8^+$/$tetramer^+$ gated T cells. The data are representative of two independent experiments.

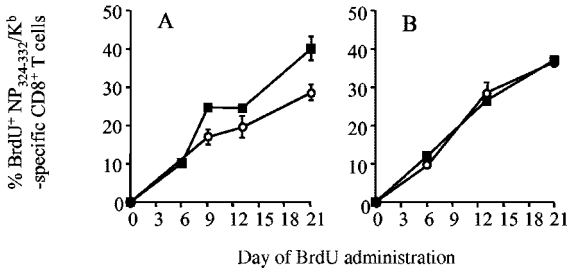

FIGURE 2. Sendai virus $NP_{324-332}/K^b$-specific memory $CD8^+$ T cells in the lung airways and spleen are maintained by homeostatic proliferation. C57BL/6 mice were intranasally infected with 500 EID_{50} Sendai virus and then given BrdU by continuous feeding in the drinking water starting at either 1 mo (*A*) or 12 mo (*B*) after infection. On various days after BrdU initiation, cells were isolated from the lung airways (○) or spleen (■) and stained with Sen-$NP_{324-332}/K^b$ tetramer-PE, anti-CD8-Tricolor, and anti-BrdU-FITC Abs. The data are shown as BrdU incorporation among $CD8^+$/$tetramer^+$ gated T cells and the error bars indicate the SD of three individual mice. The full time course was done once, but the day 6 and day 21 time points were repeated several times with similar results.

368 (Table I). This decline in the frequency of BrdU-labeled $NP_{324-332}/K^b$-specific memory cells paralleled the early decline (over the first 6 mo) in absolute numbers of cells in the lung airways that has been reported previously (5). Similarly, when mice were given BrdU in the drinking water at late time points postinfection, such as 1 year, there was a steady accumulation of BrdU-labeled $NP_{324-332}/K^b$-specific memory T cells in the lung airways over 21 days (Fig. 2*B*). This rate of accumulation was identical to that observed at 1 mo after infection (Fig. 2*A*) and was also very similar to that observed in the spleen. These observations are consistent with the idea that a subpopulation of memory $CD8^+$ T cells in the lung airways are maintained over the long term by homeostatic proliferation of the memory T cell pool. Together, the data support the hypothesis that memory $CD8^+$ T cells in the lung are comprised of a pool of nondividing cells that remain static in the lung after the resolution of the infection and a pool of slowly dividing cells that may be recruited from the lymphoid organs.

Memory T cells are able to persist in the lung airways for at least 2 wk

The BrdU data suggested that the biphasic kinetics of $NP_{324-332}/K^b$-specific memory T cell persistence in the lungs may be attributable to the deposition of a large bolus of nondividing memory cells during the acute response, which slowly declined over time, and a parallel slow continual recruitment of recently divided cells from secondary lymphoid organs. To distinguish between persistence vs continual recruitment, memory T cells were labeled in situ in the lung airways by intratracheal administration of CFSE (day 16 after Sendai virus infection). Flow cytometry was then used to follow the survival of the labeled cells over the succeeding 2 wk. Analysis 1 day after labeling indicated that ~30% of the total cells in the airways became labeled with CFSE (Fig. 3*A*). Although the range of staining intensity was quite broad, it was possible to specifically gate on CFSE-positive cells and demonstrate that they included a substantial fraction of $NP_{324-332}/K^b$-specific T cells (Fig. 3, *A* and *B*). Further analysis on days 4 and 15 postlabeling

Table I. *Memory T cells labeled with BrdU during acute infection can be recovered from the lungs >99 days later*

	BAL		Lung Tissue	
Day Postinfection	Control (%)	BrdU[a] (days 0–8, %)	Control (%)	BrdU (days 0–8, %)
33	0.7	90	3.6	87
61	0.3	85	0.9	76
99	1.2	41	3.7	52
368	1.0	1	1.1	2

[a] Mice were given BrdU in the drinking water during days 0–8 of an acute intranasal Sendai virus infection. The data represent the percentage of $tetramer^+/CD8^+$ gated T cells that are $BrdU^+$ at the indicated times postinfection.

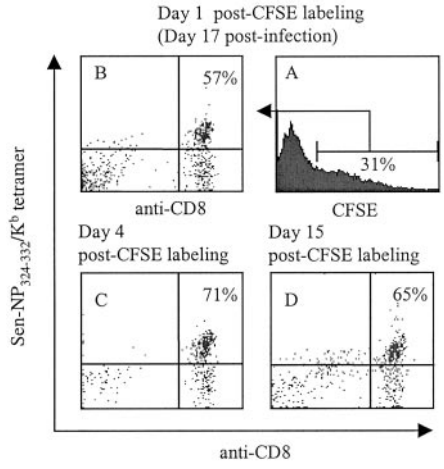

FIGURE 3. Sendai virus-specific memory $CD8^+$ T cells persist in the lung airways for >2 wk. C57BL/6 mice were infected intranasally with Sendai virus and on day 16 after infection CFSE (80 μl) was introduced directly into the lung airways via the trachea. On days 1 (*A* and *B*), 4 (*C*), and 15 (*D*) after CFSE labeling, cells were isolated from the lung airways (BAL) and stained with Sen-$NP_{324-332}/K^b$ tetramer-PE and anti-CD8-Tricolor. The data in *A* are a histogram of CFSE fluorescence with the frequency of $CFSE^+$ cells among the lymphocyte population. The data in *B–D* are gated on $CFSE^+$ lymphocytes and show Sen-$NP_{324-332}/K^b$ tetramer vs CD8 staining. The percentages in these panels indicate the frequencies of $tetramer^+$ cells among $CD8^+$ T cells. The frequencies of $CFSE^+$ lymphocytes in the spleen and MLN at 1 day after CFSE labeling were <0.7%. The data are representative of four individual experiments.

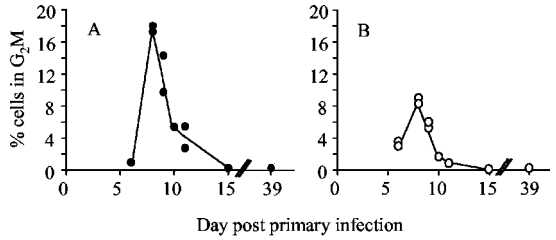

FIGURE 4. Sendai virus $NP_{324-332}/K^b$-specific memory $CD8^+$ T cells in the lung airways persist predominantly in the G_0-G_1 stage of the cell cycle. BAL and spleen cells were isolated from C57BL/6 mice at various times after Sendai virus infection and stained with Sen-$NP_{324-332}/K^b$ and anti-CD8-Tricolor. The cells were then stained with Hoechst 33342 and analyzed as described in *Materials and Methods.* The data show the percentages of Sen-$NP_{324-332}/K^b$ tetramer$^+$/$CD8^+$ (*A*) cells or Sen-$NP_{324-332}/K^b$ tetramer-negative/$CD8^+$ (*B*) cells that were in G_2-S phase of the cell cycle. The line represents the average of two experiments.

(Fig. 3, *C* and *D*, representing days 20 and 31 postinfection), demonstrated that CFSE-positive, $NP_{324-332}/K^b$-specific T cells could be readily recovered 2 wk after labeling. The absolute percentage of CFSE-positive cells in the airways declined over time from 30% at day 1 postlabeling to <5% at day 15 postlabeling (data not shown). However, the percentage of $NP_{324-332}/K^b$-specific T cells remained stable within this CFSE-labeled population. These data confirmed that a substantial number of Ag-specific memory $CD8^+$ T cells are able to persist in the lung airways for at least 2 wk. These recovered cells expressed CD69 and CD25 markers, consistent with the activated phenotype found in previous studies on memory $CD8^+$ T cells recovered from the lung airways (data not shown).

Together, the BrdU and CFSE data suggested that some memory T cells persist in the lung airways for substantial lengths of time without proliferating. Consequently, we speculated that the majority of the cells were in a resting phase of the cell cycle. To test this idea, we analyzed the DNA content of $NP_{324-332}/K^b$-specific T cells in the lung airways by flow cytometry throughout the acute infection and after memory had been established. As shown in Fig. 4*A*, ~17% of $NP_{324-332}/K^b$-specific $CD8^+$ T cells in the lung airways were in the G_2-M or S phase of the cell cycle at the peak of the infection (day 8 postinfection). However, this percentage decreased to <1% by day 15 postinfection and then persisted at this level for >24 days (Fig. 4*A*). A similar pattern of cell cycle kinetics was observed in tetramer-negative $CD8^+$ T cells, except that a lower percentage of the cells were in cycle at day 8 (Fig. 4*B*). Together these data confirm that the effector memory $CD8^+$ T cells that persist in the lung following recovery from a Sendai virus infection are not in the cell cycle.

Nonproliferating memory T cells in the lung retain the capacity to mediate a potent proliferative response to Ag

The BrdU and CFSE studies suggested that large numbers of nonproliferating memory $CD8^+$ T cells persisted in the lung airways after recovery from a Sendai virus infection. We further investigated the longevity of memory T cells in the airways using an intratracheal transfer system. BAL cells were isolated from $Thy1.2^+$ mice that had recovered from a Sendai virus infection and CFSE labeled in vitro. The labeled cells were then transferred to the airways of naive $Thy1.1^+$ mice. After 8 days, the BAL, lung tissue, MLN, and spleens were recovered from recipient mice and donor cells were identified by Thy1.2 staining. As shown in Fig. 5 (*left panels*), substantial numbers of $Thy1.2^+$ donor cells could be

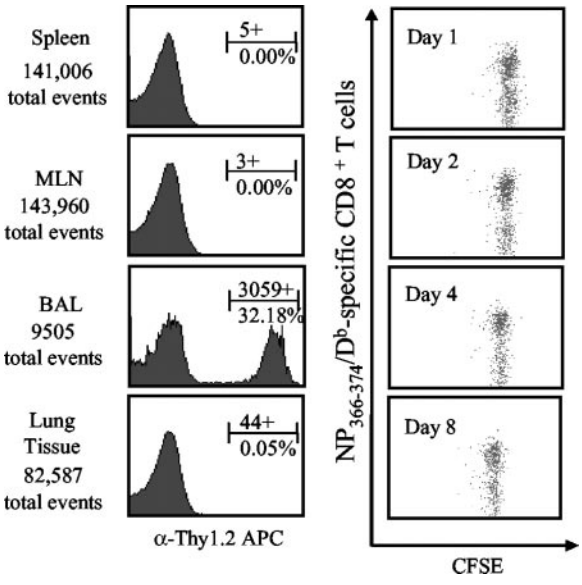

FIGURE 5. Sendai virus-specific $CD8^+$ T cells isolated from the lung airways do not proliferate after intratracheal transfer into naive mice. BAL cells were collected from C57BL/6 ($Thy1.2^+$) mice at day 31 after Sendai virus infection, labeled with CFSE, and transferred into naive C57BL/6 ($Thy1.1^+$) mice via the trachea. On day 8 posttransfer, cells isolated from the spleen, MLN, BAL, or lung tissue were analyzed for donor cell Thy1.2 expression (*left panels*). In addition, at days 1, 2, 4, and 8 after transfer, BAL cells were isolated and stained with Sen-$NP_{324-332}/K^b$ tetramer-PE, anti-CD8-CyChrome, and anti-Thy1.2-allophycocyanin (*right panels*). The data are gated on $Thy1.2^+$/$CD8^+$ lymphocytes and are representative of five independent experiments. The absolute numbers of donor cells recovered from the BAL was quite variable between experiments. However, in every experiment, donor cells were only recovered from the BAL, and not other tissues.

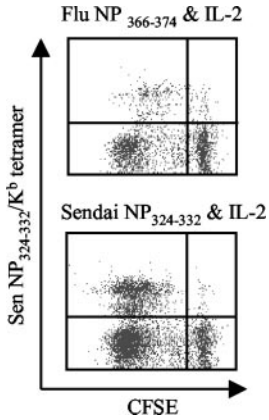

FIGURE 6. Sendai virus-specific memory $CD8^+$ T cells remain functional following intratracheal transfer. BAL cells were collected from C57BL/6 ($Thy1.2^+$) mice at day 42 after Sendai virus infection and transferred into naive C57BL/6 ($Thy1.1^+$) mice via the trachea. At 8 days posttransfer, BAL cells were reisolated from the lung airways and stimulated in vitro with IL-2 and either Flu-$NP_{366-374}$ (control) or Sen-$NP_{324-332}$ peptides. After 5 days, the cells were stained with Sen-$NP_{324-332}/K^b$ tetramer-PE, anti-CD8-Tricolor, and anti-Thy1.2-allophycocyanin. The *upper panel* represents Sen-$NP_{324-332}/K^b$ tetramer-PE staining and CFSE intensity among control peptide-stimulated $Thy1.2^+$/$CD8^+$ lymphocytes and the *lower panel* represents Sen-$NP_{324-332}/K^b$ tetramer-PE staining and CFSE intensity among Sen-$NP_{324-332}$-stimulated $Thy1.2^+$/$CD8^+$ lymphocytes. The data are representative of three independent experiments. The level of expansion of tetramer-positive cells induced by the Sen-$NP_{324-332}$ peptide was variable between experiments, but was always much greater than that induced by the control Flu-$NP_{366-374}$ peptide.

recovered from the airways, but not the lung tissue, spleens, or MLN, indicating that the donor cells had not colonized other sites. Analysis of the CFSE levels on $NP_{324-332}/K^b$ tetramer-positive cells at various days (days 1–8) posttransfer further demonstrated that the cells did not proliferate over this time frame (Fig. 5, *right panels*). Similar results were obtained when memory T cells were transferred into the lungs of mice that had previously recovered from a Sendai virus infection (data not shown). In this case, the host and donor $NP_{324-332}/K^b$-specific $CD8^+$ T cells were distinguished using the Thy1 marker. Although the memory cells from the lung airways did not proliferate in situ, they had not lost their capacity to respond to Ag. Thus, cells that had been reisolated from the lung airways 8 days after transfer proliferated in vitro in response to the $NP_{324-332}$ peptide and IL-2 (Fig. 6). These cells were also able to produce IFN-γ and mediate cytolytic activity in response to target cells coated with the $NP_{324-332}$ peptide (data not shown). Taken together, the data demonstrate that memory $CD8^+$ T cells can survive in the lung airways for substantial periods of time without proliferating and still retain the capacity to respond to Ag.

Discussion

It has recently emerged that substantial numbers of memory T cells can be recovered from peripheral sites following mucosal infections, and it has been speculated that these cells play a critical role in mediating cellular immune responses to secondary infections (3, 5–7, 9–12). These cells have an activated phenotype and resemble cells that are present during the acute phase of the immune response (5, 11). However, we currently have very little understanding of how peripheral populations of memory T cells are established and maintained or their relationship to resting memory cell populations in secondary lymphoid organs. The data presented here take advantage of a Sendai virus model of respiratory virus infection to study the persistence of memory T cells in the lung airways. We have shown that the Ag-specific effector memory $CD8^+$ T cells that remain in the lung airways following recovery from a primary infection are comprised of two populations. The first is a large population of cells that is deposited in the lung during the acute response to infection and which is characterized by minimal further division. These cells have a $t_{1/2}$ of ~40 days and their numbers decline with increasing months after infection. The steady attrition of cells probably reflects their migration out of the epithelial layer and removal via the mucociliary escalator. The second population of cells is characterized by cell division at late time points after viral clearance. Although these cells initially represent a small percentage of the memory $CD8^+$ T cell pool in the lung airways, their relative proportion increases over time, as the nondividing cells disappear. The presence of two separate populations of memory $CD8^+$ T cells in the lung provide an explanation for the kinetics of memory cell persistence described previously (5). Thus, the initial waning of cell numbers may represent the steady loss of a large bolus of cells deposited after the initial infection. The subsequent stabilization of cell numbers after 6 mo may represent steady-state recruitment and loss of memory cells, possibly driven by homeostatic proliferation of memory T cell populations in the secondary lymphoid organs followed by recruitment to the lung (19, 20). The observation that T cells can persist in an environment such as the lung airway lumina is novel and has significant implications for understanding protective cellular immune responses.

Although the BrdU data demonstrate that a subpopulation of memory $CD8^+$ T cells continues to proliferate after resolution of the infection, it is not clear where this proliferation occurs. The cells may divide in the lung airway lumen or at other distal sites before being recruited to the lung. We believe that the latter possibility is most likely for a number of reasons. First, there is substantial evidence that surfactants in the lung specifically interfere with T cell proliferation (21). Second, it seems unlikely that there are appropriate signals to drive T cell proliferation in a nonlymphoid site such as the airways of an uninfected lung. Third, we have shown that memory cells are recruited to the lung even after peritoneal infection or distal vaccination (5) (K. H. Ely, L. S. Cauley, A. D. Roberts, J. W. Brennan, T. Cookenham, and D. L. Woodland, manuscript in preparation). Fourth, the idea that cells have divided at a distal site before recruitment to the lung is consistent with the fact that memory populations in secondary lymphoid organs undergo a continual slow homeostatic turnover (19, 22–25). Thus, the memory cells in the lung appear to represent a nondividing population of cells that persists in the airways after viral clearance and a smaller population of cells that continue to divide in secondary lymphoid organs after viral clearance.

It is unclear why such large numbers of cells persist in the lung airways following recovery from respiratory virus infections. One possible explanation is that the apoptotic mechanisms that normally eliminate effector T cells in the lung may be arrested when Ag is cleared from the lung. Recent studies have suggested that IFN-γ plays a key role in regulating effector T cell numbers by potentiating apoptosis or cell removal by macrophages (26, 27). Thus, the cessation of IFN-γ production in the lung following Ag clearance may result in the accumulation and persistence of memory T cells at this site. We are currently testing this hypothesis.

Memory $CD8^+$ T cells were recovered from both the lung interstitium (including the bronchus-associated lymphoid tissue) and the lung airway lumina several weeks after infection. Since the lung airways are directly exposed to the external environment, it seems remarkable that memory cells can persist in a functionally stable state for several weeks at this site. The presence of effector memory T cells in the lung airways likely represents an early defense mechanism against a secondary viral infection. Although the absolute numbers of cells are not large, early production of IFN-γ and other antiviral cytokines may reduce the level of viral replication and reduce the viral load to be cleared by the memory response that develops in local draining lymph nodes. In this regard, localization of the cells in the airways and nasal mucosa represents an optimal location for responding to the early phase of an infection (7, 13–15). Indeed, we have previously shown that memory $CD4^+$ T cells in the lung airways can provide a substantial degree of protective immunity against a subsequent virus infection (6). In addition, previous studies have shown that cellular control of influenza virus infections wanes substantially over the course of about 6 mo, which correlates with the loss of memory $CD8^+$ T cells in the lung airways (1). This basic observation mirrors the situation in humans where cellular immunity to influenza virus appears to wane between yearly epidemics (28–32). The data in the current report argue that protective cellular immunity against this class of infection depends on a bolus of cells that is established in the lungs by the primary infection. The recruitment of new memory T cells generated by homeostatic turnover mechanisms in secondary lymphoid organs is apparently insufficient to replenish this lung population which then steadily declines over time. This observation has significant implications for the design and development of vaccines that promote cellular immunity in the lung. In this regard, we have shown that relatively long-term protective immunity can be established following some vaccination strategies, such as DNA vaccination (33). This type of vaccine may induce lymphoid memory T cell pools that exhibit increased rates of homeostatic turnover, resulting in enhanced maintenance of peripheral memory T cell pools. Thus, it is possible that the best

strategy for inducing mucosal immunity may involve a combination of vaccines that optimally induce a local pool of peripheral memory cells and a rapidly turning over pool of lymphoid memory T cells. We are currently investigating this possibility.

Acknowledgments

We thank Scottie Adams and Tim Miller of the Trudeau Institute Molecular Biology Core Facility for the generation of tetrameric reagents, and Drs. Marcy Blackman and Emilio Flaño for critically reviewing this manuscript.

References

1. Liang, S., K. Mozdzanowska, G. Palladino, and W. Gerhard. 1994. Heterosubtypic immunity to influenza type A virus in mice: effector mechanisms and their longevity. *J. Immunol. 152:1653.*
2. Flynn, K. J., G. T. Belz, J. D. Altman, R. Ahmed, D. L. Woodland, and P. C. Doherty. 1998. Virus-specific $CD8^+$ T cells in primary and secondary influenza pneumonia. *Immunity 8:683.*
3. Woodland, D. L., R. J. Hogan, and W. Zhong. 2001. Cellular immunity and memory to respiratory virus infections. *Immunol. Res. 24:53.*
4. Hou, S., P. C. Doherty, M. Zijlstra, R. Jaenisch, and J. M. Katz. 1992. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted $CD8^+$ T cells. *J. Immunol. 149:1319.*
5. Hogan, R. J., E. J. Usherwood, W. Zhong, A. A. Roberts, R. W. Dutton, A. G. Harmsen, and D. L. Woodland. 2001. Activated antigen-specific $CD8^+$ T cells persist in the lungs following recovery from respiratory virus infections. *J. Immunol. 166:1813.*
6. Hogan, R. J., W. Zhong, E. J. Usherwood, T. Cookenham, A. D. Roberts, and D. L. Woodland. 2001. Protection from respiratory virus infections can be mediated by antigen-specific $CD4^+$ T cells that persist in the lungs. *J. Exp. Med. 193:981.*
7. Wiley, J. A., R. J. Hogan, D. L. Woodland, and A. G. Harmsen. 2001. Antigen-specific $CD8^+$ T cells persist in the upper respiratory tract following influenza virus infection. *J. Immunol. 167:3293.*
8. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature 401:708.*
9. Reinhardt, R. L., A. Khoruts, R. Merica, T. Zell, and M. K. Jenkins. 2001. Visualizing the generation of memory CD4 T cells in the whole body. *Nature 410:101.*
10. Ostler, T., T. Hussell, C. D. Surh, P. Openshaw, and S. Ehl. 2001. Long-term persistence and reactivation of T cell memory in the lung of mice infected with respiratory syncytial virus. *Eur. J. Immunol. 31:2574.*
11. Masopust, D., V. Vezys, A. L. Marzo, and L. Lefrancois. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. *Science 291:2413.*
12. Marshall, D. R., S. J. Turner, G. T. Belz, S. Wingo, S. Andreansky, M. Y. Sangster, J. M. Riberdy, T. Liu, M. Tan, and P. C. Doherty. 2001. Measuring the diaspora for virus-specific $CD8^+$ T cells. *Proc. Natl. Acad. Sci. USA 98:6313.*
13. Gallichan, W. S., and K. L. Rosenthal. 1996. Long-lived cytotoxic T lymphocyte memory in mucosal tissues after mucosal but not systemic immunization. *J. Exp. Med. 184:1879.*
14. Belyakov, I. M., J. D. Ahlers, B. Y. Brandwein, P. Earl, B. L. Kelsall, B. Moss, W. Strober, and J. A. Berzofsky. 1998. The importance of local mucosal HIV-specific $CD8^+$ cytotoxic T lymphocytes for resistance to mucosal viral transmission in mice and enhancement of resistance by local administration of IL-12. *J. Clin. Invest. 102:2072.*
15. Belyakov, I. M., M. A. Derby, J. D. Ahlers, B. L. Kelsall, P. Earl, B. Moss, W. Strober, and J. A. Berzofsky. 1998. Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. *Proc. Natl. Acad. Sci. USA 95:1709.*
16. Altman, J. D., P. H. Moss, P. R. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science 274:94.*
17. Usherwood, E. J., R. J. Hogan, G. Crowther, S. L. Surman, T. L. Hogg, J. D. Altman, and D. L. Woodland. 1999. Functionally heterogeneous $CD8^+$ T-cell memory is induced by Sendai virus infection of mice. *J. Virol. 73:7278.*
18. Mohri, H., S. Bonhoeffer, S. Monard, A. S. Perelson, and D. D. Ho. 1998. Rapid turnover of T lymphocytes in SIV-infected rhesus macaques. *Science 279:1223.*
19. Tough, D. F., P. Borrow, and J. Sprent. 1996. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science 272:1947.*
20. Zhang, X., S. Sun, I. Hwang, D. F. Tough, and J. Sprent. 1998. Potent and selective stimulation of memory-phenotype $CD8^+$ T cells in vivo by IL-15. *Immunity 8:591.*
21. Paine, R., III, A. Chavis, D. Gaposchkin, P. Christensen, C. H. Mody, L. A. Turka, and G. B. Toews. 1992. A factor secreted by a human pulmonary alveolar epithelial-like cell line blocks T-cell proliferation between G_1 and S phase. *Am. J. Respir. Cell Mol. Biol. 6:658.*
22. Sprent, J. 1997. Immunological memory. *Curr. Opin. Immunol. 9:371.*
23. Tough, D. F., and J. Sprent. 1994. Turnover of naive- and memory-phenotype T cells. *J. Exp. Med. 179:1127.*
24. Schluns, K. S., W. C. Kieper, S. C. Jameson, and L. Lefrancois. 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat. Immunol. 1:426.*
25. Ku, C. C., M. Murakami, A. Sakamoto, J. Kappler, and P. Marrack. 2000. Control of homeostasis of $CD8^+$ memory T cells by opposing cytokines. *Science 288:675.*
26. Dalton, D. K., L. Haynes, C. Q. Chu, S. L. Swain, and S. Wittmer. 2000. Interferon γ eliminates responding CD4 T cells during mycobacterial infection by inducing apoptosis of activated CD4 T cells. *J. Exp. Med. 192:117.*
27. Cauley, L. S., E. E. Miller, M. Yen, and S. L. Swain. 2000. Superantigen-induced CD4 T cell tolerance mediated by myeloid cells and IFN-γ. *J. Immunol. 165:6056.*
28. Sonoguchi, T., H. Naito, M. Hara, Y. Takeuchi, and H. Fukumi. 1985. Cross-subtype protection in humans during sequential, overlapping, and/or concurrent epidemics caused by H3N2 and H1N1 influenza viruses. *J. Infect. Dis. 151:81.*
29. Frank, A. L., L. H. Taber, and J. M. Wells. 1983. Individuals infected with two subtypes of influenza A virus in the same season. *J. Infect. Dis. 147:120.*
30. Schulman, J. L. 1970. Effects of immunity on transmission of influenza: experimental studies. *Prog. Med. Virol. 12:128.*
31. Voeten, J. T., T. M. Bestebroer, N. J. Nieuwkoop, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan. 2000. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. *J. Virol. 74:6800.*
32. McMichael, A. 1994. Cytotoxic T lymphocytes specific for influenza virus. *Curr. Top. Microbiol. Immunol. 189:75.*
33. Chen, Y., E. J. Usherwood, S. L. Surman, T. L. Hogg, and D. L. Woodland. 1999. Long-term $CD8^+$ T cell memory to Sendai virus elicited by DNA vaccination. *J. Gen. Virol. 80:1393.*