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# Nonspecific Recruitment of Memory CD8<sup>+</sup> T Cells to the Lung Airways During Respiratory Virus Infections<sup>1</sup>

Kenneth H. Ely, Linda S. Cauley, Alan D. Roberts, Jean W. Brennan, Tres Cookenham, and David L. Woodland<sup>2</sup>

Previous studies have shown that heterologous viral infections have a significant impact on pre-existing memory T cell populations in secondary lymphoid organs through a combination of cross-reactive and bystander effects. However, the impact of heterologous viral infections on effector/memory T cells in peripheral sites is not well understood. In this study, we have analyzed the impact of a heterologous influenza virus infection on Sendai virus-specific CD8<sup>+</sup> effector/memory cells present in the lung airways. The data show a transient increase in the numbers of Sendai virus nucleoprotein 324–332/K<sup>b</sup>-specific CD8<sup>+</sup> memory T cells in the airways of the influenza-infected mice peaking around day 4 postinfection. Intratracheal transfer studies and 5-bromo-2'-deoxyuridine incorporation demonstrate that this increase is due to the recruitment of resting memory cells into the airways. In addition, the data show that these immigrating memory cells are phenotypically distinct from the resident memory T cells of the lung airways. A similar influx of nonproliferating Sendai virus nucleoprotein 324–332/K<sup>b</sup>-specific CD8<sup>+</sup> memory T cells is also induced by a secondary (homologous) infection with Sendai virus. Together, these data suggest that inflammation can accelerate memory T cell migration to nonlymphoid tissues and is a part of the normal recall response during respiratory infections. *The Journal of Immunology*, 2003, 170: 1423–1429.

Studies in mouse models have shown that cytolytic CD8<sup>+</sup> T cells play a central role in controlling influenza and parainfluenza virus infections in the lung (1–3). These cells are initially activated in the draining lymph node where they proliferate before acquiring effector functions and the capacity to migrate to the site of infection. Following recovery from the primary infection, large pools of memory CD8<sup>+</sup> T cells persist in the secondary lymphoid organs. These memory T cells are able to mediate a substantially accelerated recall response to secondary virus infection resulting in earlier viral clearance. Recently, it has emerged that CD8<sup>+</sup> memory T cells also persist in nonlymphoid peripheral tissues, such as the lungs, kidneys, and liver (4–6). For example, studies in the Sendai virus system have demonstrated that substantial numbers of memory CD8<sup>+</sup> T cells, specific for the immunodominant Sendai virus nucleoprotein (Sen-NP)<sup>3</sup> epitope Sen-NP<sub>324–332</sub>/K<sup>b</sup>, persist in the lung airways of mice that have recovered from acute infection (7, 8). Similar to memory CD8<sup>+</sup> T cells in secondary lymphoid organs, memory cells in the lung airways are small resting cells (9). However, they are phenotypically distinct from memory T cells in secondary lymphoid organs in that they express acute activation markers and have reduced CD11a expression (4, 7, 10). Based on their activated phenotype, memory T cells in peripheral sites are frequently referred to as effector/memory T cells (11). It has been estimated that almost half of all memory CD8<sup>+</sup> T cells established by a respiratory virus are ef-

fector/memory T cells that persist in nonlymphoid tissues following viral clearance (6).

Given the significance of memory T cell populations in protective cellular immunity, there has been considerable interest in the impact of subsequent heterologous virus infections on the stability and longevity of different memory T cell populations. Most studies have focused on memory T cells in the secondary lymphoid organs and have shown that subsequent infections induce significant changes in the size and composition of pre-existing memory T cell pools (12–15). For example, McNally et al. (16) demonstrated that heterologous viral infections can result in a reduction in the numbers of pre-existing CD8<sup>+</sup> memory T cells in the spleen. This is presumably due to competition for space, and it has been shown that the loss of memory cells in this situation is mediated by apoptosis (13, 17). However, heterologous infections can also result in the expansion of pre-existing memory T cell populations. In some instances, these responses have been attributed to T cell cross-reactivity, whereby a subset of pre-established memory cells to one virus are able to recognize an epitope on a heterologous virus. Such cross-reactive T cells have been shown to augment the clearance of the second virus. For example, prior exposure to lymphocytic choriomeningitis virus results in enhanced protection against vaccinia virus or Pichinde virus (14, 18). Other studies (16) have reported augmented CD8<sup>+</sup> memory T cell responses in the absence of cross-reactive epitopes, suggesting that nonspecific inflammation can also influence pre-existing memory populations.

Although the impact of heterologous virus infections on memory T cells in secondary lymphoid tissues is well documented, much less is known about their impact on the fate of effector/memory populations in peripheral tissues. Two recent studies (14, 19) have indicated that secondary pulmonary infection results in a change in the absolute numbers of memory T cells in the lung or lung airways, but it was not clear whether this reflected local proliferation or recruitment of cells from other sites. In addition, the specific impact of heterologous infection on the effector/memory populations already resident in the lung airways was not assessed. To address these issues, we have analyzed the impact of influenza virus infection on the fate of Sendai virus-specific effector/memory

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<sup>3</sup> Abbreviations used in this paper: Sen-NP, Sendai virus nucleoprotein; Flu-NP, influenza virus nucleoprotein; Flu-PA, influenza virus acid polymerase; MHV, murine  $\gamma$ -herpesvirus; BrdU, 5-bromo-2'-deoxyuridine; EID<sub>50</sub>, 50% egg infectious dose.

T cells in the lung airways. The data show that the heterologous infection induces the recruitment of nondividing Sendai virus-specific memory CD8<sup>+</sup> T cells into the lung airways during the first 4 days of infection, followed by their subsequent loss as the influenza infection progresses. Nonproliferating CD8<sup>+</sup> memory cells also accumulate in the lungs during the early response to a secondary (homologous) Sendai virus infection, suggesting that this influx of circulating memory CD8<sup>+</sup> T cells is a normal component of secondary cellular immune responses.

## Materials and Methods

### Viruses, mice, and infection

Sendai virus (Enders strain) (20), influenza virus A/HK-x31 (x31; H3N2) (21), vaccinia virus strain Western Reserve (22), and WUMS clone of murine  $\gamma$ -herpesvirus (MHV)-68 (23–25) were grown, stored, and titered as previously described. Female C57BL/6, B6.Pl-Thy1<sup>0</sup>/Cy (Thy1.1) and B6.SJLptpr<sup>c</sup>pep3<sup>b</sup>/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<sub>50</sub>) of Sendai virus. Mice were challenged 30–35 days after Sendai virus infection, by heterologous intranasal infection with either 300 EID<sub>50</sub> of x31, 400 PFU of MHV-68, or 10<sup>4</sup> PFU of vaccinia virus. In adoptive transfer experiments, donor mice were infected with 250 EID<sub>50</sub> of Sendai virus and recipient mice were either Sendai memory or naive.

### Bronchoalveolar lavage harvest and flow cytometry

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 collected for FACS analysis. MHC class I-peptide tetramers were generated by the Molecular Biology Core Facility at the Trudeau Institute as described previously (26). Cells were stained with APC-conjugated tetramers specific for immunodominant Sen-NP epitope (Sen-NP<sub>324–332</sub>/K<sup>b</sup>), influenza virus nucleoprotein (Flu-NP) epitope (Flu-NP<sub>366–374</sub>/D<sup>b</sup>), or influenza acid polymerase (Flu-PA) epitope (Flu-PA<sub>224–233</sub>/D<sup>b</sup>) as previously described (7). Tetramer-labeled cells were then stained with anti-CD8PerCP and FITC-conjugated mAbs specific for CD11a or Ly6C, and PE-conjugated mAbs specific for Thy1.1 (BD PharMingen, San Diego, CA) or CD45.2 (eBioscience, San Diego, CA) on ice for 20 min and fixed overnight with 1% paraformaldehyde in PBS. Samples were run on a BD Biosciences (San Jose, CA) FACScalibur flow cytometer and data were analyzed using FlowJo software (Tree Star, San Carlos, CA). The percentage of tetramer-positive cells among CD8<sup>+</sup> T cells was calculated by dividing the number of tetramer-positive CD8<sup>+</sup> events by the total number of events in the CD8<sup>+</sup> 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.

### 5-Bromo-2'-deoxyuridine (BrdU) labeling and detection

Sendai memory mice were given BrdU for 11 days in drinking water (0.8 mg/ml) beginning 1 day before infection with 300 EID<sub>50</sub> of x31 intranasally. Mice were euthanized, and lung airway cells were harvested at varying times after x31 infection as described above and 1–2 × 10<sup>6</sup> cells were stained with Sen-NP<sub>324–332</sub>/K<sup>b</sup>-APC or Flu-NP<sub>366–374</sub>/D<sup>b</sup>-APC tetramers and anti-CD8PerCP. The stained cells were washed and analyzed for BrdU content as previously described (27). Briefly, the washed cells were treated with 1 ml of FACS lysing solution (BD Biosciences) for 15 min at room temperature and fixed overnight with 1% paraformaldehyde in PBS containing 0.05% Nonidet P-40. The cells were again washed and cellular DNA was denatured with 50 Kunitz U of bovine pancreas DNase-I (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. After DNase treatment, the cells were washed in PBS with 5% FCS and 0.5% Nonidet P-40 and stained for 45 min at 4°C using anti-BrdU PE (BD Biosciences). The cells were washed twice and fixed with 1% paraformaldehyde before FACS analysis. Samples were collected and analyzed as described above.

### CFSE staining and adoptive transfer of BAL cells

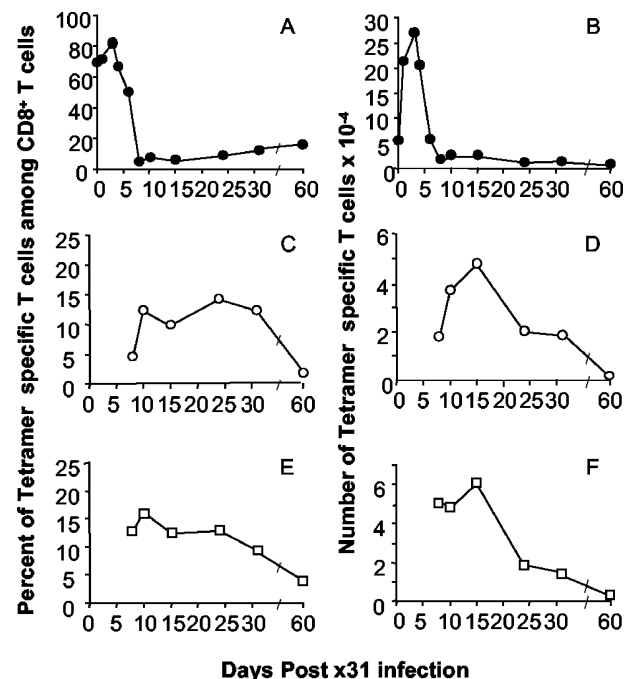
C57BL/6 Sendai memory mice were euthanized and lung airway cells were collected as described above. BAL cells were stained with 0.5  $\mu$ M CFSE for 10 min at room temperature and washed extensively. Labeled cells were resuspended and divided in a donor to recipient ratio of 5:1 in a volume of 100  $\mu$ l per mouse for intratracheal transfer. Recipient mice were anesthetized by i.p. injection of 2,2,2-tribromoethanol, and CFSE-labeled BAL

cells were transferred into either congenic Sendai memory mice or congenic naive mice by intratracheal installation using a blunted and bent 20-gauge needle. BAL cells were collected and pooled from three mice the following day to provide a transfer baseline. Remaining Sendai memory recipient mice were challenged with 300 EID<sub>50</sub> of x31 and BAL was harvested 4 days post-x31 infection; naive recipient mice were challenged with 250 EID<sub>50</sub> of Sendai virus and BAL was harvested on days 0 and 4 post-Sendai infection. Harvested cells were stained with tetramer for 1 h and with PerCP-conjugated anti-CD8 and either PE-conjugated anti-Thy1.2 or anti-CD45.1 Abs for 20 min and then fixed overnight. Stained samples were collected and analyzed as described above.

## Results

### Sendai virus specific memory cells in the lung airways increase in number following heterologous virus infection

Recent studies have shown that respiratory virus infections induce an increase in the numbers of heterologous memory CD8<sup>+</sup> T cells in the lung or lung airways (14, 19, 28). However, it was not clear whether this reflected local proliferation or recruitment of cells from other sites. To address this issue, we have analyzed the impact of a heterologous influenza virus infection on CD8<sup>+</sup> memory cells that had been established in the lungs during a prior Sendai virus infection. As shown in Fig. 1, (day 0, A and B), C57BL/6 mice that had recovered from a prior Sendai virus infection had substantial numbers of effector/memory cells specific for the immunodominant Sen-NP<sub>324–332</sub>/K<sup>b</sup> epitope in the lung airways (~50,000 cells/mouse) (7, 29, 30). These cells comprised ~70% of all CD8<sup>+</sup> T cells in the lung airways at this time (Fig. 1A), consistent with previous studies (7). Following infection with influenza virus, the numbers of Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific T cells in

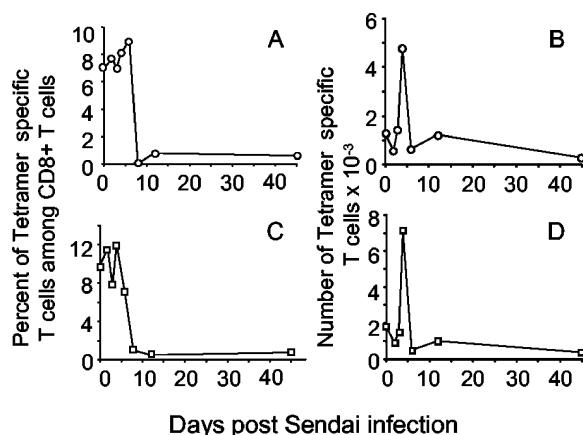


**FIGURE 1.** Sendai virus-specific memory cells in the lung airways increase in number following heterologous virus infection. Naive 6- to 8-wk-old female C57BL/6 were anesthetized and infected intranasally with 250 EID<sub>50</sub> of Sendai virus. Thirty to 35 days post-Sendai infection, mice were challenged with an intranasal infection of 300 EID<sub>50</sub> of influenza A strain x31. BAL cells were recovered at indicated time points following x31 infection and stained with tetrameric reagents specific for Sen-NP<sub>324–332</sub>/K<sup>b</sup> (●; A and B), Flu-NP<sub>366–374</sub>/D<sup>b</sup> (○; C and D), or Flu-PA<sub>224–233</sub>/D<sup>b</sup> (□; E and F), and with anti-CD8. The percentage of tetramer-positive cells among CD8<sup>+</sup> T cells was calculated as described in *Materials and Methods*. Each time point represents the number of cells per mouse calculated from a pool of three mice per time point. Data are representative of three separate time courses.

the lung airways increased dramatically over the first 4 days to ~270,000 cells (representing a >5-fold increase in cell number). After day 4, the numbers of cells decreased to levels substantially below the numbers present before the influenza virus infection. The same patterns of expansion and contraction of memory T cell numbers in the lung airways were observed in three separate experiments (data not shown). The increase in Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory T cells in the lung airways was ~4- to 5-fold at day 4 post-influenza virus infection in each experiment. Despite the increase in the absolute number of Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory T cells in the lung airways, there was no significant change in the number of these cells in the draining lymph nodes or spleen (data not shown).

The presence of Sendai virus-specific memory T cells did not impact the kinetics and magnitude of the CD8<sup>+</sup> T cell response to influenza virus infection. As shown in Fig. 1, C–F, the response of T cells specific for two dominant influenza virus epitopes (Flu-NP<sub>366–374</sub>/D<sup>b</sup> and Flu-PA<sub>224–233</sub>/D<sup>b</sup>) was consistent with a primary x31 infection (31). Following resolution of the influenza virus infection (day 60 postinfection, for example), memory cells specific for both Sendai virus (Sen-NP<sub>324–332</sub>/K<sup>b</sup>) and influenza virus (Flu-NP<sub>366–374</sub>/D<sup>b</sup> and Flu-PA<sub>224–233</sub>/D<sup>b</sup>) could be detected in the lung airways.

To investigate whether the increase in the number of Sendai virus-specific memory cells in the lungs was a unique characteristic of influenza virus infection, we reversed the order of infections. Thus, we analyzed the impact of a Sendai virus infection on pre-established populations of influenza virus-specific memory CD8<sup>+</sup> T cells. As shown in Fig. 2, the numbers of memory cells specific for two separate influenza virus epitopes (Flu-NP<sub>366–374</sub>/D<sup>b</sup> and Flu-PA<sub>224–233</sub>/D<sup>b</sup>) increased in the lung airways after Sendai virus infection, with similar kinetics to those shown for Sendai-specific memory cells after heterologous influenza infection. The peak numbers of cells for each influenza epitope (B and D) were detected on day 4 post-Sendai virus infection, and represented an increase of ~4-fold. The



**FIGURE 2.** Influenza virus-specific memory cells in the lung airways increase in number following heterologous virus infection. Naive 6- to 8-wk-old female C57BL/6 were anesthetized and infected intranasally with 300 EID<sub>50</sub> of influenza A x31 virus. Thirty to 35 days post-x31 infection, mice were challenged with an intranasal infection of 250 EID<sub>50</sub> of Sendai virus. BAL cells were recovered at indicated time points following Sendai virus infection and stained with tetrameric reagents specific for Flu-NP<sub>366–374</sub>/D<sup>b</sup> (○; A and B) or Flu-PA<sub>224–233</sub>/D<sup>b</sup> (□; C and D) and with anti-CD8. The percentage of tetramer-positive cells among CD8<sup>+</sup> T cells was calculated as described in *Materials and Methods*. Each time point represents the number of cells per mouse, calculated from a pool of three mice per time point. Data are representative of two separate experiments.

numbers of influenza virus-specific memory cells subsequently decreased to minimal levels by day 6.

Because multiple T epitopes were analyzed in this study, the results could not be readily attributed to a single cross-reactive epitope in the two viruses, and therefore suggested that the transient increase in numbers of memory cells in the lungs following heterologous infection may reflect a response to nonspecific inflammation. To further investigate this possibility, we examined the response of Sendai virus-specific memory T cells to other heterologous viral infections in the lung. Thus, Sendai virus memory mice were intranasally infected with either MHV-68 or vaccinia virus. As before, there was a substantial increase in the numbers of Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory T cells in the lung airways on day 3 following MHV-68 and vaccinia virus infection (4.4-fold and 1.7-fold expansions, respectively). Furthermore, nonspecific recruitment of Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory T cells was also observed following intranasal administration of inflammatory agents such as heat-killed *Escherichia coli* or heat-labile enterotoxin (data not shown). Together, these data demonstrated that the effects on pre-established memory cells were not specific for influenza virus infections and not dependent upon cross-reactive epitopes.

#### *Impact of heterologous infection on the phenotype of effector/memory cells in the lung airways*

We next investigated the impact of heterologous influenza virus infection on the phenotype of pre-established Sendai virus-specific memory cells. Previous studies have shown that effector/memory CD8<sup>+</sup> T cells resident in the lung airways are characterized by having an activated phenotype, CD44<sup>high</sup>CD62L<sup>low</sup>CD69<sup>high</sup>CD25<sup>high</sup>Ly6c<sup>mixed</sup> (~70% of the cells are Ly6c<sup>low</sup>) (7, 8, 32). In addition, and in contrast to memory CD8<sup>+</sup> T cells at other peripheral and lymphoid sites, these cells are CD11a<sup>low</sup>, possibly due to the fact that they have extravasated into the lung airways (10, 32). Following influenza virus infection, there were substantial changes in Ly6c and CD11a expression on Sendai virus-specific memory cells in the lung airways (Fig. 3A). By day 3 postinfection, a sizable fraction of Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory T cells were Ly6c<sup>high</sup> and CD11a<sup>high</sup>, and the ratio of Ly6c<sup>high</sup>CD11a<sup>high</sup> to Ly6c<sup>low</sup>CD11a<sup>low</sup> cells gradually increased through day 10 of the infection (Fig. 3, B and C). After day 10, the ratio of Ly6c<sup>high</sup>CD11a<sup>high</sup> to Ly6c<sup>low</sup>CD11a<sup>low</sup> cells reverted back to the phenotype characteristic of memory CD8<sup>+</sup> T cells in the lung before influenza virus infection. There were no changes in CD44, CD62L, CD69, and CD25 expression on the Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific cells following influenza infection (data not shown).

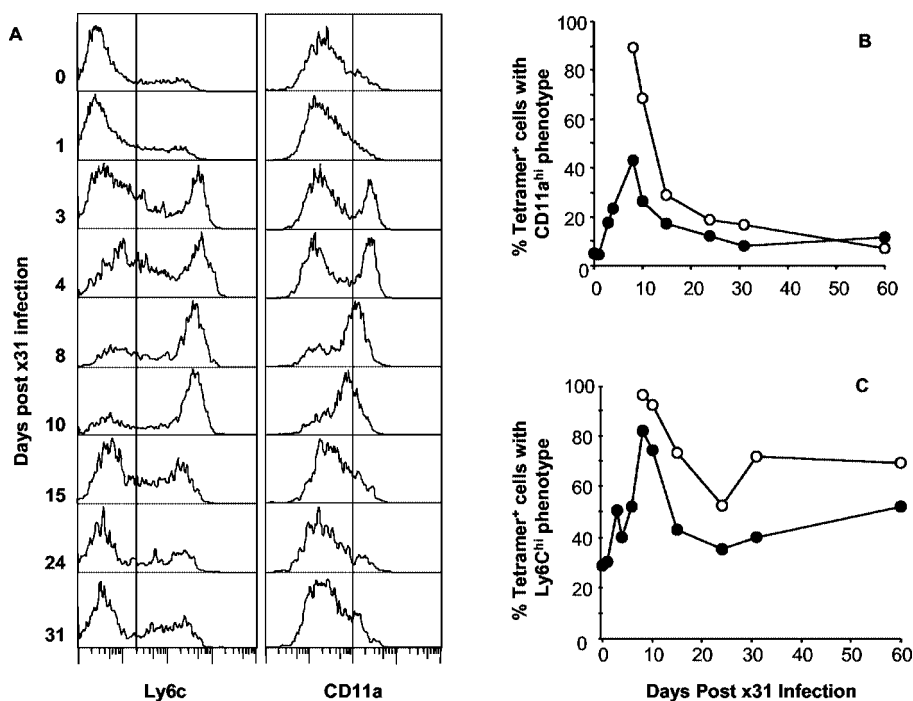
We also analyzed the phenotype of Flu-NP<sub>366–374</sub>/D<sup>b</sup>-specific cells in the lung airways. As shown in Fig. 3, B and C, almost all of the Flu-NP<sub>366–374</sub>/D<sup>b</sup>-specific cells recruited to the airways at the peak of the infection were Ly6c<sup>high</sup>CD11a<sup>high</sup>. However, the phenotype of these cells converted to a lung effector/memory phenotype after memory was established (e.g., day 60 postinfection).

#### *Increases in the number of memory cells in the lung airways reflect recruitment of cells from other sites*

One possible explanation for the changes in the absolute numbers and phenotype of Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory T cells in the lung airways at day 4 of an influenza virus infection is that the cells represent a mixture of pre-existing lung memory cells and newly recruited memory cells from the circulation. An alternative possibility is that some of the memory cells in the airways may be proliferating in response to the heterologous infection, resulting in a change in absolute number and phenotype. To distinguish these possibilities, we analyzed the fate of memory T cells resident in the lung airways using an intratracheal transfer model. Thus, memory



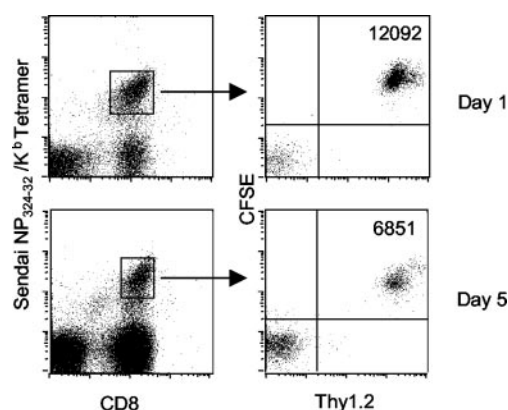
**FIGURE 3.** Sendai virus-specific memory cells in the lung airways have distinct Ly6c and CD11a phenotypes. C57BL/6 Sendai memory mice were infected with 300 EID<sub>50</sub> of influenza strain x31 30–35 days post-Sendai infection, and BAL cells were harvested on various days post-x31 infection. Nonadherent BAL cells were stained with a tetrameric reagent specific for Sen-NP<sub>324–332</sub>/K<sup>b</sup>, followed by surface staining for CD8, Ly6c, and CD11a, and analyzed by FACS. Ly6c and CD11a profiles various days post x31-infection (A) are within the Sen-NP<sub>324–332</sub>/K<sup>b</sup> by CD8<sup>+</sup> gate. Percentages of Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific CD8<sup>+</sup> T cells (●) and percentages of Flu-NP<sub>366–374</sub>/D<sup>b</sup>-specific CD8<sup>+</sup> T cells (○) that are expressing CD11a<sup>high</sup> (B) and Ly6c<sup>high</sup> (C) were calculated as described in *Materials and Methods*. Data are representative of two separate experiments.



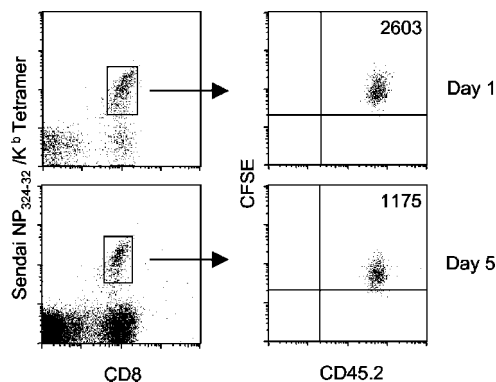
cells were isolated from the lung airways of Thy-1.2<sup>+</sup> mice (35 days post-Sendai virus infection), labeled with CFSE, and then intratracheally transferred into the lungs of Thy-1.1<sup>+</sup> memory mice that had also recovered from a prior Sendai virus infection. Previous studies have shown that ~90% of the transferred cells disappear during the first 24 h following this type of cell transfer but that the remaining 10% of the cells survive in the lung airways for several weeks (9). The transferred cells that persist for >24 h do not appear to be a selected subpopulation, because they are phenotypically indistinguishable from the original input population (data not shown). In the current study, donor (Thy-1.2<sup>+</sup>) Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory T cells were readily identified 1 day posttransfer, and these cells were all CFSE<sup>high</sup> (Fig. 4). Host (Thy-1.1<sup>+</sup>) Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory T cells were all CFSE negative. Recipient mice were then intranasally infected with influenza virus to assess the impact of a heterologous infection on lung resident memory cells. Four days postinfection (which is day 5 post-cell transfer), cells were recovered from the lung airways and analyzed for their CFSE intensity. As shown in Fig. 4, donor (Thy-1.2<sup>+</sup>) Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory T cells were readily identified at this time, and all of the cells retained high levels of CFSE, indicating that they had not proliferated in response to the heterologous infection. In addition, none of the donor cells could be detected in other organs, indicating that they had not trafficked out of the airways in substantial numbers (data not shown). We also analyzed the phenotype of the donor cells recovered on day 4 post-influenza virus infection. These cells retained the phenotype characteristic of memory CD8<sup>+</sup> T cells resident in the lung airway, i.e., CD11a<sup>low</sup>Ly6c<sup>mixed</sup> (data not shown). Together, these data indicate that Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory cells resident in the lung airways are not stimulated to proliferate or change Ly6c and CD11a phenotype following a heterologous influenza virus infection. Thus, the expansion in Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory T cell numbers in the airways on day 4 of a heterologous influenza virus infection must reflect recruitment of memory T cells from the circulation.

We also used the same intratracheal transfer approach to study the impact of a homologous Sendai virus infection on resident

Sen-NP<sub>324–332</sub>/K<sup>b</sup> memory cells in the lung airway. Thus, memory cells isolated from the lung airways of CD45.2<sup>+</sup> mice 30–35 days postinfection were labeled with CFSE and intratracheally transferred into the lungs of naive CD45.1<sup>+</sup> mice. One day posttransfer, the mice were intranasally infected with Sendai virus and subsequently analyzed on day 4 postinfection (day 5 posttransfer). As shown in Fig. 5, donor (CD45.2<sup>+</sup>) Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory T cells were readily identified at this time and all of the cells retained high levels of CFSE. There were no host (CD45.1<sup>+</sup>) Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific cells in the lung airways at this time, consistent with previous kinetic studies (2, 29). These data show



**FIGURE 4.** Transferred BAL cells from Sendai memory mice do not proliferate or migrate out of the lung airways in response to heterologous infection. C57BL/6 Sendai memory mice were euthanized 30–35 days postinfection, and lung airway cells were collected. After staining with CFSE, cells were transferred intratracheally at a donor to recipient ratio of 5:1 into Thy1.1 Sendai memory mice. Recipient mice were challenged with 300 EID<sub>50</sub> of x31 the day following transfer. BAL was harvested 1 and 5 days posttransfer and analyzed for CD8 and Sen-NP<sub>324–332</sub>/K<sup>b</sup> reactivity. Cells within the tetramer gate were analyzed for CFSE intensity and Thy1.2 expression. Numbers represent the total number of transferred Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific cells recovered per mouse. Data are representative of three separate experiments.



**FIGURE 5.** Transferred BAL cells from Sendai memory mice do not proliferate in response to homologous infection. C57BL/6 Sendai memory mice were euthanized 30–35 days postinfection, and lung airway cells were collected as described in *Materials and Methods*. CFSE-labeled BAL cells from Sendai memory mice were transferred into the lungs of naive recipient mice at a donor to host ratio of 5:1 as described in *Materials and Methods*. Recipient mice were challenged with 250 EID<sub>50</sub> of Sendai virus 1 day following transfer. BAL was harvested 1 and 5 days posttransfer and analyzed for CD8<sup>+</sup> and Sen-NP<sub>324–332</sub>/K<sup>b</sup> reactivity. Cells within the tetramer gate were analyzed for CFSE intensity and CD45.2 expression. Numbers represent the total number of transferred Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific cells recovered per mouse. Data are representative of one of three separate experiments.

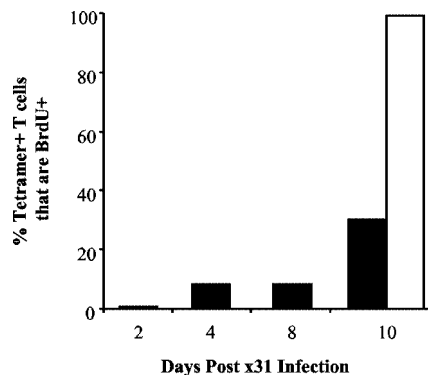
that transferred memory cells from the lung do not proliferate in the lung airways even when the cognate Ag is provided.

#### *Recruitment of memory T cells to the lung by heterologous infection does not depend on proliferation*

The previous data demonstrate that memory cells in the lung airways do not proliferate during heterologous infection indicating that the increased cell numbers reflect recruitment from the circulation. The newly recruited cells could be either resting memory cells or memory cells that had been stimulated to proliferate in the secondary lymphoid organs through a bystander activation mechanism (33–35). To distinguish between these possibilities, we analyzed the uptake of BrdU in memory cells recruited to the lung airways by a heterologous infection. Thus, mice that had recovered from a Sendai virus infection (day 40 postinfection) were intranasally infected with influenza virus and administered BrdU in the drinking water throughout the infection. On various days postinfection, the percentage of Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific memory T cells that were BrdU-positive was determined. As shown in Fig. 6, only 10% of the Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific T cells in the lung airways were BrdU<sup>+</sup> on day 4 postinfection (at this time, ~80% of the cells in the airways are recruited from the circulation). Thus, the vast majority of memory cells recruited to the lung airways at day 4 postinfection are not synthesizing DNA. At day 10 post-influenza virus infection, ~30% of the Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific T cells in the lung airways were BrdU<sup>+</sup> suggesting that, at later times, there was some degree of proliferation by Ag-specific memory cells. However, the absolute numbers of cells in the airways at this time were very low. Analysis of Flu-NP<sub>366–374</sub>/D<sup>b</sup>-specific effector cells in the lung airways at day 10 postinfection indicated that all of the cells were BrdU<sup>+</sup> (Fig. 6). This is consistent with the fact that the development of a primary influenza response is dependent on proliferation of naive T cells in the draining lymph node (36).

#### *Recruitment of memory cells is also driven by homologous virus infection*

Our previous studies had focused on the capacity of heterologous virus infections to induce the transitory recruitment of memory T

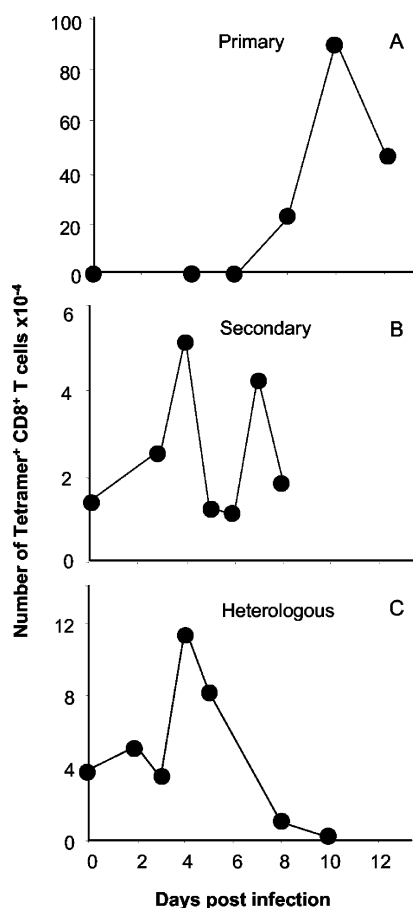


**FIGURE 6.** Recruitment of memory T cells to the lung by heterologous infection does not depend on proliferation. Sendai memory C57BL/6 mice were given BrdU in drinking water beginning the day before heterologous x31 challenge, and continuing for 11 days with daily changes of BrdU-treated water. Mice were challenged with 300 EID<sub>50</sub> of x31 intranasally and BAL was collected at the indicated time points post-x31 infection. BAL was stained with Sen-NP<sub>324–332</sub>/K<sup>b</sup>-APC (■) and Flu-NP<sub>366–374</sub>/D<sup>b</sup>-PE (□) tetramers, anti-BrdU-FITC, and anti-CD8PerCP, as described in *Materials and Methods*. The percentage of tetramer-positive cells incorporating BrdU was calculated as the number of BrdU<sup>+</sup>tetramer<sup>+</sup> cells divided by the total number of tetramer-positive cells within the CD8<sup>+</sup> gate. Data are representative of two separate experiments.

cells to the lung airways. We next asked whether this transitory recruitment of memory cells is also a normal feature of secondary T cell responses to homologous virus infections. Initial analysis of the secondary T cell response to a serologically distinct strain of influenza virus that cross-reacts at the T cell level was inconclusive. This is because the secondary T cell response was very large (peaking at day 7) and tended to mask events occurring at day 4 (2) (data not shown). However, we have recently shown that intranasal challenge of Sendai virus-immune mice with a large dose of Sendai virus results in a weak secondary T cell response in the lung, presumably because much of the virus is controlled by neutralizing Ab (L. Cauley, T. Cookenham, R. Hogan, S. Crowe, and D. Woodland, manuscript in preparation). We speculated that this system might allow us to distinguish an early peak of memory cell recruitment at day 4 postinfection. Thus, both naive C57BL/6 mice and C57BL/6 mice that had recovered from a prior Sendai virus infection were intranasally challenged with Sendai virus, and the absolute numbers of Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific T cells in the lung airways were determined on various days thereafter. As shown in Fig. 7A, the primary response was characterized by a single peak of Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific T cells at day 10 postinfection. Absolutely no Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific T cells were found in the airways at day 6 postinfection. In contrast, the secondary infection was characterized by two peaks of Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific T cells at days 4 and 7 (Fig. 7B). The peak at day 4 directly corresponded to the recruitment of Sen-NP<sub>324–332</sub>/K<sup>b</sup>-specific T cells by the heterologous infection (Fig. 7C). Taken together, these data suggest that the early recruitment of circulating, but nonproliferating CD8<sup>+</sup> memory T cells into the lung airways is a common feature of the immune response to respiratory virus infections in the lung.

## Discussion

The current studies investigate the impact of heterologous respiratory virus infections on pre-established effector/memory T cell populations in the lung airways. The data show that intranasal influenza virus infection of Sendai virus-immune mice resulted in a significant increase in the number of Sendai virus-specific memory cells in the lung airways. This increase was not due to the



**FIGURE 7.** Recruitment of memory cells is driven by homologous virus infection. Naive C57BL/6 mice were intranasally infected with 125 EID<sub>50</sub> of Sendai virus (A), or Sendai memory mice were challenged with either 30,000 EID<sub>50</sub> of Sendai virus (B) or 300 EID<sub>50</sub> of influenza x31 (C) intranasally. BAL cells were harvested at indicated times post-primary, -secondary, or -heterologous infection, and Sen-NP<sub>324-32</sub>/K<sup>b</sup>-specific T cells were enumerated. Data are representative of one of three experiments.

proliferation of effector/memory CD8<sup>+</sup> T cells already resident in the lung, but was due to the nonspecific recruitment of Sendai virus-specific memory CD8<sup>+</sup> T cells from other sites. The peak of this recruitment occurred at day 4 and did not depend on proliferation of the recruited cells. However, the absolute numbers of memory T cells in the lung airways declined after day 4 to levels lower than were present before infection, indicating that both resident effector/memory and recruited memory T cells in the lung airways were subsequently deleted. We believe the cells are lost by apoptosis or phagocytosis, because previous studies have shown that lung airway memory T cells are unable to migrate from the lung airways back into the circulation (7, 37). These observations extend previous studies in the field by distinguishing the relative contributions of resident memory cells, recruitment, and proliferation to the expansion of memory cells in the lung airways induced by heterologous virus infections (14, 19).

The mechanism underlying the early recruitment of memory cells during respiratory virus infections remains unclear. One possibility is that it is driven by cross-reactivity between a subpopulation of pre-existing memory T cells and the heterologous pathogen. Indeed, several studies have shown that viral epitopes can be tremendously cross-reactive at the T cell level (12, 14, 15, 38, 39). However, this is unlikely to be the mechanism because we also saw recruitment of memory T cells following infection with sev-

eral different viruses (influenza, vaccinia virus, or MHV-68) or by administration of nonspecific inflammatory agents, such as heat-labile enterotoxin, into the lung airways. Furthermore, studies of Stephens et al. (28) have shown that intranasal Sendai virus infection induces Ag-nonspecific recruitment to lung airways of i.v.-transferred OVA-specific Th2 cells in a murine asthma model. Thus, nonspecific recruitment of pre-established memory T cells to the lung airways appears to be a general feature of inflammation of the lung. However, the underlying mechanism of recruitment remains to be elucidated, although emerging data indicate that monocyte chemoattractant protein 1, RANTES, monocyte IFN- $\gamma$ -inducible protein, and cytokine-responsive gene 2 may play key roles (28, 40, 41). We are currently investigating the impact of inflammatory chemokine production on the nonspecific recruitment of memory CD8<sup>+</sup> T cells during heterologous infection.

The source of the Ag-specific T cells that are recruited to the lung by heterologous infection is currently unknown. However, the fact that there were minimal changes in the absolute numbers of Ag-specific cells in the spleen and local draining lymph nodes during the course of the heterologous infection suggests that the recruited cells were already in the circulation. One possibility is that there is a pool of circulating effector/memory T cells that is generated by homeostatic turnover in the secondary lymphoid organs. These circulating cells may normally home to various peripheral sites at a relatively slow basal rate. However, in the context of an acute pulmonary infection, these circulating cells may be actively recruited to the lungs, resulting in the dramatic and transient increase in cell numbers observed in these studies. Consistent with this general idea, we have recently demonstrated a low rate of recruitment of effector/memory cells to noninfected lungs (9).

The rapid deployment of memory T cells to the site of an infection may represent a significant advantage to the host by potentially exposing an invading pathogen to memory T cells at a very early stage of the infection. Because the immune system does not a priori know whether it has previously responded to a given infection, the rapid recruitment of memory cells of all specificities provides a way to quickly screen cells for reactivity to the pathogen. If the host is encountering the pathogen for the first time, these rapidly recruited T cells will not be beneficial unless there is a fortuitous cross-reactivity, as has been reported for some viruses by Selin and coworkers (14, 18, 39). In contrast, if the pathogen is mediating a secondary infection, some of the T cells recruited will be Ag specific and mediate a very early response to the pathogen. Indeed, previous studies have suggested that relatively few Ag-specific T cells present in the lung at early stages of a Sendai virus infection are able to significantly reduce the viral load present at later stages of the infection (42). Thus, the recall response to secondary virus infection might be considered to comprise three distinct phases. First, effector/memory T cells that are resident in the lung airways provide a first line of defense, rapidly producing antiviral cytokines at the site of infection when viral loads are very low. Second, the day-4 recruitment of circulating, effector/memory T cells to the lung airways will boost this early response. Because these cells are not proliferating, the response is not sustained and declines after the majority of available cells have been recruited. Finally, memory cells that have been generated by Ag-driven proliferation in the draining lymph nodes provide a sustainable source of effector cells that start to flood into the lung airways after day 5. During normal secondary cellular responses, these phases may merge to generate a comprehensive response to the challenge virus. However, these phases could be readily distinguished following a high-dose Sendai virus secondary infection of mice that had previously generated an antiviral Ab response (Fig. 7). This may



be due to substantial, but not complete, control of the virus by neutralizing Ab, resulting in the reduced recruitment of T cells to the lung after day 5 postinfection. In the context of a heterologous virus infection, only the first two phases of the memory response are apparent (neither of which recognize the pathogen) (Fig. 7C). T cells specific for the heterologous pathogen appear in the lung with primary kinetics after the expansion of naive T cells in the lymph nodes.

An interesting observation in this study is that a heterologous virus infection ultimately results in a reduction in the number of previously established memory T cells resident in the lung airways. Thus, it appears that each new infection partially replaces the previous complement of memory T cells present in the airways with new cells specific for the latest pathogen. Attrition of non-specific bystander memory T cells also has been described in the spleen following lymphocytic choriomeningitis virus infection (16). It is likely that the inflammatory response generated by respiratory virus infections induces an environment that results in rapid elimination of activated T cells from the lung (either through the induction of apoptosis or scavenging by activated macrophages). After Ag clearance, the production of new effector cells decreases and consequently the number of cells in the lung rapidly declines. We have speculated that, at some point in this process, the inflammatory status that promotes T cell removal in the lung resolves, and newly arriving effectors may be able to stabilize as lung-resident T cell memory (9).

In summary, the data presented in this study clearly demonstrate that there is a rapid, nonspecific recruitment of memory T cells to the lung airways following respiratory viral infections. We propose that this early recruitment is part of the normal recall response to respiratory virus infections and represents a mechanism to control pathogen loads until a sustained T cell response can be generated in the draining lymph nodes. A clearer understanding of the temporal development of secondary T cell responses in the lung will be of crucial importance for the development of vaccines designed to promote cellular immunity at mucosal surfaces.

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