

Renewal of Peripheral CD8⁺ Memory T Cells During Secondary Viral Infection of Antibody-Sufficient Mice¹

Linda S. Cauley, Tres Cookenham, Robert J. Hogan,² Sherry R. Crowe, and David L. Woodland³

Kinetic studies and short pulses of injected 5-bromo-2-deoxyuridine have been used to analyze the development and renewal of peripheral CD8⁺ memory T cells in the lungs during primary and secondary respiratory virus infections. We show that developing peripheral CD8⁺ memory T cells proliferate during acute viral infection with kinetics that are indistinguishable from those of lymphoid CD8⁺ memory T cells. Secondary exposure to the same virus induces a new round of T cell proliferation and extensive renewal of the peripheral and lymphoid CD8⁺ memory T cell pools in both B cell-deficient mice and mice with immune Abs. In mice with virus-specific Abs, CD8⁺ T cell proliferation takes place with minimal inflammation or effector cell recruitment to the lungs. The delayed arrival of CD8⁺ memory T cells to the lungs of these animals suggests that developing memory cells do not require the same inflammatory signals as effector cells to reach the lung airways. These studies provide important new insight into mechanisms that control the maintenance and renewal of peripheral memory T cell populations during natural infections. *The Journal of Immunology*, 2003, 170: 5597–5606.

CD8⁺ T cells play a central role in the control of respiratory virus infections, such as influenza and parainfluenza viruses (1–3). These cells possess a number of effector functions, such as cytolytic activity and secretion of antiviral cytokines, that mediate clearance of the virus (4). Following recovery from infection, virus-specific CD8⁺ memory T cells persist in the lymphoid organs in large numbers and are able to mediate accelerated responses to secondary infections (1, 5).

Recently, it has emerged that substantial numbers of CD8⁺ memory T cells also reside in nonlymphoid tissues after viral infections (6–9). It has been estimated that these peripheral memory cells account for as many as one-half of the total CD8⁺ memory T cell pool, and their location in peripheral tissues may be an important asset in combating mucosal infections (6, 8–10). Interestingly, peripheral CD8⁺ memory cells have phenotypic and functional characteristics that are distinct from those of lymphoid memory cells (sometimes referred to as central memory cells), including the expression of acute activation markers and constitutive cytolytic activity (6–8, 11). Based on their partially activated state and anatomical distribution, peripheral memory cells have been referred to as effector/memory T cells (12). The functional and phenotypic characteristics of virus-specific CD4⁺ memory cells are less well defined (10, 13). However, a similar dichotomy of lymphoid and nonlymphoid CD4⁺ memory cells has been suggested in other models (14–17).

How memory T cells develop during viral infections and the relationship between effector/memory cells and lymphoid memory cells are poorly understood. The developmental pathway of memory T cells has been the subject of intense investigation. The observation that in vitro generated effector cells acquire the phenotypic characteristics of lymphoid memory cells after transfer to naive animals supports a linear developmental pathway of effector cells to memory T cells (18–20). This appears to be a default pathway, because cognate interactions with processed Ags are not required (21, 22). Effector cells can also attain memory status during viral infections (23). However, it has been suggested that intermediates, which did not attain full effector function after primary stimulation, can also become long-lived memory cells (14). In support of this suggestion, it was recently shown that Th1 cells that do not produce IFN- γ have greater potential to become long-lived memory cells than IFN- γ -secreting Th1 cells (24). How transitional populations of memory cells would be maintained or renewed during recurrent viral infections is not known. Other evidence also suggests that acquisition of full effector function is not necessarily a prerequisite for memory development (25, 26). For example, heat-killed *Listeria monocytogenes* has been used to prime lymphoid CD8⁺ memory T cells without generating large effector cell populations in vivo (25). In addition, environmental cytokines can promote the transition of newly activated T cells to a lymphoid memory phenotype in vitro, without the acquisition of full effector function (26, 27). To what extent each of these developmental pathways is used during natural infections is not known.

In this study, we have used a murine respiratory virus model to analyze the proliferative response of CD8⁺ memory T cells during their development. Using kinetic studies and short pulses of injected 5-bromo-2-deoxyuridine (BrdU),⁴ we show that long-lived CD8⁺ memory T cells are generated throughout the effector response to primary viral infection, at a rate roughly proportional to the size of the total responding CD8⁺ T cell population. Identical

Trudeau Institute, Saranac Lake, NY 12983

Received for publication January 13, 2003. Accepted for publication March 28, 2003.

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 National Institutes of Health Grants R01 HL-69502 (to D.L.W.) and P01 HL-63925 (to D.L.W.), and the Trudeau Institute.

² Current address: Department of Homeland Security and Infectious Diseases, Southern Research Institute, Birmingham, AL 35205.

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@trudeauintstitute.org

⁴ Abbreviations used in this paper: BrdU, 5-bromo-2-deoxyuridine; BAL, bronchoalveolar lavage; EID₅₀, 50% egg infectious dose; MLN, mediastinal lymph node; NP, nucleoprotein.

patterns of incorporated BrdU in each tissue indicate simultaneous proliferation by peripheral and lymphoid memory T cells during their development. The same approach has also been used to analyze CD8⁺ T cell proliferation during secondary infection with the same virus. We show extensive renewal of virus-specific CD8⁺ memory T cells in both peripheral and lymphoid tissues. In mice that have virus-specific Abs, this replacement occurs in the absence of extensive inflammation or effector cell populations in the lungs. Our data suggest that a majority of resident CD8⁺ memory T cells in the lungs are replaced during secondary infection, by circulating memory T cells that continue to enter the lungs after the resolution of inflammation. These data provide important new insights into the mechanisms that control memory T cell development and renewal in the context of a natural infection.

Materials and Methods

Mice and viral stocks

Female C57BL/6 and B cell-deficient (B6.129S2-Igh-6tmlCgn) mice were purchased from Taconic (Germantown, NY) or The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions. At 8–12 wk of age, mice were anesthetized by i.p. injection with avertin (2, 2, 2-tribromoethanol) before intranasal infection with 125 or 250 50% egg infectious doses (EID₅₀) of Enders stain of Sendai virus for primary infections and 1,100–10,000 EID₅₀ for secondary infections, as specified. Sendai virus stocks were grown in chicken eggs, titered, and stored, as described previously (2).

Sample preparation for flow cytometry

Cells were collected from the lung airways of groups of three or four animals by bronchoalveolar lavage (BAL) five times in HBSS. Single cell suspensions were prepared from lung tissues, spleens, and mediastinal lymph nodes (MLN) by passage through cell strainers. The spleen cells were depleted of erythrocytes by treatment with buffered ammonium chloride solution, and adherent cells were removed from BAL and spleen preparations by plastic adherence for 2 h at 37°C. BAL cells were spun through 40% Percoll to remove low density surfactant particles. Cells from dissociated lung tissues were resuspended in 80% isotonic Percoll and overlaid with 40% isotonic Percoll for centrifugation at 400 × *g* for 25 min. Cells were recovered from the 40/80 Percoll interface and washed with HBSS before further purification by centrifugation over lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada).

Tetramer analysis and flow cytometry

MHC class I tetramers and peptides specific for the Sendai virus nucleoprotein (NP)_{324–332}/K^b epitope have been described previously (1, 5). T cells were stained with APC-conjugated NP_{324–332}/K^b tetramers for 1 h at room temperature and then stained with PE-conjugated anti-CD8 Abs or PerCP-conjugated anti-CD4 Abs (BD Pharmingen, San Diego CA) for 30 min at 4°C. Fixed samples were analyzed on a BD Biosciences FACS-Calibur flow cytometer and analyzed using CellQuest software (BD Biosciences, San Diego, CA).

BrdU analysis

BrdU (Sigma-Aldrich, St. Louis, MO) was administered in a single 200 μl i.p. injection (0.8 mg) in PBS, or added to the drinking water (0.8 mg/ml) and replaced daily. Organs were harvested at the times indicated, and cell suspensions were processed, as above. After surface staining for T cell markers, BrdU content was analyzed, as described previously (28). Briefly, the cells were washed and treated with 1 ml FACS lysing solution (BD Biosciences) for 15 min at room temperature, then fixed overnight with 1% paraformaldehyde/PBS containing 0.05% Nonidet P-40. The cells were washed again, and cellular DNA was denatured with 50 Kunitz U of bovine pancreas DNase-1 (Sigma-Aldrich) for 30 min at 37°C. After DNase treatment, the cells were washed in PBS supplemented with 5% FCS and 0.5% Nonidet P-40 and stained for 45 min at 4°C using FITC-conjugated anti-BrdU Abs (BD Biosciences).

In vivo cytotoxicity assay

Cytolytic activity was analyzed in vivo, using a modified version of the method of Ritchie et al. (29). Briefly, spleen cells from uninfected C57BL/6 mice were pulsed with peptides (10 μM) specific for the Sendai virus epitope (NP_{324–332}) or influenza virus epitope (NP_{366–374}) for 2 h at

37°C. The peptide-pulsed cells were divided into two equal aliquots and labeled with CFSE or red bodipy dyes (Molecular Probes, Eugene, OR) for 10 min at 37°C. After extensive washing, the red- and green-labeled spleen cells with different peptides were mixed at a ratio 1:1. Mice were given 2.5 × 10⁶ cells by i.v. injection. Twenty-four hours later, the spleens of the recipient mice were analyzed for red- or green-labeled donor cells, as well as NP_{324–332}/K^b-specific CD8⁺ T cells by four-color FACS analysis.

Intracellular cytokine staining

Nonadherent cells were cultured for 5 h at 37°C in the presence of CFSE-labeled syngeneic spleen cells, with peptides at 1 μg/ml and brefeldin A at 20 μg/ml. T cells were stained with anti-CD8 and anti-IFN-γ Abs in the presence of brefeldin A (10 μg/ml), as previously described (6). The influenza virus-specific peptide (hemagglutinin_{192–207}) was used as a specificity control. The CFSE-labeled feeder cells were excluded during analysis.

Results

CD8⁺ T cell proliferation during memory development

Ag-specific CD8⁺ T cell populations undergo massive expansion during the immune response to respiratory virus infections. A large percentage of the responding CD8⁺ cells are subsequently deleted during the contraction of the effector response, leaving residual populations of virus-specific memory cells in both lymphoid and nonlymphoid tissues, including the lungs (6–8). A key transitional step in this process is the decline in rate of T cell proliferation and stabilization as long-lived memory cells. To better understand how memory T cells are generated during this transition, we have analyzed the proliferation kinetics of developing CD8⁺ memory cells during acute viral infection. To do this, groups of C57BL/6 mice were given short pulses of BrdU by i.p. injection on different days after intranasal infection with Sendai virus, as illustrated in Fig. 1A. This protocol labels cells that are actively synthesizing DNA, and preliminary studies with tumor cells placed in the peritoneum indicate that a bulk of the labeling occurs within 24 h of the BrdU injection, with very little labeling thereafter (data not shown). The mice were then left until day 30 after infection, when the effector response had contracted, and CD8⁺ T cells specific for the immunodominant epitope (NP_{324–332}/K^b) (30) were collected from the parenchyma and airways of the lungs and analyzed for BrdU content, as described (28). Because all the animals were analyzed on the same day of the response, there were similar numbers of virus-specific CD8⁺ memory cells in the tissues of each animal. However, the frequency of BrdU⁺ T cells, within the NP_{324–332}/K^b-specific cell population, varied according to the day of BrdU injection (Fig. 1B). The highest percentages of BrdU⁺ cells were recovered from the airways and lung parenchyma of animals injected 8–10 days after primary infection (Fig. 1B). The numbers of BrdU⁺ CD8⁺ cells were substantially reduced in mice that were given BrdU later than 10 days after infection, which correlated with the approximate time of Ag clearance in previous studies (31). The presence of incorporated BrdU on day 30 after infection demonstrates that a large proportion of the surviving memory population stopped dividing before their BrdU was displaced. The kinetics of response also indicates that there was minimal proliferation after viral clearance, and demonstrates that developing memory T cells can retain their BrdU during the contraction of the effector response.

We next compared the patterns of incorporated BrdU in CD8⁺ memory T cells from the lungs with memory cells from the secondary lymphoid organs, using the same protocol and day 30 analysis as before. The total numbers of NP_{324–332}/K^b-specific CD8⁺ T cells in each tissue are shown in Table I, and the frequencies of BrdU⁺ cells within each NP_{324–332}/K^b-specific CD8⁺ T cell population are shown in Fig. 2, A–D. Identical patterns of incorporated BrdU in each of the four tissues (BAL, lung parenchyma, MLN,

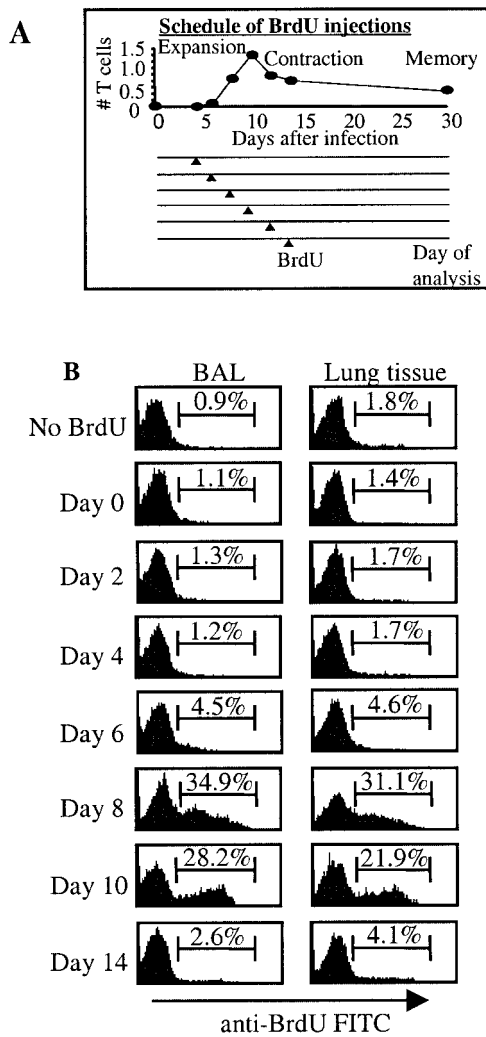


FIGURE 1. BrdU incorporated during the acute response to Sendai virus infection is retained in peripheral memory cells. Groups of C57BL/6 mice were intranasally infected with 125 EID₅₀ of Sendai virus and then administered 800 μg BrdU/PBS by i.p. injection on different days postinfection (as illustrated in the schedule of injections (A)). The schematic shows the numbers of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells in the MLN during a representative primary infection. Thirty days after infection, each group of animals was sacrificed, and the NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells in the airways (BAL) and lung parenchyma were analyzed for BrdU content (B). CD8⁺ T cells were stained with APC-conjugated class I tetramers, PE-conjugated anti-CD8, and FITC-conjugated anti-BrdU Abs. The numbers indicate the percentages of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells that were BrdU⁺ at the time of analysis. One of four independent experiments is shown.

and spleens) indicate that developing CD8⁺ memory cells from peripheral and lymphoid tissues proliferated at the same stage of the response.

Other experiments, in which BrdU was administered by continuous feeding in the drinking water of mice during the first 8 days of the primary response, resulted in 90% BrdU⁺ NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells in the lungs (32), indicating that most peripheral memory cells were generated during this time period. In resting animals, the incorporated BrdU remained relatively stable in Ag-specific CD8⁺ T cells in the lung airways for at least 2 mo after infection (32). This further suggests that virus-specific effector/memory cells are maintained in peripheral tissues in a relatively inactive state after viral clearance. Because some investiga-

tors have found that BrdU can be toxic to T cells in certain circumstances, we also analyzed the numbers of virus-specific CD8⁺ memory cells in peripheral and lymphoid tissues. ELISPOT analysis on day 30 after infection showed no difference in the numbers of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells that produced IFN-γ, in animals that were injected with BrdU 9 days after virus infection, as compared with PBS-treated controls (data not shown).

CD8⁺ T cell proliferation during acute viral infection

In the experiments described above, we used a day 30 analysis to specifically analyze proliferation in T cells that survived the contraction of the effector response. Other studies have shown that in vitro generated effector cells adopt a memory phenotype after transfer (18, 19, 22), indicating that they may be an important source of memory cells. However, effector cells are highly susceptible to cell death and killed in large numbers at the site of inflammation. Because of this susceptibility to cell death, it has been postulated that effector cells that are generated late in the immune response are most likely to become memory T cells (33). If this were the case, we would expect to find different patterns of incorporated BrdU during acute viral infection and in long-lived memory cells. To investigate this possibility, we compared the BrdU content of virus-specific CD8⁺ memory T cells on day 30 after infection (Fig. 2, A–D), with the kinetics of T cell proliferation during acute viral infection (Fig. 2, E–H). In this study, C57BL/6 mice were treated with BrdU on different days after Sendai virus infection, as previously. Each group of mice was sacrificed 24 h after the BrdU injections, and the BAL, lung parenchyma, MLN, and spleens were analyzed for total numbers of BrdU⁺ NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells.

After a primary Sendai virus infection, the first virus-specific CD8⁺ T cells can be detected in the MLN on day 6 after viral infection (data not shown). In this study, mice that were injected with BrdU on day 6 after infection had detectable populations of BrdU⁺ virus-specific CD8⁺ T cells in each of the tissues (BAL, lung, MLN, and spleen) by day 7. The numbers of these cells increased until day 9 after infection (Fig. 2, E–H), which was consistent with the kinetics of the T cell response in other studies. There were large numbers of BrdU⁺ CD8⁺ T cells in the lung airways, indicating the presence of a large effector cell population at the site of infection (Fig. 2E). Mice that were given BrdU on day 5 after viral infection, but were not analyzed until after the contraction of the effector response (day 30), also revealed a small percentage of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells that contained BrdU. A direct comparison between the kinetics of BrdU incorporation during acute viral infection (Fig. 2, E–H) and the percentage of CD8⁺ memory cells that retained BrdU on day 30 (Fig. 2, A–D) revealed a close correlation at each time point of the analysis. The only exception was the day 10 time point, in which there was a small shift in the kinetics of BrdU incorporation between the two analyses (~24 h). However, the shift was of insufficient magnitude to indicate that effector cells that were generated

Table I. Numbers of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells on day 30 after infection

Infection	No. of Mice	No. of cells × 10 ⁻⁴ (SD)			
		BAL	Lung	MLN	Spleen
Primary ^a	15	5 (2.3)	2.3 (1.3)	4.8 (0.6)	170 (56)
Secondary ^b	18	2.2 (0.8)	2.7 (1.9)	3.9 (1.3)	300 (1.30)

^a C57BL/6 mice infected with 250 EID₅₀ Sendai virus.

^b C57BL/6 mice were reinfected with 10,000 EID₅₀ Sendai virus on day 60 after primary infection.

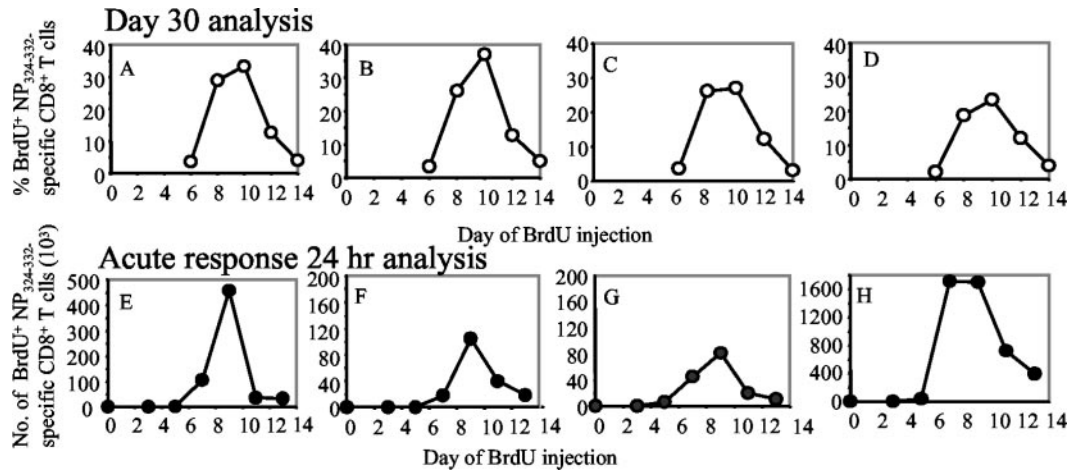


FIGURE 2. Similar patterns of BrdU incorporation in developing peripheral and lymphoid CD8⁺ memory T cells. Groups of C57BL/6 mice were given BrdU by i.p. injection on different days after Sendai virus infection. Thirty days after infection, each group of mice was sacrificed, and the BAL (A), lung parenchyma (B), MLNs (C), and spleens (D) were analyzed for numbers of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells that contained BrdU. Three separate time courses, with three mice per group, gave virtually identical results. To analyze T cell proliferation during acute viral infection, C57BL/6 mice were given BrdU on different days after Sendai virus infection, as indicated. Twenty-four hours after BrdU injection, each group of mice was sacrificed and analyzed for NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells that contained BrdU in BAL (E), lung parenchyma (F), MLN (G), and spleen (H). Two separate time courses, with four mice per time point, gave very similar results.

late in the response had a significant selective advantage in becoming long-lived memory cells, and may have been due to some ongoing cell proliferation in the memory cell population. It was clear that a percentage of T cells that proliferated early in the response (day 6) stopped dividing before their BrdU was displaced and survived to become long-lived memory cells, while other cells that proliferated several days later (day 10) were deleted in large numbers. Overall, these data indicate that T cells that proliferated at each stage of the response had a similar chance of becoming long-lived memory cells (~20% in the tissues analyzed). Similar results were recently reported using CFSE analysis after lymphocytic choriomeningitis virus infection (23).

Renewal of peripheral CD8⁺ memory T cells during secondary infection

Analysis of the primary response indicated that the proportion of proliferating CD8⁺ T cells that survived to become long-lived memory cells was roughly proportional to the size of the total responding cell population during acute viral infection (Fig. 2). We next investigated how surviving memory cells were reused during secondary infection, and whether there was a similar correlation between the kinetics of T cell proliferation during acute viral infection and the pattern of incorporated BrdU in long-lived memory cells. In the first series of experiments, B cell-deficient mice (μ MT) were reinfected with Sendai virus (300 EID₅₀) on day 60 after primary infection. Groups of six mice were then given BrdU on different days after infection, as in Fig. 1. Three mice from each group were sacrificed 24 h after their BrdU injection (Fig. 3, A–D). All the others were analyzed in groups on day 30 after infection (Fig. 3, E–H).

In the mice that were analyzed 24 h after BrdU injection, the highest numbers of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells that contained BrdU were found in mice given BrdU on day 6 after secondary infection, which was consistent with the faster kinetics of secondary CD8⁺ T cell responses in other studies (1). Similar patterns of incorporated BrdU were also found in the day 30 analysis, indicating a close correlation between the size of the total responding cell population during acute viral infection (Fig. 3, A–D) and the percentage of memory cells that retained BrdU on

day 30. A maximum percentage of 40–50% BrdU⁺ T cells in each tissue indicated extensive renewal of the peripheral and lymphoid memory T cell pools. Identical patterns of incorporated BrdU in each tissue also indicated that peripheral and lymphoid memory cells proliferated at the same stage of the response.

Renewal of peripheral CD8⁺ memory T cells in Ab-sufficient animals

Sendai virus infections induce protective Ab responses that last for the life of the animal (34). However, protective cellular immunity is relatively short-lived and correlates closely with the numbers of virus-specific CD8⁺ memory T cells in the lungs (3, 35). Because of this decline in protective cellular immunity, boosting the numbers of memory T cells in peripheral tissues is an important priority in vaccine development. For this reason, we investigated whether secondary infection of mice with virus-specific Abs would induce proliferation and renewal of virus-specific CD8⁺ memory T cells. Thus, we challenged Sendai virus-immune mice with a large dose virus (10,000 EID₅₀), which is ~10 times the lethal dose for 50% of naive animals (LD₅₀), on day 60 after primary viral infection. Groups of six mice were then given BrdU by i.p. injection on different days after secondary infection, as before. Three mice from each group were analyzed 24 h after their BrdU injections (Fig. 4, A–D). All the other animals were analyzed on day 30 after infection (Fig. 4, E–H).

In the mice that were analyzed 24 h after their BrdU injections, there were large numbers of BrdU⁺ NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells in the lymphoid organs (Fig. 4, C and D). The kinetics and magnitude of the response were similar to the response in B cell-deficient mice. This demonstrated extensive virus-specific CD8⁺ T cell proliferation after secondary viral infection in presence of immune Abs. There were similar numbers of BrdU⁺ NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells in the lymph organs at the peak of the response to secondary viral infection, to the numbers in the lymph organs at the peak of the response to primary infection (Fig. 2, G and H). However, there were only very small numbers of BrdU⁺ CD8⁺ T cells in the lungs at each time point of the analysis (Fig. 4, A and B). As a result, there were 16 times fewer BrdU⁺ NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells in the lung airways than in the MLN at the

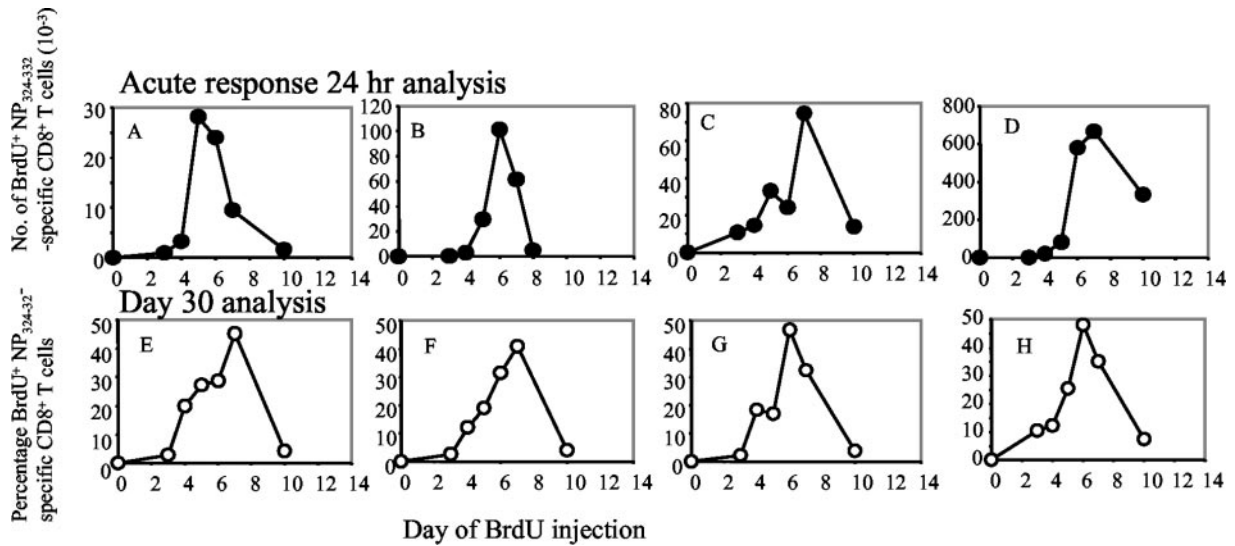


FIGURE 3. Renewal of peripheral CD8⁺ memory T cells during secondary infection. To analyze CD8⁺ T cell proliferation during secondary infection, B cell-deficient mice were reinfected with 300 EID₅₀ Sendai virus on day 60 after primary infection. BrdU was administered to groups of six mice on different days after secondary infection, as indicated. Twenty-four hours after BrdU injection, three mice from each group were analyzed for the total numbers of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells in BAL (A), lung parenchyma (B), MLN (C), and spleen (D). The other three mice in each group were left until day 30 after infection and analyzed for the percentages of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells that contained BrdU in the BAL (E), lung parenchyma (F), MLN (G), and spleen (H). The disorganized structure and high percentage of autofluorescent cells in the spleens of B cell-deficient mice reduced the quality of staining at some time points in D and H.

peak of the secondary response. This compared with a 5-fold larger number of BrdU⁺ NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells in the airways, as compared with the MLN, at the peak of the response to primary infection (Fig. 2, E and G). This indicated that relatively small numbers of effector cells entered the lungs after secondary infection, as compared with the primary response, and suggested that there may be very different inflammatory responses in the lungs after primary and secondary infections of Ab-sufficient mice.

Large populations of BrdU⁺ CD8⁺ T cells were also detected in the mice that were analyzed on day 30 after secondary infection (Fig. 4, E-H). By this time, there were identical patterns of BrdU incorporation in each tissue, including the lung airways. The kinetics of the response, and maximum values of BrdU incorporation on day 30 (50%), were remarkably similar to the values found in B cell-deficient mice (Fig. 3). Because there were very similar numbers of virus-specific CD8⁺ T cells in each tissue on day 30 after primary and secondary viral infections (Table I), the high

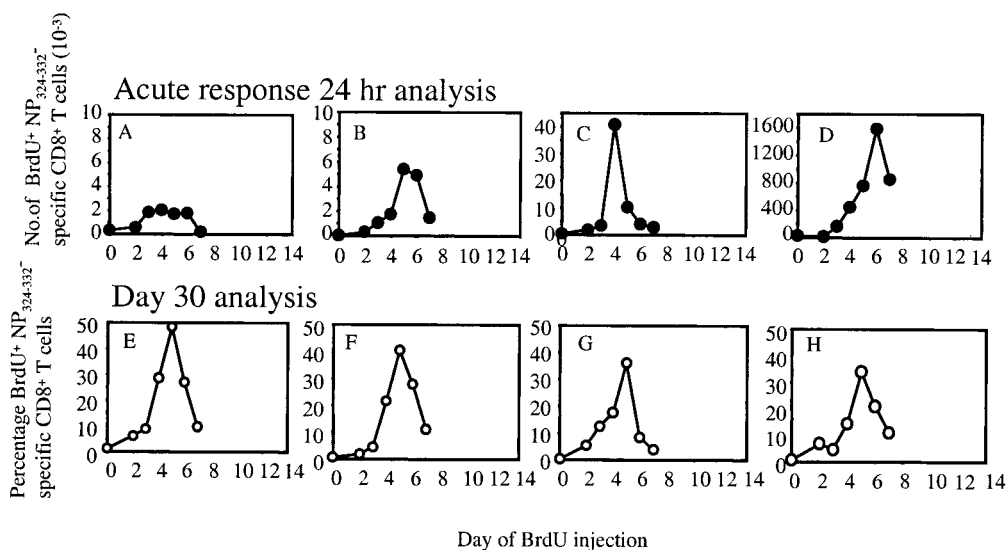


FIGURE 4. Renewal of peripheral CD8⁺ T memory cells in Ab-sufficient mice. To analyze T cell proliferation in the presence of neutralizing Abs, groups of six C57BL/6 mice were given a large dose of Sendai virus (10,000 EID₅₀) on day 60 after primary infection. BrdU was administered on different days after secondary infection, as in Fig. 3. Twenty-four hours after BrdU injection, three mice from each group were analyzed for the total numbers of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells in BAL (A), lung parenchyma (B), MLNs (C), and spleen (D). The remaining three animals in each group were left until day 30 after infection and analyzed for the percentages of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells that contained BrdU in the BAL (E), lung parenchyma (F), MLN (G), and spleens (H). Two similar time courses gave virtually identical results.

frequencies of BrdU⁺ NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells in each tissue (50%) indicated extensive renewal of both peripheral and lymphoid CD8⁺ memory T cell pools after secondary infection. This renewal took place in the presence of immune Abs.

Because we used a lethal dose of virus for secondary challenge, a 100% survival rate indicated that all the animals were successfully immunized during primary viral infection. This high dose of virus induced extensive renewal of both peripheral and lymphoid memory T cell populations. However, it was unclear whether renewal of virus-specific CD8⁺ memory T cells would also occur in the presence of immune Abs using a lower dose of virus. Relatively low levels of T cell proliferation may be sufficient to substantially increase the numbers of virus-specific memory cells in circulation. We used a slightly different protocol to investigate this issue. For this experiment, virus-specific CD8⁺ memory T cells were labeled by adding BrdU to the drinking water (0.8 mg/ml) during days 4–17 after primary infection, when most memory cells are generated (32). On day 17 after primary infection, the mice were returned to normal drinking water for the remainder of the experiment. In the absence of reinfection, 85–95% of the NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells remained BrdU⁺ until day 68 after primary viral infection (Fig. 5). On day 60, groups of five BrdU-treated mice were reinfected with varying doses of Sendai virus, as shown. Eight days after secondary infection, the mice were sacrificed, and the percentages of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells that retained their BrdU⁺ in the BAL, lungs, MLN, and spleens were compared with the percentages in control animals that did not get a secondary viral infection (Fig. 5). Decreased percentages of BrdU⁺ cells were found in all four tissues of each group of mice that received a secondary dose of Sendai virus. This indicated detectable levels of T cell proliferation at each dose of virus tested. The greatest decreases in percentages of BrdU⁺ cells were detected in the BAL and lungs. Because there was no detectable increase in numbers of virus-specific CD8⁺ memory

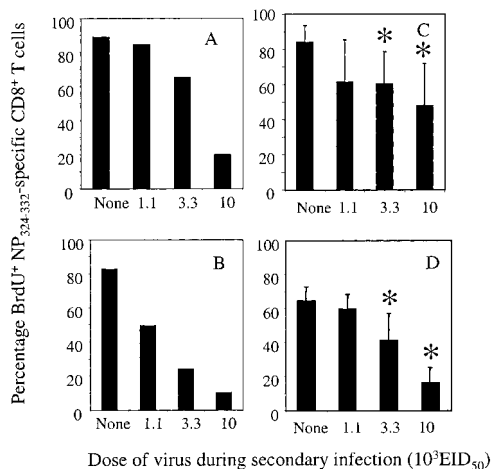


FIGURE 5. Reduced frequencies of BrdU⁺ CD8⁺ memory T cells after secondary infection of Ab-sufficient mice. To analyze memory CD8⁺ T cell proliferation in response to varying doses of virus, BrdU was added to the drinking water (0.8 μg/ml) of C57BL/6 mice between days 4 and 17 after primary infection. On day 17, the mice were returned to normal drinking water. Sixty days after primary infection, groups of five mice were reinfected with varying doses of Sendai virus (none, 1,100 EID₅₀, 3,300 EID₅₀, or 10,000 EID₅₀). On day 8 after secondary infection, all the mice were sacrificed, and the BAL, lungs, MLN, and spleens were analyzed for changes in the percentages of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells that contained BrdU. Due to low numbers, the cells from the lung airways (A) and lung parenchyma (B) were analyzed in pools from five animals. The MLN (C) and spleens (D) were analyzed from individual animals (*, *p* < 0.05 in Student's *t* test).

cells in the lungs (data not shown), there was also some deletion during the renewal process.

Delayed recruitment of CD8⁺ memory T cells to the lung airways during the secondary infection

Although large numbers of virus-specific CD8⁺ T cells incorporated BrdU in the lymph organs after secondary infection of immune competent animals (Fig. 4, C and D), relatively few BrdU⁺ cells were detected in the lung airways during acute viral infection (Fig. 4A). By day 30 after infection, there were similar percentages of BrdU⁺ cells in each tissue (Fig. 4, E–H), indicating that peripheral memory cells ultimately reached the lung airways. This indicated that 24 h may not be sufficient time for developing peripheral CD8⁺ memory T cells to reach the lung airways of immune competent animals, after BrdU incorporation. Thus, we further investigated the kinetics of T cell recruitment to the lung airways by following the rate of BrdU incorporation in each tissue at short time intervals after injection. The data shown in Figs. 2 and 4 were used to identify the maximum periods of BrdU incorporation during primary and secondary infections of C57BL/6 mice. BrdU was then injected during the peak proliferative phase of the response (i.e., day 7 after primary infection and day 4 after secondary infection). At varying times after BrdU injection, groups of three mice were sacrificed and analyzed for virus-specific CD8⁺ T cells in the BAL, lungs, MLN, and spleens, during the subsequent 72 h (Fig. 6).

When BrdU was injected on day 7 after primary viral infection, a majority of the virus-specific CD8⁺ T cells had incorporated BrdU within 6 h of the injection, and there was very little difference between the individual tissues. Because naive T cells are believed to initiate cell division in the peripheral lymph organs before migrating to the lung airways (36), and some evidence suggests that lung environment may not support CD8⁺ T cell proliferation (32), these data indicated a very rapid transit time from the lymph organs to the lungs. In contrast, when BrdU was injected on day 4 after secondary infection, there were very different patterns

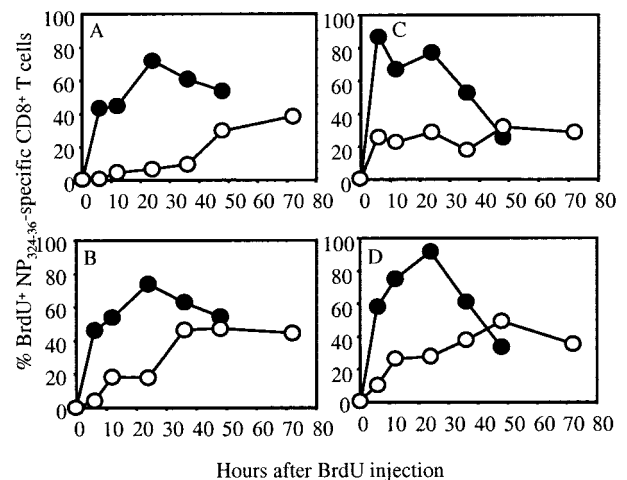


FIGURE 6. Delayed recruitment of Ag-specific CD8⁺ T cells to the lung airways during secondary infection. C57BL/6 mice were infected with primary (250 EID₅₀) or secondary (10,000 EID₅₀) doses of Sendai virus at 60-day intervals. Each animal was given a single BrdU injection on either day 7 after primary infection (●) or day 4 after secondary infection (○). Groups of three mice were sacrificed at varying times after BrdU injection, as shown, and the BAL (A), lung parenchyma (B), MLN (C), and spleens (D) were analyzed for the percentages of NP₃₂₄₋₃₃₂/K^b-specific CD8⁺ T cells that contained BrdU. Three independent experiments gave similar results.

of BrdU incorporation in the different tissues. As expected, very few virus-specific CD8⁺ T cells that contained BrdU reached the lung airways of the immune mice between 0 and 24 h after injection (Fig. 6A), and the percentage remained below 10% until 48 h after injection. This pattern of BrdU incorporation differed substantially from the patterns in the other tissues. At 6 h after BrdU injection, there were high percentages of BrdU⁺ NP_{324–332}/K^b-specific T cells in the MLN and spleens. Smaller percentages of BrdU⁺ T cells were also detected in the lung parenchyma by 12–24 h after injection. These data suggested that the lymph organs were an important site of T cell proliferation, not only in naive animals, but also during the renewal of second-generation CD8⁺ memory T cells in mice with immune Abs. Importantly, although most of the second-generation memory cells incorporated BrdU during days 5 and 6 after infection, the peripheral memory cells were not recruited to the airways of the immunocompetent animals until 2 or more days later.

Circulating CD8⁺ effector T cells accumulate in the spleen after secondary infection

Efficient priming of CD8⁺ memory T cells without effector cell generation was recently reported using heat-killed *Listeria* (25). We therefore considered the possibility that, in the presence of virus-specific Abs, CD8⁺ memory T cells had also been generated in the absence of effector cells. However, as shown in Fig. 7A, when mice were analyzed 24 h after the BrdU injections, disproportionately large numbers of nonproliferating virus-specific CD8⁺ T cells were found in the spleens on day 8 after secondary infection. It is likely that these cell populations included residual nonproliferating memory cells that were generated during primary viral infection, in addition to the nascent cell populations that were generated after secondary infection. To determine whether the virus-specific CD8⁺ T cells in the spleens included effector cells, we analyzed for cytolytic activity *in vivo* (29). Spleen cells from uninfected mice were pulsed with peptides and labeled with CFSE or red bodipy. After transfer, CFSE-labeled spleen cells that had been pulsed with Sendai virus-specific peptides (NP_{324–332}) were deleted from day 8 infected mice, while red bodipy-labeled spleen cells that had been pulsed with influenza virus-specific peptides remained (Fig. 7E). The reciprocal experiment gave identical results (Fig. 7G), and neither transferred cell population was deleted from uninfected control mice (Fig. 7, D and F). In other experiments, spleen cells that were recovered from Sendai virus-infected mice 8 days after secondary infection produced IFN- γ in response to *in vitro* stimulation with Ag (Fig. 7H). Together these data indicated that both effector cells and CD8⁺ memory T cells were generated during secondary infection of immune competent mice. The high numbers of BrdU-negative cells in the spleens on day 8 indicated that CD8⁺ cells that had proliferated earlier in the response entered the circulation in large numbers during acute viral infection. These cells were presumably disseminated throughout the entire periphery of the animals (8, 16) and remained in the circulation for at least 24 h. After proliferation, large numbers of nondividing cells were later filtered out in the spleens in which effector cells are suggested to leave the circulation (33). Together, these data indicated that an absence of circulating effector cells was not responsible for the delayed recruitment of BrdU⁺ T cells to the lung airways during secondary infection of immune competent animals.

Altered recruitment of inflammatory cells to the lung airways following primary and secondary viral infections

The difference in numbers of BrdU⁺ CD8⁺ T cells to the lung airways after primary and secondary infections of immune com-

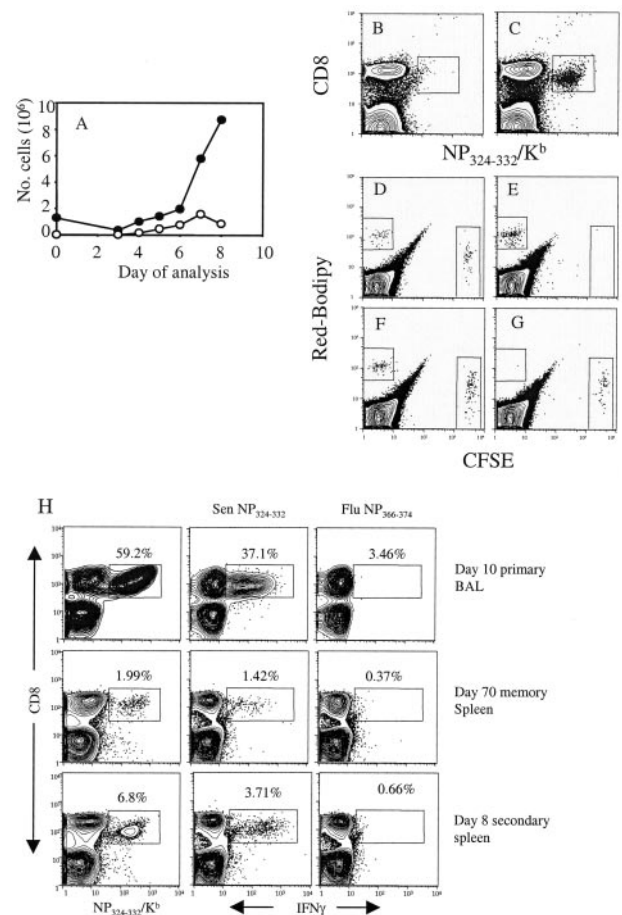


FIGURE 7. Effector cells accumulate in the spleens after secondary infection of immune competent animals. *A*, C57BL/6 mice were infected with 10,000 EID₅₀ Sendai virus on day 60 after primary infection. BrdU was given by i.p. injection on the days indicated, and the mice were sacrificed 24 h after BrdU injection. Total NP_{324–332}/K^b-specific CD8⁺ T cells (●) and BrdU⁺ NP_{324–332}/K^b-specific CD8⁺ T cells (○) are shown. To measure cytolytic activity *in vivo*, uninfected (*B*, *D*, and *F*) or day 8 secondary infected mice (*C*, *E*, and *H*) were given CFSE-labeled NP_{324–332}-pulsed spleen cells, mixed 1:1 with red bodipy-labeled influenza virus NP_{366–374}-pulsed cells (*D* and *E*). Other mice were given red bodipy-labeled spleen cells with NP_{324–332} peptides, and CFSE-labeled cells with influenza NP_{366–374} peptides (*F* and *G*). The spleen cells of the recipient mice were analyzed for NP_{324–332}/K^b-specific CD8⁺ T cells and donor spleen cells 24 h after transfer. Five individual animals gave identical results. We also investigated whether the virus-specific CD8⁺ T cells in the spleen were able to make IFN- γ *in vitro* (*H*). Spleen cells were recovered 70 days after primary infection or day 8 after secondary infection and stimulated *in vitro* with the Sendai virus-specific peptide NP_{324–332} or the influenza virus control peptide NP_{366–374}. Nonadherent cells were analyzed for NP_{324–332}/K^b-specific CD8⁺ T cells and IFN- γ expression. BAL cells, harvested 10 days after primary infection, were used as a positive control.

petent animals (Fig. 6A) suggested that there may be fundamentally different inflammatory responses in the lungs during the two infections. To further investigate this difference, BAL cells were collected at the peak of the response to each infection, and analyzed using H&E-stained cytopins for standard differential analysis (Fig. 8). BAL cells that were recovered 9 days after primary infection (Fig. 8A) were a heterogeneous mixture of macrophages (63%) with dense vacuolated cytoplasm, neutrophils (17%) with highly segmented nuclei, lymphocytes (12%), and small numbers of other unidentified cells (8%). A similar composition of BAL

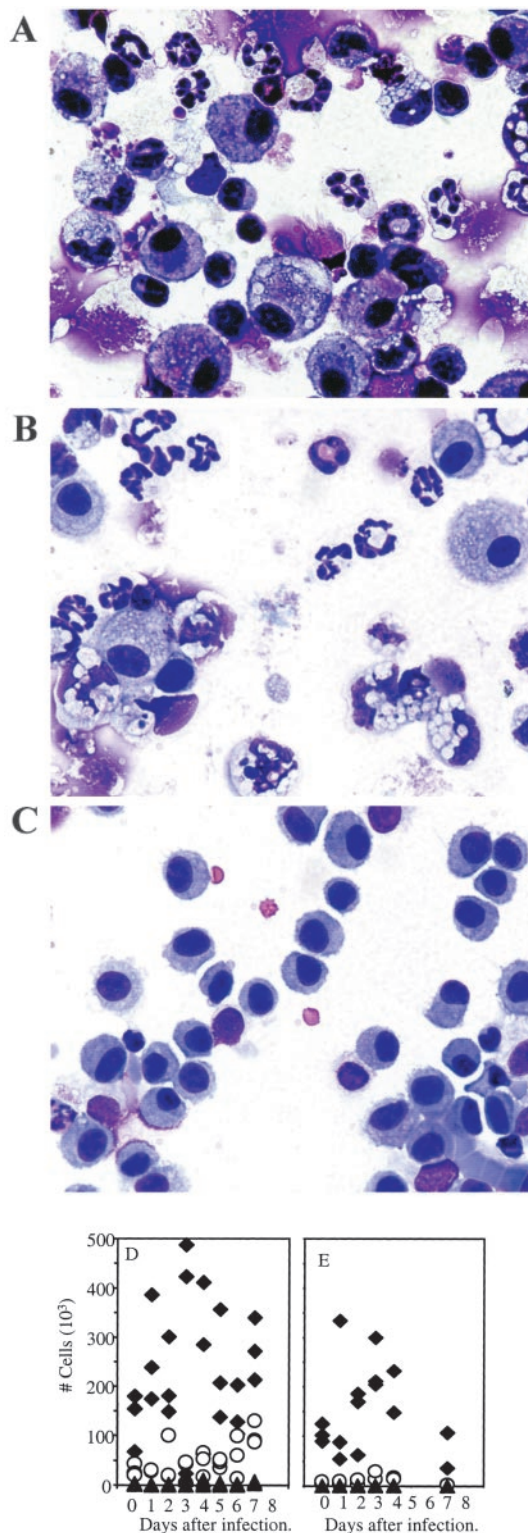


FIGURE 8. Composition of BAL cells after primary and secondary Sendai virus infections. BAL cells were attached to microscope slides by low speed centrifugation and stained with H&E dyes. Examples of BAL cells that were stained 9 days after primary Sendai virus infection (A), 4 days after secondary infection (300 EID₅₀) of B cell-deficient mice (B), and 4 days after secondary infection (10,000 EID₅₀) of Ab-sufficient C57BL/6 mice (C) are shown. Macrophages/monocytes (◆), neutrophils (▲), and lymphocytes (○) from individual mice were counted at different times after secondary infection of B cell-deficient mice (D) and Ab-sufficient C57BL/6 mice (E).

was also recovered from B cell-deficient mice 4 days after secondary infection (Fig. 8B). However, much smaller numbers of BAL cells were recovered from Ab-sufficient mice (Fig. 8C), which were primarily macrophages (90%), with a mixed population of other cells, including small numbers of lymphocytes. Very few neutrophils were detected (less than 2%). Other time points gave similar results (Fig. 8, D and E). The difference in composition of the BAL cells confirmed very different levels of inflammation in the lungs during primary and secondary infections in Ab-sufficient mice. These data indicate that different inflammatory signals control the recruitment of effector cells and peripheral CD8⁺ memory cells to the lung airways during viral infection.

Discussion

Short BrdU pulses have been used to analyze the proliferative response of Sendai virus-specific CD8⁺ T cells, during the development and renewal of lymphoid and peripheral memory populations. The data show that BrdU that is incorporated during the acute phase of the immune response is retained by long-lived resting memory cells for several weeks after infection, and is not substantially displaced by low level homeostatic turnover (37, 38). We have used the patterns of BrdU incorporation generated before the contraction of the effector response to determine the kinetics of T cell proliferation during memory development. We find an almost perfect correlation between the kinetics of T cell proliferation during acute viral infection, and the pattern of BrdU incorporation in long-lived CD8⁺ memory T cells. This tight correlation indicates that CD8⁺ memory T cells are generated at all times during the effector response, and there is no evidence that T cells that proliferate late in the response have a selective advantage in becoming memory cells. Similar results have recently been reported for lymphocytic choriomeningitis virus infection (23). Furthermore, a substantial proportion of memory cells that proliferate early in the response stop dividing before they lose their BrdU. Identical patterns of incorporated BrdU in the lungs, MLN, and spleens also indicate that CD8⁺ memory cells from lymphoid and peripheral tissues proliferate at the same time.

The initiating events of a primary immune response have been well characterized. Adaptive immunity develops when specialized APCs carry processed viral Ags to the lymph organs, where they are presented to naive T cells (33). After proliferation, differentiated effector cells migrate to the lungs in response to inflammation. Effector cells are highly susceptible to cell death, and killed in large numbers at the site of inflammation. However, the response to a secondary infection is less well defined. Memory cells have less rigorous requirements for costimulatory signals than naive T cells, and can respond to a variety of different APCs (39, 40). These differences might suggest quite different responses by naive T cells and virus-specific memory cells during primary and secondary infections.

In this study, we have compared CD8⁺ T cell responses to primary and secondary Sendai virus infections in mice capable of making immune Abs. Although primary Sendai virus infection induces strong anti-viral Ab responses in C57BL/6 mice (34), we find extensive proliferation by virus-specific CD8⁺ T cells soon after secondary infection. The kinetics and magnitude of this response are similar to the response of B cell-deficient mice; however, a majority of the proliferating CD8⁺ T cells are detected in the lymph organs with very reduced inflammatory responses and only very small numbers of BrdU⁺ CD8⁺ T cells in the lungs during acute viral infection. This did not appear to be due to a shortage of effector cells in the circulation, because virus-specific CD8⁺ T cells accumulated the spleens later in the response (Fig.

8). Cytolytic activity in vivo and IFN- γ production in vitro indicated that functional effector cells were also present in the spleen. A majority of the T cells in the spleens were BrdU negative, indicating that they remained in the circulation for at least 24 h after dividing. Together these data indicate that the signals that induced effector cell migration to the lungs during primary viral infection were not present after secondary infection of immune competent animals. However, because there were identical patterns of incorporated BrdU in each tissue on day 30 after infection, it was clear that some second-generation peripheral memory cells reached the lung airways later in the response. These data are consistent with an active process of recruitment by short-lived effector cells to the lungs during inflammation. Disseminated peripheral CD8⁺ memory cells continued to enter the lungs after inflammation had resolved, indicating a response to different signals in the lungs, or a more passive process of recruitment from the circulation.

It is possible that developing memory cells are refractory to the signals that attract effector cells to the inflammatory site, and might thus represent an intermediate level of differentiation (14, 41). Differences in the homing potential by subsets of human memory T cell have previously been suggested (42), and also in animal models using transfer studies (20). More recently, it was shown that Th1 cells that do not produce IFN- γ have a greater potential to become long-lived memory cells than IFN- γ -secreting Th1 cells (24). How these homing differences might influence T cell responses during natural infections is not known. In a situation in which developing memory cells failed to respond to the signals that draw effector cells to the inflammatory site, the kinetics of BrdU incorporation by the developing memory cells would remain independent of the effector response in the lungs, as shown in this study. The advantage of this situation would be to protect developing memory cells from deletion at the site of inflammation.

The observation that live virus can induce CD8⁺ T cell proliferation in the presence of immune Abs has important implications for the development of vaccines designed to boost cellular immunity. Protective cellular immunity to respiratory virus infections declines as the numbers of memory cells in the lung decrease (3, 35). It is therefore important to find safe methods of boosting the numbers of peripheral memory cells in the lungs. In our study, a large dose of virus was used for secondary challenge, without any mortality or morbidity. Lower doses of virus also induced detectable levels of cell division in peripheral CD8⁺ memory T cell populations. This assay may be more sensitive if lower (more physiological) doses of virus were used during primary challenge, or later time intervals were used for secondary challenge.

In summary, our data show that CD8⁺ memory T cells are generated at all times during the acute response to Sendai virus infection, and find little evidence that straggling effector cells or inflammation play a major role in molding the memory response. Because BrdU is not displaced from memory cells that proliferate in the early response, these data also indicate that many memory cells stop dividing as the effector cells are deleted. A relatively fixed portion of the responding CD8⁺ T cells is diverted toward memory development during acute viral infection, providing a potential explanation for the correlation between the clonal burst size and the numbers of memory cells detected in other studies (43). In addition, we have shown that secondary infection of mice with virus-specific Abs can induce extensive renewal of CD8⁺ memory T cell populations in both peripheral and lymphoid tissues. This replacement takes place in the absence of substantial inflammation or effector cell populations in the lungs. Because Sendai virus is not a systemic infection, our data indicate that a majority of the T cell proliferation takes place in the secondary lymphoid organs, where the earliest BrdU incorporation is detected (Fig. 6C). Rel-

atively low levels of T cell proliferation may be sufficient to substantially augment the numbers of memory T cells in peripheral tissues between viral infections. In this study, we have shown detectable levels of CD8⁺ T cell proliferation in response to three different doses of virus, using a single time point for secondary infection (day 60 after primary infection). Longer time intervals, or exposure to other viral infections, between primary and secondary challenge may result in T cell proliferation in response to smaller doses of virus.

Acknowledgments

We thank Scottie Adams and Tim Miller of the Trudeau Institute Molecular Biology Core Facility for the generation of tetrameric MHC reagents, and Jean Brennan of the Imaging and Microscopy Core Facility for help with the cytospin data. We also thank Drs. Marcy Blackman, Eulogia Roman, and Markus Mohrs for helpful comments in the preparation of this manuscript.

References

1. 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.
2. 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.
3. 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.
4. Doherty, P. C. 1996. Cytotoxic T cell effector and memory function in viral immunity. *Curr. Top. Microbiol. Immunol.* 206:1.
5. 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.
6. 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.
7. 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.
8. Masopust, D., V. Vezys, A. L. Marzo, and L. LeFrancis. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 291:2413.
9. 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.
10. 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.
11. Walzer, T., C. Arpin, L. Beloeil, and J. Marvel. 2002. Differential in vivo persistence of two subsets of memory phenotype CD8 T cells defined by CD44 and CD122 expression levels. *J. Immunol.* 168:2704.
12. Sallusto, F., and A. Lanzavecchia. 2001. Exploring pathways for memory T cell generation. *J. Clin. Invest.* 108:805.
13. Cauley, L. S., T. Cookenham, T. B. Miller, P. S. Adams, K. M. Vignali, D. A. Vignali, and D. L. Woodland. 2002. Cutting edge: virus-specific CD4⁺ memory T cells in nonlymphoid tissues express a highly activated phenotype. *J. Immunol.* 169:6655.
14. Lanzavecchia, A., and F. Sallusto. 2000. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* 290:92.
15. Geginat, J., F. Sallusto, and A. Lanzavecchia. 2001. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4⁺ T cells. *J. Exp. Med.* 194:1711.
16. 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.
17. Campbell, D. J., and E. C. Butcher. 2002. Rapid acquisition of tissue-specific homing phenotypes by CD4⁺ T cells activated in cutaneous or mucosal lymphoid tissues. *J. Exp. Med.* 195:135.
18. Hu, H., G. Huston, D. Duso, N. Lepak, E. Roman, and S. L. Swain. 2001. CD4⁺ T cell effectors can become memory cells with high efficiency and without further division. *Nat. Immun.* 2:705.
19. Opferman, J. T., B. T. Ober, and P. G. Ashton-Rickardt. 1999. Linear differentiation of cytotoxic effectors into memory T lymphocytes. *Science* 283:1745.
20. Harris, N. L., V. Watt, F. Ronchese, and G. Le Gros. 2002. Differential T cell function and fate in lymph node and nonlymphoid tissues. *J. Exp. Med.* 195:317.
21. Swain, S. L., H. Hu, and G. Huston. 1999. Class II-independent generation of CD4 memory T cells from effectors. *Science* 286:1381.

22. Murali-Krishna, K., L. L. Lau, S. Sambhara, F. Lemonnier, J. Altman, and R. Ahmed. 1999. Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* 286:1377.
23. Kaech, S. M., S. Hemby, E. Kersh, and R. Ahmed. 2002. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell* 111:837.
24. Wu, C. Y., J. R. Kirman, M. J. Rotte, D. F. Davey, S. P. Perfetto, E. G. Rhee, B. L. Freidag, B. J. Hill, D. C. Douek, and R. A. Seder. 2002. Distinct lineages of T(H)1 cells have differential capacities for memory cell generation in vivo. *Nat. Immun.* 3:852.
25. Lauvau, G., S. Viji, P. Kong, T. Horng, K. Kerksiek, N. Serbina, R. A. Tuma, and E. G. Pamer. 2001. Priming of memory but not effector CD8 T cells by a killed bacterial vaccine. *Science* 294:1735.
26. Manjunath, N., P. Shankar, J. Wan, W. Weninger, M. A. Crowley, K. Hieshima, T. A. Springer, X. Fan, H. Shen, J. Lieberman, and U. H. von Andrian. 2001. Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. *J. Clin. Invest.* 108:871.
27. 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.
28. 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.
29. Ritchie, D. S., I. F. Hermans, J. M. Lumsden, C. B. Scanga, J. M. Roberts, J. Yang, R. A. Kemp, and F. Ronchese. 2000. Dendritic cell elimination as an assay of cytotoxic T lymphocyte activity in vivo. *J. Immunol. Methods* 246:109.
30. Cole, G. A., T. L. Hogg, and D. L. Woodland. 1995. T cell recognition of the immunodominant Sendai virus NP324–332/Kb epitope is focused on the center of the peptide. *J. Immunol.* 155:2841.
31. Usherwood, E. J., T. L. Hogg, and D. L. Woodland. 1999. Enumeration of antigen-presenting cells in mice infected with Sendai virus. *J. Immunol.* 162:3350.
32. Hogan, R. J., L. S. Cauley, K. H. Ely, T. Cookenham, A. D. Roberts, J. W. Brennan, S. Monard, and D. L. Woodland. 2002. Long-term maintenance of virus-specific effector memory CD8⁺ T cells in the lung airways depends on proliferation. *J. Immunol.* 169:4976.
33. Sprent, J., and C. D. Surh. 2002. T cell memory. *Annu. Rev. Immunol.* 20:551.
34. Sangster, M., L. Hyland, R. Sealy, and C. Coleclough. 1995. Distinctive kinetics of the antibody-forming cell response to Sendai virus infection of mice in different anatomical compartments. *Virology* 207:287.
35. Woodland, D., K. Ely, S. Crowe, M. Tighe, J. Brennan, A. Harmsen, and L. Cauley. 2002. Antiviral memory T-cell responses in the lung. *Microb. Infect.* 4:1091.
36. Roman, E., E. Miller, A. Harmsen, J. Wiley, U. H. V. Adrian, G. Huston, and S. L. Swain. 2002. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. *J. Exp. Med.* 196:957.
37. Tough, D. F., and J. Sprent. 1994. Turnover of naive- and memory-phenotype T cells. *J. Exp. Med.* 179:1127.
38. Flynn, K. J., J. M. Riberdy, J. P. Christensen, J. D. Altman, and P. C. Doherty. 1999. In vivo proliferation of naive and memory influenza-specific CD8⁺ T cells. *Proc. Natl. Acad. Sci. USA* 96:8597.
39. Berard, M., and D. F. Tough. 2002. Qualitative differences between naive and memory T cells. *Immunology* 106:127.
40. Dutton, R. W., L. M. Bradley, and S. L. Swain. 1998. T cell memory. *Annu. Rev. Immunol.* 16:201.
41. Sallusto, F., A. Langenkamp, J. Geginat, and A. Lanzavecchia. 2000. Functional subsets of memory T cells identified by CCR7 expression. *Curr. Top. Microbiol. Immunol.* 251:167.
42. 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.
43. Hou, S., L. Hyland, K. W. Ryan, A. Portner, and P. C. Doherty. 1994. Virus-specific CD8⁺ T-cell memory determined by clonal burst size. *Nature* 369:652.