Differential contributions of central and effector memory T cells to recall responses

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Although the absolute number of memory CD8$^+$ T cells established in the spleen following antigen encounter remains stable for many years, the relative capacity of these cells to mediate recall responses is not known. Here we used a dual adoptive transfer approach to demonstrate a progressive increase in the quality of memory T cell pools in terms of their ability to proliferate and accumulate at effector sites in response to secondary pathogen challenge. This temporal increase in efficacy occurred in CD62L$^{lo}$ (effector memory) and CD62L$^{hi}$ (central memory) subpopulations, but was most prominent in the CD62L$^{hi}$ subpopulation. These data indicate that the contribution of effector memory and central memory T cells to the recall response changes substantially over time.

A central feature of the immune system is the capacity of memory T cells to mediate faster, stronger, and more effective responses to secondary pathogen challenge than naive T cells (1, 2). In the case of CD8$^+$ T cells, increased numbers, higher activation status, reduced stimulatory requirements, more rapid induction of effector functions, and altered homing patterns of memory T cells contribute to enhanced recall responses (2–4). In addition, it has been established that memory CD8$^+$ T cell pools are relatively long-lived, and maintained by a process of cytokine-driven homeostatic T cell proliferation (5, 6). However, the relative efficacy of these long-lived memory T cell pools at mediating recall responses has not been addressed fully (7).

Recently, it has emerged that memory CD8$^+$ T cells are heterogeneous in terms of their phenotype, function, and anatomic distribution; this led to the idea that they can be divided into two major subtypes, effector and central memory T cells (8–17). Central memory T cells (CD62L$^{hi}$/CCR7$^{hi}$) tend to localize in secondary lymphoid tissues, whereas effector memory T cells (CD62L$^{lo}$/CCR7$^{lo}$) localize in lymphoid and peripheral tissues (16–18). The relative contribution of these different subpopulations to recall responses is poorly understood (19, 20). One possibility is that effector memory cells present an immediate, but not sustained, defense at pathogen sites of entry, whereas central memory T cells sustain the response by proliferating in the secondary lymphoid organs and producing a supply of new effectors (21–23). Consistent with this idea, effector memory T cells resident in the lung airways were shown to mediate early control of a secondary respiratory virus challenge (11, 24). In addition, effector memory cells have been reported to be comparatively inefficient at mediating recall responses in terms of proliferation and accumulation at inflammatory sites (18). However, other studies indicated that effector memory cells can mediate potent recall responses to pathogens which raises questions regarding the relative contributions of this subset to recall responses (23, 25, 26). In the current report, we take advantage of a dual transfer system in which the proliferative capacity of different populations of memory cells can be compared directly in vivo. The data show that CD62L$^{lo}$ and CD62L$^{hi}$ memory cell subpopulations contribute to recall responses to Sendai virus infection in the lung. However, the relative contributions of these cells change over time; CD62L$^{lo}$ cells dominate at early time points, whereas CD62L$^{hi}$ cells dominate at late time points. These changes correspond to a general increase in the proliferative capacity of memory CD8$^+$ T cells over time.

RESULTS

The capacity of memory CD8$^+$ T cells to mediate proliferative recall responses improves over time

To compare the capacity of recently generated and long-term memory CD8$^+$ T cells to proliferate in response to Sendai virus infection in vivo, we took advantage of a dual transfer ap-
This involves isolating populations of memory T cells from different congenic strains of mice (B6-CD90.1 and B6-CD45.1), cotransferring them into naive recipient C57BL/6 mice (CD90.2/CD45.2), and analyzing their response to a subsequent intranasal Sendai virus infection. At the peak of the response (between days 9 and 11), the relative contributions of donor 1, donor 2, and host T cells that were specific for the immunodominant nucleoprotein (NP)\textsubscript{324-332}/K\textsuperscript{b} epitope can be determined using an MHC/peptide tetramer reagent. The advantage of this approach is that the response of two donor T cell populations can be compared directly under identical conditions in the same infected animals.

In initial experiments we compared NP\textsubscript{324-332}/K\textsuperscript{b}-specific memory CD8\textsuperscript{+} T cells that had been established by Sendai virus infection either 1 mo or >12 mo earlier (phenotypic analysis of the cells at these time points revealed similar patterns of adhesion molecule and chemokine receptor expression, Table I). Memory cells were isolated from the spleens of donor mice by negative enrichment of CD3\textsuperscript{+} T cells followed by flow cytometric sorting for cells that expressed high levels of CD44. These CD3\textsuperscript{+}/CD44\textsuperscript{hi} cells were mixed such that the ratio of NP\textsubscript{324-332}/K\textsuperscript{b}-specific CD8\textsuperscript{+} T cells in the 1-mo and 12-mo donor populations was 1:1 (a total of 6,000 NP\textsubscript{324-332}/K\textsuperscript{b}-specific cells) and they were injected intravenously into recipient mice. 11 d following a subsequent intranasal Sendai virus infection, NP\textsubscript{324-332}/K\textsuperscript{b}-specific T cells were detected readily by tetramer staining in the lung airways (bronchoalveolar lavage), lung parenchyma, pleural cavity lavage (PCL), mediastinal lymph nodes (MLNs), and spleens of the recipient mice (Fig. 1, A and B). Analysis of CD45.1 and CD90.1 expression on the NP\textsubscript{324-332}/K\textsuperscript{b}-specific CD8\textsuperscript{+} T cells from the donor (CD45.1/CD90.1) and recipient (CD45.2/CD90.2) which were infected intranasally with Sendai virus 1 d later. 11 d after infection, various tissues were isolated and stained with anti-CD8, NP\textsubscript{324-332}/K\textsuperscript{b} tetramer, and antibodies specific for each of the congenic markers. The flow cytometric profiles in the lower rows of each panel are gated on the tetramer\textsuperscript{+}/CD8\textsuperscript{+} population in the oval gate from the flow cytometric profiles in the upper rows. Panels A and B represent two independent experiments, each of which involved four recipient mice. Each recipient mouse received pooled donor memory cells that were isolated from the equivalent of two mice and each recipient received 6,000 tetramer-positive cells (3,000 from each donor). The flow-cytometric data in both panels are from single representative recipient mouse with the exception of the lung airways (Airways) and lung parenchyma (Lung) which are from a pool of four recipient mice.
cells allowed us to distinguish the relative contributions of each of the donors and the host to the response (27). The total number of NP324-332/Kb-specific T cells derived from the two donors (CD90.1/CD45.2 and CD90.2/CD45.1) in these tissues typically was $>4 \times 10^6$ per recipient mouse, which indicated a substantial expansion of the 6,000 donor cells that initially were transferred in these experiments (unpublished data). In addition, the data revealed that although both donor populations proliferated strongly, the 12-mo memory donor memory cells made up a greater proportion of the response in all tissues analyzed (Fig. 1). This relative dominance of 12-mo memory CD8$^+$ T cells (ranging from 1.5- to 13-fold) was consistent in three separate experiments (Fig. 2). The same results were obtained when we used an intracellular IFN-γ assay to identify NP324-332/Kb-specific T cells (the numbers of cells that synthesized IFN-γ directly correlated with the numbers of tetramer cells, unpublished data). Thus, the cells recruited to the lung airways are functional in terms of their capacity to secrete antiviral cytokines.

The differential response of 1-mo and 12-mo memory CD8$^+$ T cells was not due to an unanticipated influence of the congenic markers because similar results were obtained when the congenic markers were reversed (compare Fig. 1 A with Fig. 1 B). Nor could the difference in the response be attributed to a difference in the kinetics of the response, because similar patterns were obtained at days 7 and 14 after infection (unpublished data; references 25, 26). The dominance of 12-mo memory CD8$^+$ T cells also could not be attributed to the absence of CD4$^+$ T cell help. Strong CD4$^+$ T cell responses that were specific for an immunodominant Sendai virus hemagglutinin-neuraminidase (HN)419-433/A1 epitope (from young and aged donors) were detected in all tissues at the peak of the response using a multimer reagent that specifically identifies these cells (unpublished data). Finally, the data could not be explained by expansions of CD8$^+$ T cells that have been observed in aging mice (28). Although we noted that some mice that were infected 24 mo earlier with Sendai virus had CD8 expansions (defined by expression of a single TCR Vβ element)—some of which were NP324-332/Kb-tetramer positive (unpublished data)—we did not observe these in the 12-mo donor mice that were used in these studies. Thus, these data indicated that 12-mo memory cells have a greater capacity to proliferate than 1-mo memory cells under these conditions.

### Deficient generation of memory CD8$^+$ T cells in aged mice

The observation that the capacity of memory CD8$^+$ T cells to proliferate and accumulate at effector sites substantially improved (on a per cell basis) over time was surprising, especially given reports that a variety of immune defects tend to accumulate in aged mice (29, 30). Thus, we compared the proliferative capacities of CD8$^+$ memory T cells that had been generated recently in young or aged mice using the dual adoptive transfer system described above. 2-mo-old B6-CD90.1 mice and 20-mo-old C57BL/6 donor mice were infected intranasally with Sendai virus, and spleen cells were isolated 1 mo later. CD3$^+$/CD44hi T cells from the young and aged mice were sorted by flow cytometry, mixed at a 1:1 ratio with respect to NP324-332/Kb-specific T cells, and transferred into B6-CD44.1 congenic recipients (the number of NP324-332/Kb-specific T cells was estimated by flow cytometry using a tetramer reagent). These experiments were done on unmanipulated animals, and the results were consistent over the time period of these studies.

### Table I. Percentage of CD62Lhi and CD62Llo NP324-332/Kb-specific splenocytes expressing various adhesion molecules and chemokine receptors

| Marker | CD62Lhi | CD62Llo |
|--------|---------|---------|
| Ly6C   | 88      | 97      |
| CD27   | 88      | 77      |
| CD49a  | 73      | 54      |
| CD49b  | 77      | 68      |
| CD49d  | 87      | 87      |
| CCR5   | 51      | 30      |
| CCR7   | 70      | 96      |
| CXCR3  | 68      | 24      |
| CXCR4  | 24      | 8       |

Single cell suspensions of splenocytes from C57BL/6 mice were stained as described in Materials and methods. Numbers represent the percentage of NP324-332/Kb-specific CD8$^+$ T cells expressing the marker within CD62Lhi or CD62Llo gates. Data are representative of two separate experiments.
specific T cells in the 20-mo-old mice was \( \sim 2.4 \text{-fold} \) less than in the 2-mo-old mice, consistent with earlier reports that memory is established less effectively in old mice; unpublished data; reference 7). 1 d later, the mice were infected intranasally with Sendai virus and analyzed at days 7 and 11 after infection. Memory cells that were generated in aged mice were less efficient than memory cells that were generated in young mice at mediating recall responses in the lungs and other tissues (Figs. 2 and 3 A, and not depicted). Similar data were obtained when the young B6-CD45.1 congenic mice were used as donors, and which ruled out an unexpected effect of the congenic markers (Figs. 2 and 3). These data demonstrate that on a per cell basis, memory cells that are generated in older mice have a significantly lower proliferative capacity than memory cells that are generated in young mice. This pattern of response is in striking contrast to the enhanced responsiveness of long-term memory CD8\(^+\) T cells that initially had been generated in young mice (\( P = 0.0033 \) in the lung airways and \( P = 0.0029 \) in the lung tissues, as determined by a two-tailed Student’s t test; Fig. 2).

The improved capacity of long-term memory cells to mediate proliferative responses occurs predominantly in the CD62L\(^{hi}\) subpopulation

Thus far, the data suggest that memory CD8\(^+\) T cells that are generated in young mice increase their capacity to medi-
ate proliferative recall responses over time. One possible explanation for this effect is that it reflects the progressive accumulation of a subpopulation of antigen-specific cells with an enhanced capacity to proliferate. For example, we and others have reported that there is a gradual shift in the composition of the memory T cell pool from an effector memory to a central memory phenotype (8, 11, 18, 31). In the case of NP<sub>324-332</sub>/K<sub>b</sub>-specific T cells, the population progresses from a mixed phenotype in terms of CD62L and CD43 at 1 mo after infection to a predominantly CD62L<sup>hi</sup>/CD43<sup>lo</sup> phenotype at 20 mo after infection (Fig. 4). Therefore, we speculated that the increase in the proliferative capacity of the memory T cell pool over time occurred specifically in a subpopulation of memory T cells. To address this issue, we compared the proliferative responses of CD62L<sup>hi</sup> memory cells that had been generated 1 mo or 14 mo earlier. In parallel experiments, we also compared CD62L<sup>lo</sup> memory cells from the same sets of mice. Spleen cells from B6-CD45.1 (1 mo after infection) or B6-CD90.1 (14 mo after infection) congenic mice were enriched for CD3<sup>+</sup> cells on a negative selection column, and then stained with NP<sub>324-332</sub>/K<sub>b</sub> tetramer and antibodies specific for CD8, CD62L, and CD44. Cells were subjected to flow cytometric sorting to isolate CD44<sup>hi</sup>/CD62L<sup>lo</sup> and CD44<sup>hi</sup>/CD62L<sup>hi</sup> populations (top profiles of the figure) with 95–99.9% purity (middle profiles of the figure). The frequency of NP<sub>324-332</sub>/K<sub>b</sub>-specific cells in each sorted population is indicated in the bottom profiles of the figure. The bars in the profiles delineate regions and the associated numbers represent the percentage of gated cells in the indicated region.

**Figure 4. Phenotypic changes in memory CD8<sup>+</sup> T cells occur over time.** Mice were infected intranasally with Sendai virus; spleens, MLNs, and total lung tissue were recovered at 1, 6, 12, and 20 mo after infection. Single-cell suspensions were prepared and stained with NP<sub>324-332</sub>/K<sub>b</sub> tetramer and antibodies specific for CD8, CD62L, and CD43. The flow cytometric profiles show CD62L and CD43 profiles of tetramer<sup>+</sup>/CD8<sup>+</sup> gated cells. Data are representative of three independent experiments for each time point.

**CD62L<sup>hi</sup> cells play an increasingly dominant role in recall responses over time**

In a previous study we showed that CD62L<sup>lo</sup> cells mounted stronger proliferative responses to intranasal Sendai virus infection than CD62L<sup>hi</sup> cells (25). These data were generated with splenic memory T cells that were isolated at short times after infection (1–5 mo). In light of the data presented above, we speculated that the progressive increase in the proliferative capacity of CD62L<sup>hi</sup> memory CD8<sup>+</sup> T cells over time would result in a switch in this pattern, such that CD62L<sup>hi</sup>...
cells would mount stronger responses than CD62Llo cells at late time points. To address this issue, we compared the relative responsiveness of CD62Llo and CD62Lhi cells that had been generated >1 yr before analysis. Spleen cells were isolated from B6-CD45.1 or B6-CD90.1 mice that had recovered from an intranasal Sendai virus infection 1 yr earlier (CD45.1/CD90.2) or 14 yr earlier (CD45.2/CD90.1) and negatively enriched for CD3+ T cells on a column and then sorted into CD44hi/CD62Llo and CD44hi/CD62Lhi populations by FACS (as illustrated in Fig. 5). Total memory cells (CD3 enriched, CD44 sorted) also were isolated from C57BL/6 mice (syngeneic with the recipient mice) that had recovered from a previous Sendai virus infection. 1-mo and 14-mo CD44hi/CD62Llo memory cells (plus filler cells) or 1-mo and 14-mo CD44hi/CD62Lhi memory cells (plus filler cells) were combined such that the mix included equal numbers of NP324-332/Kb-specific cells derived from each of the congenic donors and the syngeneic filler population (the number of NP324-332/Kb-specific cells in each population was determined in a separate staining experiment). The mixed population of cells was transferred i.v. into C57BL/6 recipient mice (CD45.2/CD90.2) which were infected intranasally with Sendai virus 1 d later. 11 d after infection, various tissues were isolated and stained with anti-CD8, NP324-332/Kb tetramer, and antibodies specific for each of the congenic markers. The flow cytometric profiles in the bottom row of each transfer group are gated on the tetramer+CD8+ population in the oval gate from the flow cytometric profiles in the top row. The data represent a linked experiment involving four recipient mice (two each for the CD62Llo and CD62Lhi analysis). Each recipient mouse received donor memory cells isolated from the equivalent of five mice and each recipient received ~3,000 tetramer-positive cells (1,000 from each congenic donor and 1,000 from the syngeneic C57BL/6 mice). The flow cytometric data in both panels are from a single representative recipient mouse.

Figure 6. CD62Lhi and CD62Llo long-term memory CD8+ T cells mediate stronger recall responses than the corresponding populations of recently generated memory CD8+ T cells. Spleen cells from congenic donor mice that had recovered from an intranasal Sendai virus infection 1 mo earlier (CD45.1/CD90.2) or 14 mo earlier (CD45.2/CD90.1) were negatively enriched for CD3+ T cells on a column and then sorted into CD44hi/CD62Llo and CD44hi/CD62Lhi populations by FACS (as illustrated in Fig. 5). Total memory cells (CD3 enriched, CD44 sorted) also were isolated from C57BL/6 mice (syngeneic with the recipient mice) that had recovered from a previous Sendai virus infection. 1-mo and 14-mo CD44hi/CD62Llo memory cells (plus filler cells) or 1-mo and 14-mo CD44hi/CD62Lhi memory cells (plus filler cells) were combined such that the mix included equal numbers of NP324-332/Kb-specific cells derived from each of the congenic donors and the syngeneic filler population (the number of NP324-332/Kb-specific cells in each population was determined in a separate staining experiment). The mixed population of cells was transferred i.v. into C57BL/6 recipient mice (CD45.2/CD90.2) which were infected intranasally with Sendai virus 1 d later. 11 d after infection, various tissues were isolated and stained with anti-CD8, NP324-332/Kb tetramer, and antibodies specific for each of the congenic markers. The flow cytometric profiles in the bottom row of each transfer group are gated on the tetramer+CD8+ population in the oval gate from the flow cytometric profiles in the top row. The data represent a linked experiment involving four recipient mice (two each for the CD62Llo and CD62Lhi analysis). Each recipient mouse received donor memory cells isolated from the equivalent of five mice and each recipient received ~3,000 tetramer-positive cells (1,000 from each congenic donor and 1,000 from the syngeneic C57BL/6 mice). The flow cytometric data in both panels are from a single representative recipient mouse.
The increasing per cell efficacy of CD62L<sup>hi</sup> cells, coupled with their increasing representation in the splenic memory T cell pool (Fig. 4), explains the substantial increase in the overall efficacy of long-term memory T cells in the spleen. Taken together, these data demonstrate that T cell memory is very dynamic with respect to its composition and capacity to mediate proliferative recall responses.

**DISCUSSION**

The longevity and stability of CD8<sup>+</sup> T cell memory is poorly understood. Although it was reported that pools of CD8<sup>+</sup> memory T cells that were generated in young mice persist for the life of the animal, little is known about the relative capacity of these cells to mediate proliferative responses in vivo (7, 18, 25). Here we used a dual adoptive transfer approach to compare directly the capacity of different memory cell populations to proliferate in the same recipient animals. The data show that there is a progressive increase in the quality of memory T cell pools in terms of their ability to proliferate and accumulate at effector sites in response to secondary virus challenge. This increase in efficacy occurred in CD62L<sup>lo</sup> and CD62L<sup>hi</sup> memory T cell subpopulations and was more prominent in the CD62L<sup>hi</sup> subpopulation. There also was a progressive change in the composition of memory T cell pool from a predominantly CD62L<sup>lo</sup> population to a predominantly CD62L<sup>hi</sup> population. Thus, the long-term memory T cell population is characterized by an increase in the efficacy of cells to mediate proliferative responses on a per cell basis, and the accumulation of cells that mediate the strongest responses. Taken together, the data demonstrate that T cell memory improves over time on a per cell basis and it seems to take at least 1 yr for stable T cell memory to be established fully (18, 32).

Although it is clear that memory cells can be distinguished into central and effector subtypes, the relevance of these subtypes for recall responses has been controversial. A study by Wherry et al., using the lymphocytic choriomeningitis virus system, reported that CD62L<sup>lo</sup> memory CD8<sup>+</sup> T cells play the prominent role in recall responses (18). Other studies have underscored the strong proliferative capacity of central memory cells in a variety of infections. In contrast, there is substantial evidence that effector memory T cells can proliferate extensively to antigen in vitro and in vivo. For example, we showed that effector memory cells are substantially better than central memory cells at mediating proliferative recall responses to intranasal Sendai virus infection (25). Similarly, it was reported that effector memory CD62L<sup>lo</sup> cells mediate the strongest protection against *Plasmodium berghei* (23). The data presented here reconcile these apparently contradictory observations by revealing that time has a significant impact on the proliferative capacity of different memory subpopulations. As illustrated in Fig. 9, there is a progressive switch in the relative efficacy of CD62L<sup>lo</sup> and CD62L<sup>hi</sup> memory CD8<sup>+</sup> T cells to mediate proliferative responses. Thus, disparate results regarding the relative contributions of effector and central memory T cells to recall responses may be explained, in part, by the time after infection at which the cells are analyzed. The changes in T cell efficacy seem to reflect a general stabilization of the memory T cell pool over time. At early times after infection, effector memory T cells dominate the memory pool. These cells provide potent protective immunity, primarily because of their presence at peripheral sites (where they can make first contact with the invading pathogen), but also as a result of an enhanced capacity to mediate proliferative responses. However, at late times after infection, central memory T cells dominate the memory pool. Although these cells generally are not present at peripheral sites, they become the more potent responders to infection in terms of proliferative potential and provide the more durable immunity. Consistent with this, in the influenza and Sendai virus systems, it has been reported that the protective efficacy of cellular memory initially wanes over the first 6 mo after infection before stabilizing for the life of the animal (11, 33). These observations are explained best by a progressive loss of effector memory T cell pools over that time, and the subsequent establishment of a stable pool of central memory T cells.

The mechanisms that underlie the increasing proliferative efficacy of memory T cell subpopulations are not understood. Although it is possible that all of the memory cells gradually
improve their capacity to proliferate in response to antigen, a more likely explanation is that a specific subpopulation of cells with strong proliferative potential becomes the predominant population with increasing time. For example, this subpopulation may become progressively enriched in the CD62Lhi population, which accounts for the greater increase in responsiveness in this population compared with the CD62Llo population. One possibility is that proliferative efficacy is linked to low levels of CD43 expression, because CD43lo cells become progressively enriched in the CD62Lhi subpopulation (Fig. 4). We are testing this possibility. Another issue that we considered is that CD4+ regulatory cells were present in the transferred populations and were modifying the response of antigen-specific CD8+ T cells (34, 35). However, we showed previously that removal of CD4+ T cells before transfer does not affect the outcome of these dual transfer experiments, and indicates that regulatory cells were not affecting the response of the donor cells differentially (25; unpublished data).

An alternative explanation for the progressive increase in proliferative efficacy is that there are changes in TCR usage and avidity for antigen. Although it has been reported that most processed antigen is cleared rapidly after the resolution of a Sendai virus infection, it cannot be excluded that small amounts of antigen persist in a form that continually influences the memory T cell pool over the long term (36). However, we did not observe differences in functional avidity of NP324-332/Kb-specific cells in an intracellular cytokine assay, and the characteristic pattern of Vβ usage by NP324-332/Kb-specific T cells is stable over time (37; unpublished data). Thus, it seems unlikely that the effects observed depend on persistent antigen. A related possibility is that expansions of memory CD8+ T cells seen in aged mice may have a direct effect on proliferative efficacy (28, 38). Messaoudi et al. reported that these expansions can reduce the efficacy of the naive T cell response to viral challenge, presumably by affecting the numbers of precursor cells that are available (39). In this regard, we noted that some mice that were infected 24 mo earlier with Sendai virus had CD8 expansions (defined by expression of a single TCR Vβ element), some of which were NP324-332/Kb-tetramer–positive (unpublished data).

Figure 8. Long-term CD62Lhi/CD8+ memory T cells mediate stronger recall responses than long-term CD62Llo/CD8+ memory T cells. Spleen cells from congenic donor mice (CD45.1/CD90.2 or CD45.2/CD90.1) that had recovered from an intranasal Sendai virus infection 15 mo earlier were negatively enriched for CD3+ T cells on a column and then sorted into CD44hi/CD62Llo and CD44hi/CD62Lhi populations by FACS (as illustrated in Fig. 5). CD62Lhi and CD62Llo memory T cells were combined such that the number of NP324-332/Kb–specific cells derived from each donor population was equivalent (the number of NP324-332/Kb–specific cells in each population was determined in a separate staining experiment). The mixed population of donor cells was transferred i.v. into C57BL/6 recipient mice (CD45.2).

CD90.2) which were infected intranasally with Sendai virus 1 d later. 11 days after infection, various tissues were isolated and stained with anti-CD8, NP324-332/Kb tetramer, and antibodies specific for each of the congenic markers. The flow cytometric profiles in the bottom row of each panel are gated on the tetramer–CD8+ population in the oval gate from the flow cytometric profiles in the top row. The data are from a linked reciprocal experiment involving eight recipient mice (four each for each combination analysis). Each recipient mouse received donor memory cells that were isolated from the equivalent of three mice, and each recipient received ~4,000 tetramer–positive cells (2,000 from each donor). The flow cytometric data in both panels are from single representative recipient mouse.
These perturbations in the memory T cell pool were not a problem for the current study because they were not observed in the 12-mo donor mice used. However, it will be interesting to determine precisely how these expansions (antigen-specific or not) affect memory recall responses.

The capacity of the immune system to remember an earlier infection with a given pathogen is a hallmark of adaptive immunity. The data presented here indicate that this capacity is maintained for long periods of time and is enhanced in the face of an age-related decline in the capacity of the immune system to respond to new pathogens (30). This is consistent with data in the human that suggest that cellular immunity is relatively long-lived (3). However, it is clear that there are changes in the distribution of cells that may affect the overall efficacy of the memory T cell pool in terms of its capacity to mediate pathogen clearance. For example, there is a significant loss of effector memory cells from peripheral sites over time which may reduce the immediate response of memory T cells to secondary challenge; this may counteract the enhanced capacity of memory cells in the secondary lymphoid organs to generate proliferative responses (11, 40). In addition, it has been reported that memory T cell pools may be buffeted continually by infections with unrelated pathogens that express cross-reactive T cell epitopes (41). This results in the specific expansion of T cells that are specific for some epitopes and the loss of T cells that are specific for others (42, 43). How these changes influence the overall recall efficacy of a pool of memory T cells is not known. Clearly, a better understanding of the relative roles of these different memory T cell subsets, the temporal changes that affect their relative stability and efficacy, and the factors that are involved in their initial generation is essential for the development of vaccines that are designed to promote cellular immunity.

**MATERIALS AND METHODS**

**Viruses, mice, and infection.** Sendai virus (Enders strain) was grown, stored, and titered as described previously (44). Male C57BL/6J, B6.PI-Thy1a/H11001, and B6.SJL-Pep3/BoyJ (CD45.1) mice were purchased from The Jackson Laboratory. Aging C57BL/6 mice (19–20 mo) were obtained from the National Institute of Aging’s aged rodent colonies. Mice (6–8 wk-CD90.1 and CD45.1 donors, or 19–20-mo C57BL/6J donors) were anesthetized by i.p. injection of 2,2,2-tribromoethanol and injected intranasally with 250 50% egg infectious doses of Sendai virus. Animals were housed under specific pathogen-free conditions from 1 mo to 20 mo after Sendai infection. All animal studies were approved by the Institutional Animal Care and Use Committee.

**Preparation and adoptive transfer of donor cells.** Lymphocyte suspensions were obtained by pressing spleens through a 70-μ nylon mesh (Bally Ribbon), and erythrocytes were depleted with buffered ammonium chloride (5 min at room temperature). Cells were plated on goat antimouse IgG H+L (Jackson Immunoresearch Laboratories)-coated Primaria flasks (Falcon) for 1 h at 37°C to remove B cells and macrophages, and then were enriched for CD3+ cells using negative selection T Cell Enrichment Columns (R&D Systems). For the transfer experiments with CD62Lhi and CD62Llo subpopulations, enriched cells were stained with anti–CD26-L–FITC and anti–CD44-PE (anti–CD8-PE/CY5 was included in some experiments) and sorted into CD44hi/CD62Llo and CD44hi/CD62Lhi populations using a FACSVantage (Becton Dickinson) cell sorter with DIVA enhancement software. A sample of the cells was analyzed to determine the percentage of NP324–332/Kb-specific T cells present in the sorted populations using an APC-conjugated tetramer. The sorted CD44hi/CD62Lhi and CD44hi/CD62Llo populations from the B6.SJL–Pep3/BoyJ (CD45.1), B6.PI–Thy1a/H11001 (CD90.1), or C57BL/6J donor animals were recombined such that the number of NP324–332/Kb-specific T cells in each donor population was equal. The recombined cell populations were transferred intravenously into naïve recipient mice via tail vein injection. 1 d later, recipient mice were challenged intranasally with 250 50% egg infectious doses of Sendai virus, and T cell responses were analyzed on days 7, 11, and 14 after infection. Control experiments demonstrated that transferred Sendai virus-specific memory cells did not proliferate in uninfected mice or in response to influenza virus infection (reference 25; unpublished data). We also analyzed the distribution of the transferred cells in uninfected mice at different times after transfer (unpublished data). In several experiments we determined that CD62Lhi and CD62Llo cells were established in the MLN and spleen at a ratio of ~2:1 (range: 1.6:1 to 2.8:1). We did not detect any donor cells in the lung tissue or airways under these conditions.

Donor mice older than 8 mo were analyzed for CD8+ T cell expansions that can occur with increasing age (28, 45). Any mice that exhibited abnormal VB distributions or unexpectedly high frequencies of tetramer-positive cells were excluded from the experiment.

**Analysis of the T cell response.** Bronchoalveolar lavage (airways), lung parenchymal tissue (lung), PCL, MLNs, and spleens were harvested from recipient mice and lymphocyte populations were prepared. Cells were blocked first with mAbs to FcRγII/I receptor and then stained with combinations of APC-conjugated NP324–332/Kb tetramer, anti–CD45.1-FITC, anti–CD45.2-FITC, anti–CD90.1-PE, anti–CD90.2-PE, anti–CD8-PerCP, anti–CD62L-FITC, and anti–CD43-PE. Samples were run on a FACSCalibur flow cytometer (Becton Dickinson), and the data were ana-

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**Figure 9.** Comparison of the relative responses of CD62Lhi versus CD62Llo memory T cells isolated at 1, 5, and 15 mo after infection. The data show the ratio of NP324–332/Kb-specific cells derived from each of the respective donors on day 11 after Sendai virus infection. Data are derived from the lung airways (Airways) and lung parenchyma (Lung) as indicated. Filled circles indicate data from independent experiments in which the samples were pooled from three individual mice. Open symbols indicate data from four independent experiments, each involving four recipient mice (triangles, inverted triangles, circles, and squares distinguish the independent experiments in which each individual animal was analyzed separately. The figure includes data from the experiment illustrated in Fig. 8.
lyzed using Flowjo software (Tree Star). The absolute number of antigen-specific T cells in each tissue was calculated from the percentage of tetramer-positive cells in a total live cell gate and the number of trypan blue excluding cells isolated per mouse tissue. The NP324–332/Kb tetramer was generated by the Molecular Biology Core Facility at the Trudeau Institute (46) and CCL19-Fc, a gift from S. Krautwald, was used to identify CCR7+ cells (47).

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REFERENCES

1. Woodland, D.L. 2003. Cell-mediated immunity to respiratory virus infections. Curr. Opin. Immunol. 15:430–435.
2. Dutton, R.W., L.M. Bradley, and S.L. Swain. 1998. T cell memory. Annu. Rev. Immunol. 16:201–223.
3. Hammarlund, E., M.W. Lewis, S.G. Hansen, L.I. Strelow, J.A. Nelson, G.J. Sexton, J.M. Hanifin, and M.K. Slifka. 2003. Duration of antiviral immunity after smallpox vaccination. Nat. Med. 9:1131–1137.
4. Seder, R.A., and R. Ahmed. 2003. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat. Immunol. 4:835–842.
5. Tan, J.T., B. Ernst, W.C. Kieper, E. LeRoy, J. Sprent, and C.D. Surh. 2004. Defective generation but normal maintenance of memory T cells in old mice. Eur. J. Immunol. 32:1567–1573.
6. Usherwood, E.J., R.J. Hogan, G. Crowther, S.L. Surman, T.L. Hogg, J.D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8+ T cells: a reevaluation of bystander activation during viral infection. Immunity. 8:177–187.
7. Tripp, R.A., S. Hou, and P.C. Doherty. 1995. Temporal loss of the transient and effector functions. Immunity. 3:225–234.
8. Usherwood, E.J., T.L. Hogg, and D.L. Woodland. 1994. The MHC class II-restricted T cell response to Sendai virus infection in C57BL/6 mice: nature of the Th1 and Th2 responses to infection? J. Immunol. 152:1653–1661.
9. Yang, R.A. Lempicki, I. Sereti, and H.C. Lane. 2003. Cutting edge: L-selectin expression distinguishes small resting memory CD4+ T cells from naive to effector-alterations with aging. J. Immunol. 170:5870–5875.
10. Rouse, B.T., and S. Suvas. 2004. Regulatory cells and infectious agents: detentes cordiale and contraire. Nat. Rev. Immunol. 4:841–855.
11. Mills, K.H. 2004. Regulatory T cells: friend or foe in immunity to infection? Nat. Rev. Immunol. 4:841–855.
12. Kaech, S.M., S. Hemby, E. LeRoy, J. Sprent, and C.D. Surh. 1998. Functionally heterogeneous virus-specific CD8+ T cells in primary and secondary influenza pneumonia. J. Immunol. 158:638–641.
13. Yang, R.A. Lempicki, I. Sereti, and H.C. Lane. 2003. Cutting edge: L-selectin expression distinguishes small resting memory CD4+ T cells from naive to effector-alterations with aging. J. Immunol. 170:5870–5875.
14. Kaech, S.M., S. Hemby, E. LeRoy, J. Sprent, and C.D. Surh. 1998. Functionally heterogeneous virus-specific CD8+ T cells in primary and secondary influenza pneumonia. J. Immunol. 158:638–641.
15. Yang, R.A. Lempicki, I. Sereti, and H.C. Lane. 2003. Cutting edge: L-selectin expression distinguishes small resting memory CD4+ T cells from naive to effector-alterations with aging. J. Immunol. 170:5870–5875.
16. Kaech, S.M., S. Hemby, E. LeRoy, J. Sprent, and C.D. Surh. 1998. Functionally heterogeneous virus-specific CD8+ T cells in primary and secondary influenza pneumonia. J. Immunol. 158:638–641.
17. Yang, R.A. Lempicki, I. Sereti, and H.C. Lane. 2003. Cutting edge: L-selectin expression distinguishes small resting memory CD4+ T cells from naive to effector-alterations with aging. J. Immunol. 170:5870–5875.
18. Kaech, S.M., S. Hemby, E. LeRoy, J. Sprent, and C.D. Surh. 1998. Functionally heterogeneous virus-specific CD8+ T cells in primary and secondary influenza pneumonia. J. Immunol. 158:638–641.
a single immunodominant epitope elicits an extremely diverse repertoire of T cells. *Int. Immunol.* 6:1767–1775.

38. Callahan, J.E., J.W. Kappler, and P. Marrack. 1993. Unexpected expansions of CD8-bearing cells in old mice. *J. Immunol.* 151:6657–6669.

39. Messaoudi, I., J. Lemaoult, J.A. Guevara-Patino, B.M. Metzner, and J. Nikolich-Zugich. 2004. Age-related CD8 T cell clonal expansions constrict CD8 T cell repertoire and have the potential to impair immune defense. *J. Exp. Med.* 200:1347–1358.

40. Woodland, D.L., R.J. Hogan, and W. Zhong. 2001. Cellular immunity and memory to respiratory virus infections. *Immunol. Rev.* 24:53–67.

41. Brehm, M.A., A.K. Pinto, K.A. Daniels, J.P. Schneck, R.M. Welsh, and L.K. Selin. 2002. T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. *Nat. Immunol.* 3:627–634.

42. Chen, H.D., A.E. Fraire, I. Joris, M.A. Brehm, R.M. Welsh, and L.K. Selin. 2001. Memory CD8⁺ T cells in heterologous antiviral immunity and immunopathology in the lung. *Nat. Immunol.* 2:1067–1076.

43. Selin, L.K., M.Y. Lin, K.A. Kraemer, D.M. Pardoll, J.P. Schneck, S.M. Varga, P.A. Santolucito, A.K. Pinto, and R.M. Welsh. 1999. Attrition of T cell memory: selective loss of LCMV epitope-specific memory CD8 T cells following infections with heterologous viruses. *Immunity.* 11:733–742.

44. 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.* 369:652–654.

45. Ku, C.C., J. Kappler, and P. Marrack. 2001. The growth of the very large CD8⁺ T cell clones in older mice is controlled by cytokines. *J. Immunol.* 166:2186–2193.

46. Altman, J.D., P.H. Moss, P.R. Goulder, D.H. Barouch, M.G. McHeyzer-Williams, J.L. Bell, A.J. McMichael, and M.M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science.* 274:94–96.

47. Unsoeld, H., and H. Pircher. 2005. Complex memory T-cell phenotypes revealed by coexpression of CD62L and CCR7. *J. Virol.* 79:4510–4513.