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CUTTING EDGE

Cutting Edge: Effector Memory CD8$^+$ T Cells Play a Prominent Role in Recall Responses to Secondary Viral Infection in the Lung[1]

Alan D. Roberts and David L. Woodland[2]

The relative contributions of CD62L^{high} (central) memory and CD62L^{low} (effector) memory T cell populations to recall responses are poorly understood, especially in the respiratory tract. In this study, we took advantage of a dual-adoptive transfer system in the mouse to simultaneously follow the recall response of effector and central memory subpopulations to intranasal parainfluenza virus infection. Using MHC class I and class II multimers, we tracked the responses of Ag-specific CD8$^+$ and CD4$^+$ memory T cells in the same animals. The data show that effector memory T cells mounted recall responses that were equal to, or greater than, those mounted by central memory T cells. Moreover, effector memory T cells were more efficient at subsequently establishing a second generation of memory T cells. These data contrast with other studies indicating that central memory CD8$^+$ T cells are the prominent contributors to systemic virus infections. The Journal of Immunology,* 2004, 172: 6533–6537.

T lymphocytes play a central role in controlling respiratory virus infections (1). Following clearance of the infection, populations of Ag-specific memory T cells are established that have the capacity to mediate accelerated and more vigorous responses to secondary viral challenge (1). Memory T cells persist in both lymphoid and peripheral tissues and can be resolved into two major subsets based on their expression of lymph node homing receptors (CD62L and CCR7), referred to as central and effector memory T cells (1–10). Whereas central memory T cells (CD62L^{high}CCR7$^+$) are predominantly found in lymphoid tissues, effector memory T cells (CD62L^{low}CCR7$^-$) are found in both lymphoid and peripheral tissues (10, 11). The relative contribution of these different subpopulations to recall responses is poorly understood (12, 13). 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 (14–16). Consistent with this idea, effector memory T cells in the lung airways have been shown to mediate early control of respiratory virus challenge (4, 17). Furthermore, Wherry et al. (11) analyzed the recall response to systemic lymphocytic choriomeningitis virus (LCMV)[3] (1) infection and showed that CD62L^{high} (central) memory CD8$^+$ T cells respond more vigorously to secondary challenge than CD62L^{low} (effector) memory T cells in terms of their expansion and capacity to clear virus. However, there is little information on how these memory T cell subsets (either CD4$^+$ or CD8$^+$) contribute to the recall response to mucosal, rather than systemic, infections. To address this issue, we have investigated the proliferative response of CD4$^+$ and CD8$^+$ memory T cells to Sendai virus infection in the lung. The data show that CD62L^{low} (effector) memory T cells are able to generate a recall response that is at least as strong as that mediated by CD62L^{high} (central) memory T cells.

Materials and Methods

Viruses, mice, and infection

Male C57BL/6, B6.Pl-*Thy1^a*/Cy (Thy1.1), and B6.SJL-*Ptprc^a Pep3*/BoyJ (CD45.1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions. Anesthetized mice were intranasally infected with 250 50% egg infectious doses of Sendai virus (Enders strain) (2).

Adoptive transfers

Spleens from B6.Pl-*Thy1^a*/Cy (Thy1.1) and B6.SJL-*Ptprc^{a}Pep3*/BoyJ (CD45.1) donor mice that had recovered from a prior Sendai virus infection were depleted of erythrocytes, panned on goat anti-mouse IgG H+L (Jackson ImmunoResearch Laboratories, West Grove, PA)-coated Primaria flasks (Falcon; BD Discovery Labware, Bedford MA) to remove B cells and macrophages, and then further enriched for either CD8$^+$ or CD3$^+$ T cells using negative selection columns (R&D Systems, Minneapolis, MN). Cells were then stained with CD62L-FITC, CD44-PE, CD8-PE/CY5 (CD8$^+$ enriched), or CD62L-FITC and CD44-PE (CD3$^+$ enriched) for sorting on a FACSVantage (BD Immunocytometry Systems, San Jose, CA) cell sorter with DIVA enhancement software. Sort gates were set for CD44$^+$CD8$^+$ (or just CD44$^+$) and CD62L (as illustrated in Fig. 1). The CD62L^{low}- and CD62L^{high}-sorted cell populations were then mixed, labeled with 0.5 μM CFSE for 10 min at room temperature, washed, and adoptively transferred i.v. into naive C57BL/6 recipient mice (typically three recipients per experiment). One day later, mice were challenged intranasally with 250 50% egg infectious doses of Sendai virus. At various days postinfection (see Table I), bronchoalveloar lavage (BAL), mediastinal lymph nodes (MLN), lungs, and spleens were harvested, and lymphocyte populations

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[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; BAL, bronchoalveloar lavage; MLN, mediastinal lymph node; NP, nucleoprotein; HN, hemagglutinin-neuraminidase.

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were stained as described previously (1, 2, 4, 8). The data were acquired using a FACSCalibur flow cytometer (BD Immunocytometry Systems) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Results and Discussion

Previous studies have shown that populations of $CD8^+$ memory T cells specific for the immunodominant nucleoprotein $(NP)_{324-332}/K^b$ epitope are established in various organs following the recovery of C57BL/6 mice from an intranasal Sendai virus infection (2). Typically, 200,000 $NP_{324-332}/K^b$-specific cells persist in the spleen, and the majority of these express an effector memory phenotype based on CD62L expression (2). To investigate the contribution of these memory cell subsets to pulmonary recall responses, we compared their capacity to respond to a Sendai virus challenge in an adoptive transfer system. $CD8^+CD44^+CD62L^{low}$ or $CD8^+CD44^+CD62L^{high}$ memory cells were isolated from Thy1.1 mice by flow-cytometric cell sorting (the purity of the cells was always $\geq$95%, as illustrated by a typical sort in Fig. 1). Sorted cells were then CFSE labeled and i.v. transferred to recipient C57BL/6 (Thy1.2) mice before intranasal Sendai virus infection 1 day later. The numbers of donor and host $NP_{324-332}/K^b$-specific T cells were subsequently determined in different organs at various times postinfection. In preliminary studies (data not shown), we observed substantial expansion of $NP_{324-332}/K^b$-specific T cells in all tissues tested, peaking between days 9 and 11 postinfection. Typically, the $CD62L^{low}$ memory T cells generated responses

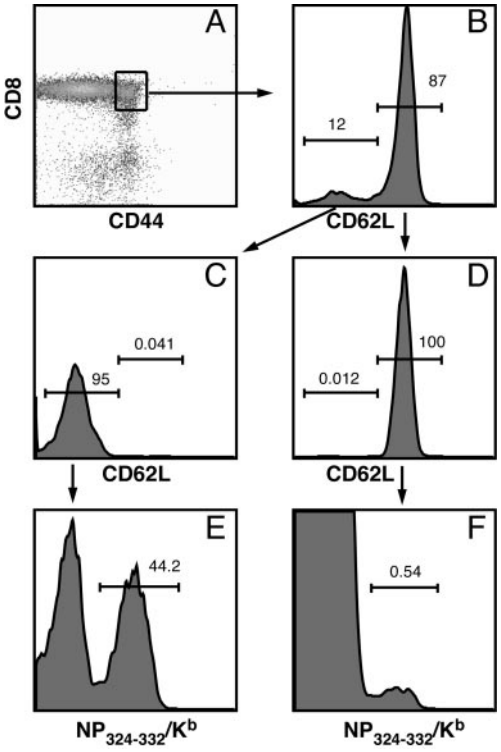

FIGURE 1. Isolation of memory cell subsets. CD8-enriched spleen cells were flow-cytometrically separated into memory T cell subsets by gating on $CD44^{high}CD8^+$ cells (*A*) and then sorting on the basis of CD62L expression (*B*). Sorted populations were >95% pure (*C* and *D*). Samples of the sorted populations were stained with $NP_{324-332}/K^b$ multimer to determine the percentage of $NP_{324-332}/K^b$-specific T cells in each sorted population (*E* and *F*). In some experiments (experiments 5 and 6), the cells were isolated and purified using the same protocol, with the exception that the CD8 Ab was excluded. The numbers in *B–F* indicate the percentages of cells in the marked regions.

equivalent to, or better than, $CD62L^{high}$ memory T cells in terms of the numbers of donor $NP_{324-332}/K^b$-specific T cells recovered (data not shown). This expansion was Ag dependent, because Sendai virus-specific memory cells did not expand in uninfected or influenza virus-infected mice.

Although these preliminary data demonstrated a strong expansion of $CD62L^{low}$ memory T cells, variation between individual recipients made it difficult to directly compare the expansion of the $CD62L^{low}$ and $CD62L^{high}$ subsets. To get around this problem, we developed a dual-transfer protocol using $CD44^+CD62L^{low}$ and $CD44^+CD62L^{high}$ memory T cells isolated from CD45.1 (Ly-5.1) and CD90.1 (Thy1.1) donors (as illustrated in the diagram in Fig. 2). Sorted cells were labeled with CFSE and then mixed together such that the number of $NP_{324-332}/K^b$-$CD62L^{low}$ and $NP_{324-332}/K^b$-$CD62L^{high}$ memory T cells was at the original splenic ratio, or at a 1:1 ratio. The cells were then transferred i.v. into C57BL/6 (CD45.2/CD90.2) recipient mice, which were intranasally infected with Sendai virus 1 day later. At various times postinfection, the numbers of $NP_{324-332}/K^b$-specific donor T cells in various tissues were determined on the basis of CD45.1 and CD90.1 expression. Data from six individual experiments and a representative example of the flow-cytometric analysis are presented in Table I and Fig. 2.

In general, there was a very strong expansion of Ag-specific donor cells to Sendai virus challenge, ranging from 7 to 80 times the input cell number at day 11 postchallenge. Furthermore, all of the Ag-specific donor T cells were CFSE negative, indicating that they had undergone at least seven divisions since transfer (Fig. 2). The advantage of the dual-transfer approach was that we were able to directly compare the expansion of the two subsets of transferred cells within individual mice. This revealed that there was a very strong expansion of the $CD62L^{low}$ subset during the acute response to Sendai virus challenge that was greater than that of the $CD62L^{high}$ subset in all experiments (i.e., the ratio of $CD62L^{low}$ to $CD62L^{high}$ donor cells recovered was increased relative to the input ratio; $p = 0.006$ in a binomial test). This was true regardless of whether the two subsets were initially transferred at a ~1:1 ($CD62L^{low}$ to $CD62L^{high}$) ratio, or their original ratio in the donor mice (usually between 4:1 and 10:1, $CD62L^{low}$ to $CD62L^{high}$). The strong expansion of the $CD62L^{low}$ subset was observed in all tissues tested (spleen, MLN, lung parenchyma, and lung airways) and at both days 7 and 11 postinfection (Table I and data not shown). Similar data were also obtained when we analyzed the cells for cytokine production following stimulation with the $NP_{324-332}$ peptide in an intracellular IFN-γ assay (data not shown).

The strong expansion of $CD62L^{low}$ memory T cells could not be attributed to an unanticipated effect of one of the genetic markers, because the same results were obtained regardless which marker was used to distinguish each T cell subset (Fig. 2, compare *A* and *B*). Similarly, the strong expansion of $CD62L^{low}$ cells was not limited to recently established donor memory cells, because the data in experiments 5 and 6 were generated with donor memory cells that had been isolated 5 mo postinfection (Table I). However, it was possible that the isolation procedure affected the subsequent survival or response of the cells after transfer. Thus, we compared the survival of transferred $CD62L^{low}$ and $CD62L^{high}$ memory donor cells in the absence of infection, or after an irrelevant influenza virus infection (infected on day 1 posttransfer). Although the absolute

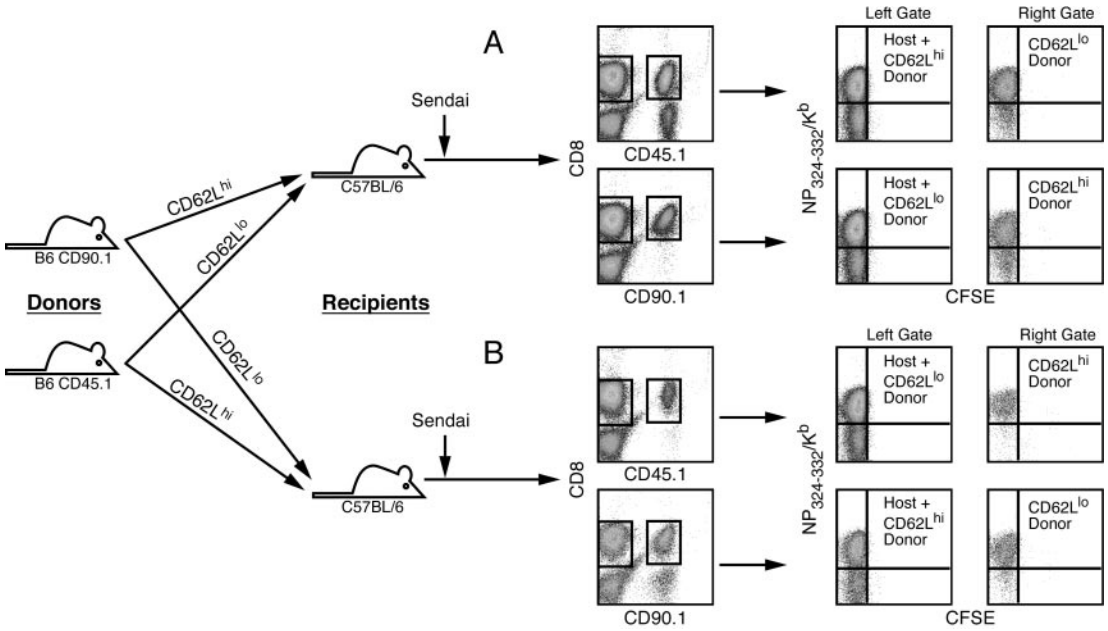

FIGURE 2. Expansion of $CD62L^{low}$ (effector) memory $CD8^+$ T cells. Mixtures of CFSE-labeled $CD44^+CD62L^{high}$ and $CD44^+CD62L^{low}$ memory T cells were transferred into two groups of C57BL/6 recipient mice. The first group of mice (*A*) received a mixture of $CD44^+CD62L^{low}$ T cells from CD45.1 donors and $CD44^+CD62L^{high}$ T cells from CD90.1 donors. The second group of mice (*B*) received $CD44^+CD62L^{low}$ T cells from CD90.1 donors and $CD44^+CD62L^{high}$ T cells from CD45.1 donors. Recipient C57BL/6 mice (CD45.2/CD90.2) were then intranasally infected with Sendai virus 1 day later. Eleven days postinfection, cells were recovered from the lung airways and stained for the expression of CD8 and each of the congenic markers (CD45.1 and CD90.1, *left column*). The boxes indicate gates that identify host or donor CD8 cells, which are then analyzed for multimer ($NP_{324-332}/K^b$) and CFSE expression (*middle* and *right-hand columns*). The data in this figure are derived from experiments 5 and 6 in Table I.

numbers of memory cells recovered after 3 wk were relatively low, both $CD62L^{low}$ and $CD62L^{high}$ donors were readily identified in the spleen and lymph nodes, and each subset had retained its original phenotype (data not shown). This is consistent with earlier studies indicating that ligation with the CD62L Ab does not affect the precursor frequency or recall of memory T cells (18). Together, these data suggest that the isolation procedure does not favor the survival of either effector or central memory T cells in this transfer model.

We next considered the possibility that $CD4^+$ T cells affected the recall response of memory $CD8^+$ T cell subsets. This was an important issue, because many of our transfer studies were with memory T cells that had been purified on the basis of $CD8^+$ expression. However, some of the transfer studies used cells isolated on the basis of CD44 and CD62L, but not CD8 (Table I, experiments 5 and 6). With this protocol, not only both effector and central $CD8^+$ memory T cell populations, but also a full complement of memory $CD4^+$ T cells are present. Again, $CD62L^{low}$ memory T cells responded strongly to acute Sendai virus challenge and mediated responses that were at least as good, if not better than, $CD62L^{high}$ memory T cells. To confirm that $CD4^+$ T cells were indeed transferred and responding in these experiments, we used a multimer reagent that specifically detects T cells specific for the immunodominant hemagglutinin-neuraminidase $(HN)_{419-433}/A^b$ epitope (8). As shown in Fig. 3, there was a strong expansion of $HN_{419-433}/A^b$-specific donor T cells during the acute response to Sendai virus challenge. It should be noted that the data in Fig. 3 are from the same experiment shown in Fig. 2 and represent responses that were occurring concomitantly with the CD8 responses in the same animals. Thus, the preferential expansion of $CD62L^{low}$ memory $CD8^+$ T cells cannot be attributed to an absence of memory $CD4^+$ T cells. Interestingly, the recovery of $HN_{419-433}/A^b$-specific donor T cells was also biased to cells derived from the $CD62L^{low}$ subset during the acute infection (day 11) (Table I). These data indicate that effector memory $CD4^+$ T cells also respond vigorously to secondary virus challenge and that they are at least as efficient as, if not superior, to central memory cells in terms of their ability to mediate recall responses.

Given the strong proliferative response of Ag-specific $CD62L^{low}$ memory T cells to Sendai virus challenge, we next investigated whether these cells contributed to the re-establishment of memory T cells. In three different experiments, there was a strong bias in the spleen toward the re-establishment of memory T cells from the $CD62L^{low}$ donor population (Table I, experiments 3, 4, and 6). A similar bias was observed in the lung airways in experiments 3 and 4 (in experiment 6, the ratio of cells recovered reflected the input ratio). Analysis of the $CD4^+$ T cell response also indicated that $HN_{419-433}/A^b$-specific T cells from the $CD62L^{low}$ subset were superior to those from the $CD62L^{high}$ subset in terms of establishing memory, although the absolute numbers were relatively low.

Taken together, the data clearly show that $CD62L^{low}$ memory T cells respond vigorously to secondary Sendai virus challenge in vivo, both in terms of the absolute numbers of cells recovered, and in the capacity of the cells to migrate to the lung airways. Moreover, the strong expansion of $CD62L^{low}$ memory T cells occurs in the face of a vigorous response by $CD62L^{high}$ memory T cells. These findings differ from those of Wherry et al. (11) and suggest that the relative contributions of $CD62L^{low}$ (effector) and $CD62L^{high}$ (central) memory T cells to Sendai virus and LCMV infection may be different. It is possible that

Table I. *Preferential contribution of CD62L^{low} memory cells in the recall response to Sendai virus infection*

Expt.	Donor Cells	Day of Analysis	Input Donor Cells (Number of Ag-Specific Cells)[a] CD62L^{low}	Input Donor Cells (Number of Ag-Specific Cells)[a] CD62L^{high}	Input Donor Cells (Number of Ag-Specific Cells)[a] Ratio low:high	Donor Cells Recovered from Spleen (Number of Ag-Specific Cells)[b] CD62L^{low}	Donor Cells Recovered from Spleen (Number of Ag-Specific Cells)[b] CD62L^{high}	Donor Cells Recovered from Spleen (Number of Ag-Specific Cells)[b] Ratio low:high	Donor Cells Recovered from BAL (Number of Ag-Specific Cells)[b] CD62L^{low}	Donor Cells Recovered from BAL (Number of Ag-Specific Cells)[b] CD62L^{high}	Donor Cells Recovered from BAL (Number of Ag-Specific Cells)[b] Ratio low:high	Total Donor Cells Recovered (Number)[c]
$NP_{324-332}/K^b$ response												
1	CD8	7	24,300	4,200	6	1,600	200	8	700	100	7	9,600
2	CD8	7	25,800	5,100	5	3,000	200	15	400	50	8	25,600
3	CD8	7	3,300	3,300	1	8,000	300	27	410	50	8	17,400
1	CD8	11	23,100	4,300	5	78,000	4,000	20	39,700	2,700	15	195,000
2	CD8	11	35,400	2,300	15	60,100	600	100	199,400	700	280	685,400
4	CD8	11	3,300	3,800	1	108,200	32,500	3	21,900	12,800	2	237,000
5	CD4 + CD8	11	4,200	3,000	1	319,000	78,500	4	62,100	37,500	2	557,700
6	CD4 + CD8	11	5,100	2,500	2	272,300	47,100	6	31,500	11,500	3	397,000
3	CD8	35	3,300	3,300	1	1,700	100	17	2,000	<50	>40	5,500
4	CD8	35	3,300	3,800	1	16,800	400	42	400	<50	>8	19,800
6	CD4 + CD8	40	5,100	2,500	2	120,200	12,200	10	13,936	8,500	2	177,400
$HN_{419-433}/A^b$ response[d]												
5	CD4 + CD8	11	≃600	≃700	1	2,500	200	13	2,900	<50	>58	7,200
6	CD4 + CD8	11	≃900	≃500	2	900	<50	>18	1,200	<50	>24	3,100
6	CD4 + CD8	40	≃900	≃500	2	800	<50	>16	500	<50	>10	1,800

[a] The data in these three columns show the absolute number of Ag-specific donor cells transferred (either $NP_{324-332}/K^b$ - or $HN_{419-433}/A^b$-specific) that were CD62L^{low} (first column), CD62L^{high} (second column), and the ratio of these cell numbers (third column).

[b] The data in these columns show the numbers of Ag-specific donor cells recovered (either $NP_{324-332}/K^b$- or $HN_{419-433}/A^b$-specific) in the indicated tissues from either the CD62L^{low} or CD62L^{high} donors and the ratio of these two numbers.

[c] This column indicates the total number of donor cells recovered from the spleen, MLN, lung tissue, and BAL.

[d] Given the relatively low numbers of $HN_{419-433}/A^b$-specific T cells in the donor populations and the limited number of donor cells available, the input numbers of $HN_{419-433}/A^b$-specific T cells were calculated from frequencies determined prior to enrichment (i.e., they are not direct measurements).

this reflects the fact that Sendai virus establishes a mucosal infection, whereas LCMV establishes a systemic infection, even when initially introduced through the nose. However, there are also technical differences in the experiments that may explain the different outcomes. Whereas Wherry et al. (11) used isolated subsets of memory T cells in their transfers, we used blended effector and central memory populations corresponding to the normal memory T cell pool. In addition, we used low numbers of nontransgenic T cells, potentially affecting the level of expansion achieved. Finally, we did not use multimer in the sorting protocol to avoid an affect on the viability or function of the transferred cells.

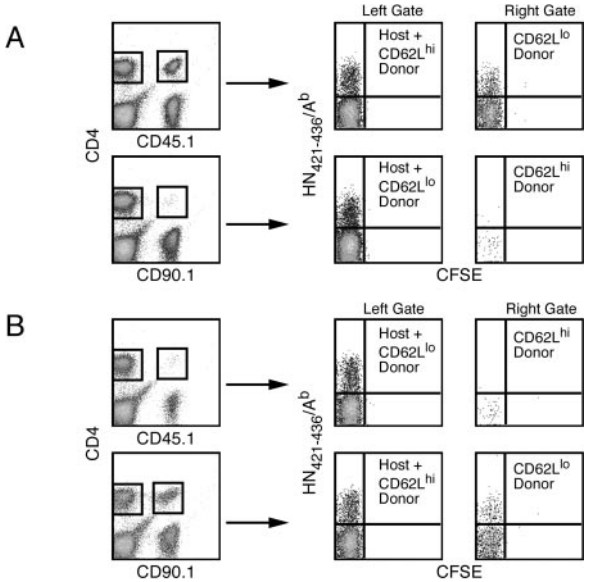

FIGURE 3. Expansion of CD62L^{low} (effector) memory CD4$^+$ T cells. Mixtures of CFSE-labeled CD44$^+$CD62L^{high} and CD44$^+$CD62L^{low} memory T cells were transferred into two groups of C57BL/6 recipient mice (using the same basic protocol illustrated in Fig. 2). Recipient mice were then intranasally infected with Sendai virus 1 day later. Eleven days postinfection, cells were recovered from the lung airways and stained for the expression of CD4 and each of the congenic markers (CD45.1 and CD90.1, *left column*). The boxes indicate gates that identify host or donor CD4 cells, which are then analyzed for multimer ($HN_{419-433}/A^b$) and CFSE expression (*middle* and *right-hand columns*). The data in this figure are derived from experiments 5 and 6 in Table I.

The finding that CD62L^{low} effector memory T cells make substantial contributions to recall responses argues against the idea that the primary function of these cells is to immediately engage the pathogen at the site of infection until the central memory T cell population has expanded and produced new effector cells. Rather, it appears that CD62L^{low} effector memory T cells may be involved in all aspects of the response, including the aggressive generation of new effectors. Given that CD62L^{low} central memory T cells tend to be excluded from the lymph nodes, this suggests that the reactivation of memory T cell responses in the lung does not necessarily depend on local draining lymph nodes. Indeed, we have shown that strong T cell responses can be initiated in the complete absence of encapsulated lymph nodes (19). Although the data presented in this study demonstrate that CD62L^{low} memory T cells in the spleen are able to mediate strong recall responses to respiratory virus infections, the proliferative capacity of effector and central memory from other sites is unclear. However, it should be noted that CD62L^{low} (effector) memory T cells isolated from the lung airways can mediate strong recall responses to secondary challenge (20). In addition, although both effector and central memory T cells expanded vigorously in our studies, we were not able to compare their protective efficacy, because both

populations were present in the same animals. Further studies will be required to understand the functional contributions of central and effector memory T cells to secondary respiratory virus challenge.

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