The route of priming influences the ability of respiratory virus–specific memory CD8+ T cells to be activated by residual antigen

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After respiratory virus infections, memory CD8+ T cells are maintained in the lung airways by a process of continual recruitment. Previous studies have suggested that this process is controlled, at least in the initial weeks after virus clearance, by residual antigen in the lung-draining mediastinal lymph nodes (MLNs). We used mouse models of influenza and parainfluenza virus infection to show that intranasally (i.n.) primed memory CD8+ T cells possess a unique ability to be reactivated by residual antigen in the MLN compared with intraperitoneally (i.p.) primed CD8+ T cells, resulting in the preferential recruitment of i.n.-primed memory CD8+ T cells to the lung airways. Furthermore, we demonstrate that the inability of i.p.-primed memory CD8+ T cells to access residual antigen can be corrected by a subsequent i.n. virus infection. Thus, two independent factors, initial CD8+ T cell priming in the MLN and prolonged presentation of residual antigen in the MLN, are required to maintain large numbers of antigen-specific memory CD8+ T cells in the lung airways.

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Abbreviations used: EID_{50}, 50% egg infectious dose; i.n., intranasal; MLN, mediastinal LN; NP, nucleoprotein; T_{CM}, central memory T cell; T_{EM}, effector memory T cell.

In recent years, there has been considerable progress in understanding the mechanisms regulating the tissue-specific migration of lymphocytes to peripheral sites. An evolving concept is that environmental factors at the site of initial priming induce the expression of tissue-selective homing molecules on activated lymphocytes. In support of this, numerous studies have demonstrated a pivotal role for antigen-presenting cells in the programming of lymphocyte trafficking patterns during priming (Mora et al., 2003; Iwata et al., 2004; Sigmundsdottir et al., 2007). In contrast, several recent studies suggest a pliable property of memory T cells in terms of their tissue tropism. Adoptive transfer and parabiosis studies have shown that the location of initial priming has little impact on the ability of circulating effector memory T cells (T_{EM}s) to migrate to different nonlymphoid sites (Klonowski et al., 2004; Masopust et al., 2004). One explanation for this pleotropic homing ability is that activated CD8+ T cells disseminate from LNs draining the site of infection to distant LNs, where they acquire additional tissue-homing molecules associated with the local microenvironment (Liu et al., 2006). Moreover, the migration of circulating central memory T cells (T_{CM}s) to nonlymphoid tissues also results in functional and phenotypic conversion to tissue-resident T_{EM} phenotype (Laouar et al., 2005, 2007; Kohlmeier et al., 2007; Marzo et al., 2007). Together, these studies demonstrate that the site of initial priming, the continued maturation of activated T cells in nondraining lymphoid tissues, and the local environment within nonlymphoid tissues all contribute the migratory properties of memory CD8+ T cells.

Studies in both humans and mice have shown that substantial numbers of T_{EM} persist in the lung airways after the resolution of respiratory virus infections. The numbers of T_{EM} in the lung airways gradually decline over the first 6 mo after infection and then stabilize as a relatively small population of memory T cells that is maintained in the lung airways indefinitely (Ostler et al., 2001; Hogan et al., 2001a; Wiley et al., 2001; de Bree et al., 2005; van Panhuys et al., 2005). This decline and stabilization in the

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number of memory T cells in the lung airways correlates with a progressive decline in cell-mediated protection from a secondary challenge (Liang et al., 1994; Kündig et al., 1996; Hogan et al., 2001b; Ray et al., 2004; Bachmann et al., 2005a,b). Unlike memory T cell populations that reside in other anatomical locations, lung airway memory T cells are not directly maintained through cytokine-driven homeostatic proliferation within the lung airways. Rather, antigen-specific memory T cells present in the lung airways represent a dynamic population that is maintained by continual recruitment from the systemic memory T cell pool under steady-state conditions (Ely et al., 2006). The accumulation of memory T cells in the airways under steady-state conditions is determined by migration from the circulation and cell death within the airways, a process which we refer to as continual recruitment. A recent study has demonstrated that residual antigen is maintained in the local draining LNs for several months after respiratory virus infection, and it has suggested a model in which recent stimulation by residual antigen is required for continual recruitment of memory $CD8^+$ T cells to the airways (Zammit et al., 2006). In addition, we previously demonstrated that systemic memory $CD8^+$ T cells generated after a respiratory virus infection could migrate to the airways in the absence of cognate antigen, albeit at low levels (Kohlmeier et al., 2007). However, it is not known how the route of priming impacts the ability of these antigen-dependent and -independent mechanisms to promote the recruitment of memory $CD8^+$ T cells to the lung airways.

To better understand the mechanisms regulating the continual recruitment of memory $CD8^+$ T cells to the lung airways, we investigated the localization of memory $CD8^+$ T cells that had been elicited by intranasal (i.n.) versus i.p. infection. The data show that i.n.-primed memory $CD8^+$ T cells were preferentially recruited and maintained in the lung airways compared with i.p.-primed $CD8^+$ T cells, and the defective recruitment of i.p.-primed memory $CD8^+$ T cells to the lung airways was not corrected by the presence of cognate residual antigen in the mediastinal LN (MLN). Importantly, the ability of virus-specific memory $CD8^+$ T cells to be activated by residual antigen in the MLN was restricted to i.n.-primed cells, and this activation resulted in multiple phenotypic changes which are associated with lung airway-resident cells. Collectively, the data suggest that not only the prolonged presentation of cognate antigen in the MLN but also T cell priming in the LNs that drain the respiratory tract during the primary response are required for the continual recruitment of memory $CD8^+$ T cells to the lung airways.

RESULTS AND DISCUSSION

Defective recruitment of virus-specific $CD8^+$ T cells in the lung airways after i.p. infection

To investigate the factors that regulate the recruitment of memory $CD8^+$ T cells to the lung airways, we needed an experimental approach that would generate memory T cells at a distal site to the lung. We speculated that an i.p. infection offered a means to generate substantial numbers of effector and memory $CD8^+$ T cells in the absence of a pulmonary infection. To confirm that virus is not transferred to the lung in the course of an i.p. infection, we analyzed the immune response and viral loads after i.p. infection with Sendai virus (Fig. 1 A). Even in the absence of a productive lung infection, i.p. infection elicited a robust Sendai-specific $CD8^+$ T cell response in the spleen and lung parenchyma (Fig. 1 B). Notably, the numbers of Sendai-specific $CD8^+$ T cells in the spleen and lung parenchyma were similar between i.p.- and i.n.-infected mice during the memory stages, indicating no requirement for i.n. virus

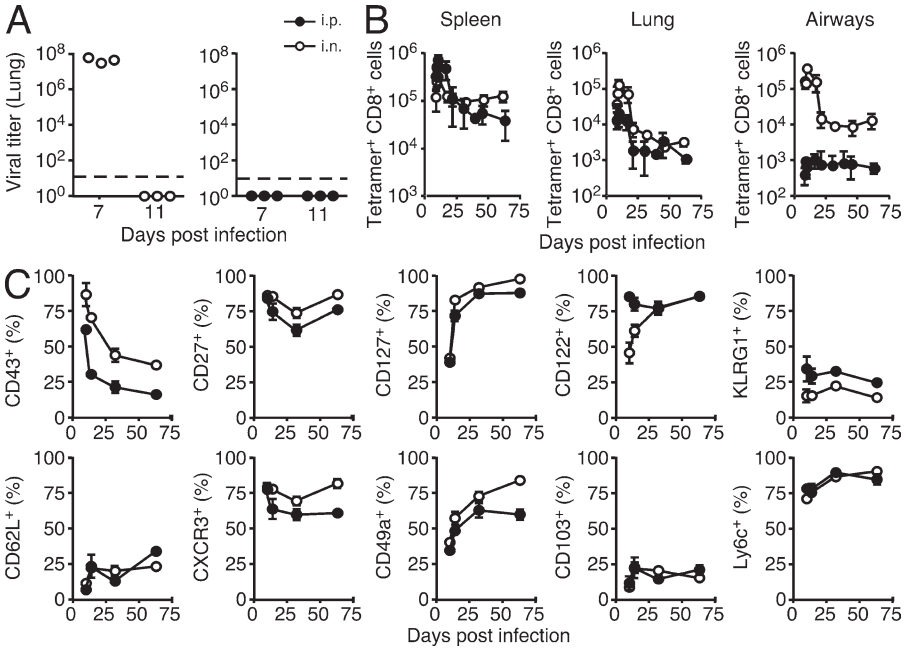

Figure 1. i.p.-primed memory $CD8^+$ T cells display defective recruitment to the lung airways. C57BL/6 mice were infected either i.p. (10^4 50% egg infectious dose [EID_{50}]) or i.n. (250 EID_{50}) with Sendai virus. (A) The virus titers in the lung at days 7 and 11 after infection determined by plaque assay. The dashed line indicates the lower limit of detection. (B) Cells were isolated from the spleen, lung tissues (cells located in the airways are not included), and lung airways at the indicated time points and stained with anti-CD8 and Sendai nucleoprotein $(NP)_{324-332}/K^b$ tetramer (n = 3–10 at each time point). Shown are the actual numbers of $CD8^+$tetramer$^+$ cells in each tissue. (C) C57BL/6 mice were infected either i.p. or i.n. with Sendai virus. Cells were isolated from the spleen at the indicated time points and stained with anti-CD8, the indicated marker-specific mAb, and Sendai $NP_{324-332}/K^b$ tetramer (n = 3–10 at each time point). The percentage of tetramer$^+CD8^+$ cells expressing each marker is indicated. Error bars indicate SD.

infection in the steady-state recruitment of antigen-specific memory CD8$^+$ T cells to the lung parenchyma (Klonowski et al., 2004). The i.p. infection protocol also elicited memory CD8$^+$ T cells that were phenotypically similar to those elicited by i.n. infection (Fig. 1 C).

In contrast to the lung parenchyma, the ability of i.p.- and i.n.-primed memory CD8$^+$ T cells to localize to the lung airways was vastly different. As shown in Fig. 1 B, after i.n. infection a robust peak of recruitment to the lung airways was observed at day 10 after infection, followed by sustained continual recruitment of antigen-specific memory CD8$^+$ T cells at least 2 mo after infection. After i.p. infection, however, very few Sendai-specific CD8$^+$ T cells were present in the lung airways during both the acute and memory phases. This limited recruitment of i.p.-primed cells to the lung airways is not a unique feature of Sendai virus infection or peritoneal infection because an identical migratory pattern was also observed after i.p. infection with influenza virus or after subcutaneous Sendai virus infection and peptide vaccination (unpublished data). Collectively, we conclude that antigen-specific memory CD8$^+$ T cells originally primed via nonpulmonary routes show a severe defect in recruitment to the lung airways under steady-state conditions.

i.p.-primed memory CD8$^+$ T cells are not activated by residual antigen in the MLN

Based on previously published studies, we hypothesized that the defective recruitment of i.p.-primed memory CD8$^+$ T cells to the lung airways was the result of a lack of residual antigen in the lung-draining LNs (Zammit et al., 2006). In support of this hypothesis, Sendai-specific memory CD8$^+$ T cells in the MLN after i.n. infection were mostly CD69$^+$, suggesting recent antigen stimulation, whereas memory CD8$^+$ T cells in the MLN after i.p. infection were mostly CD69$^-$ (Fig. 2 A). In addition, after i.n. infection the majority of CD69$^+$ memory CD8$^+$ T cells in the MLN also expressed high levels of the integrins CD49a and CD103, which are associated with retention in the lung and extravasation through mucosal epithelium, respectively (Ray et al., 2004; Sung et al., 2006). In contrast to the MLN, both the frequency of cells expressing these integrins and the relative intensity of expression were low on i.n.-primed memory CD8$^+$ T cells in the spleen and CD69$^-$ memory CD8$^+$ T cells in the MLN (Fig. 2, A and B, top). In the case of i.p. infection, up-regulation of these integrins was not observed on antigen-specific cells regardless of CD69 expression (Fig. 2, A and B, bottom). However, residual antigen is present in the MLN of i.p.-primed mice (Fig. S1), suggesting that the limited activation of i.p.-primed memory CD8$^+$ T cells was a consequence of the infection route and not a lack of residual antigen.

The rapid migration of antigen-bearing dendritic cells from the lung to the MLN is a well characterized process after respiratory virus infection

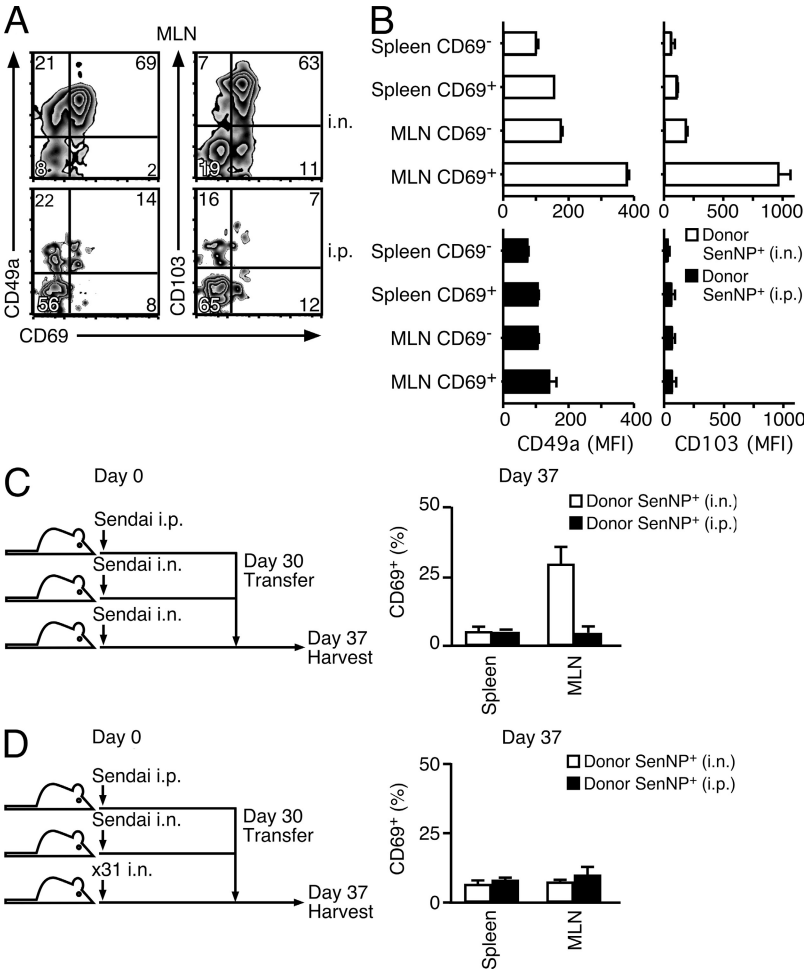

Figure 2. i.p.-primed memory CD8$^+$ T cells are not activated by cognate residual antigen in the lung-draining LNs. (A and B) Mice were infected either i.p. or i.n. with Sendai virus and rested for 30 d. (A) Tetramer$^+$ cells in the MLN of i.n.-primed or i.p.-primed mice were analyzed for the expression of CD69, CD49a, and CD103 on day 30 after infection. (B) Mean fluorescence intensity (MFI) of CD49a and CD103 on resting (CD69$^-$) or activated (CD69$^+$) cells in the spleen and MLN on i.n.-primed and i.p.-primed tetramer$^+$CD8$^+$ cells. The data are from 10–20 mice (mean ± SD). (C and D) Donor mice (CD45.1$^+$CD90.2$^+$ and CD45.2$^+$CD90.2$^+$) were infected either i.p. or i.n. with Sendai virus and rested for 30 d. After 30 d, CD8$^+$ enriched splenocytes from each donor population were combined such that the number of Sendai $NP_{324-332}/K^{b+}$ cells derived from each donor was equivalent and intravenously transferred into recipient mice (CD45.2$^+$CD90.1$^+$) that had been infected with either Sendai virus (C) or influenza x31 virus (D) 30 d before donor cell transfer. 7 d after transfer, cells were isolated from the MLN and spleen and stained with anti-CD8, congenic marker, and Sendai $NP_{324-332}/K^b$ tetramer. The data show the frequency of CD69$^+$ cells among CD8$^+$tetramer$^+$ cells in each tissue on day 7 after transfer. The parentheses indicate the manner by which the donor cells were originally primed. The data are representative of two independent experiments. Error bars indicate SD.

(Legge and Braciale, 2003; Belz et al., 2004). Although it has been previously shown that the peritoneal cavity can also drain to the MLN, it was possible that the differences observed between i.n.-primed and i.p.-primed memory CD8$^+$ T cells in this tissue were a result of the differential localization or altered activation status of APCs within the LN (Marco et al., 1992). Thus, we used a dual transfer approach to directly compare the ability of i.n.-primed and i.p.-primed memory CD8$^+$ T cells to be reactivated by residual antigen under identical conditions in vivo. Surprisingly, after transfer into the same congenic host only i.n.-primed memory CD8$^+$ T cells were activated by residual antigen, as measured by expression of CD69 (Fig. 2 C). Importantly, this activation required cognate antigen stimulation, as neither i.n.-primed nor i.p.-primed Sendai-specific cells expressed CD69 after transfer into mice that had been primed with influenza x31 (Fig. 2 D).

To determine whether the inability of i.p.-primed cells to recognize residual antigen was a consequence of differential T cell localization within the MLN, we used a TCR transgenic system to visualize antigen-specific i.n.- and i.p.-primed cells in the same host (Fig. 3 A). As shown in Fig. 3 B, both i.n.-primed (CD90.1$^+$, top) and i.p.-primed (CD45.1$^+$, bottom) OT-I cells localized to the T cell– and dendritic cell–rich areas in the MLN. Furthermore, consistent with our previous observations, i.p.-primed OT-I cells in the MLN failed to express CD69, CD49a, and CD103 (Fig. S2). Therefore, despite being localized to similar areas of the LN, i.n.- and i.p.-primed cells have a differential ability to be reactivated in the MLN. Collectively with Fig. 2, these data demonstrate that the route of initial priming dictates the ability of memory CD8$^+$ T cells to recognize residual antigen.

Cognate antigen is insufficient to restore steady-state recruitment of i.p.-primed memory CD8$^+$ T cells to the lung airways

The finding that i.p.-primed memory CD8$^+$ T cells are not activated by residual antigen in the MLN suggested that even if cognate antigen were present, i.p.-primed cells would exhibit less steady-state recruitment to the lung airways than i.n.-primed cells. To test this hypothesis, we performed dual adoptive transfer studies to directly compare i.n.- and i.p.-primed memory CD8$^+$ T cells under identical conditions in the same host. At 1 wk after transfer, the relative recruitment of i.n.-primed cells to the airways was much higher than that of i.p.-primed cells (~60 fold; Fig. 4 A and Fig. S3). In contrast, the relative recruitment to the MLN, lung parenchyma, and pleural cavity was similar between i.n.- and i.p.-primed cells, indicating that the inability of i.p.-primed cells to be

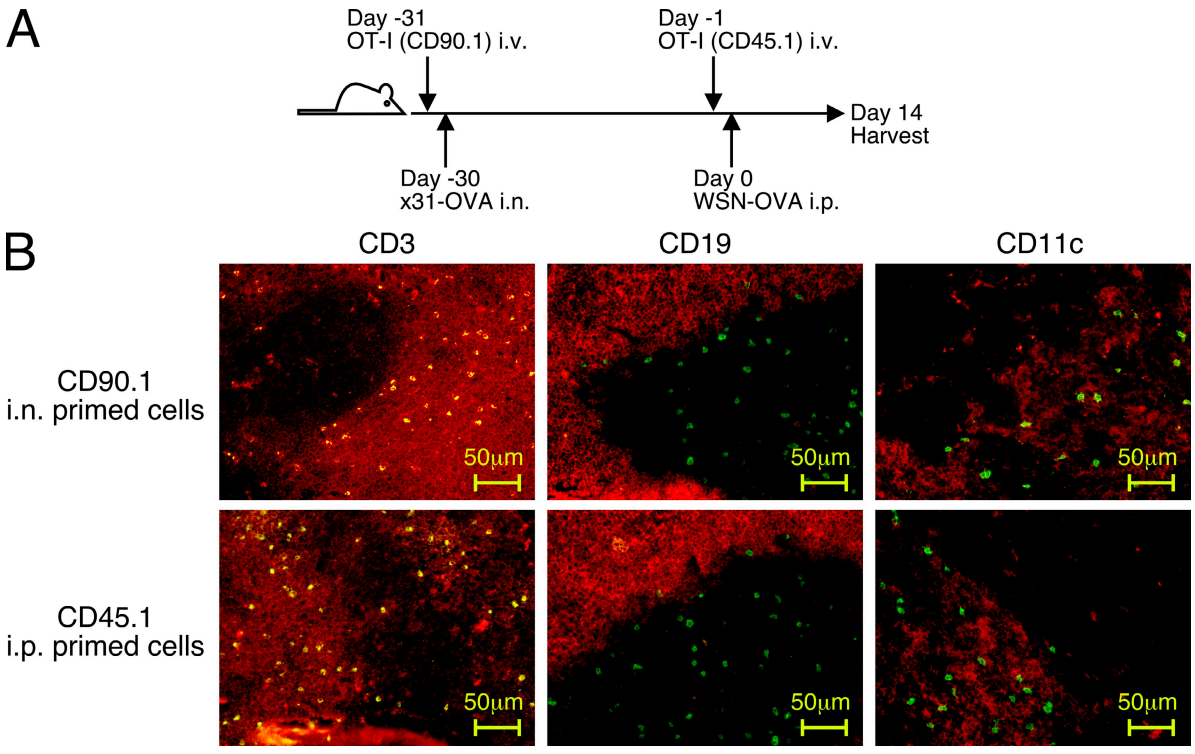

Figure 3. Similar distribution of i.p.- and i.n.-primed antigen-specific CD8$^+$ T cells within the MLN. C57BL/6 mice were infected i.n. with x31-OVA$_I$ and, 30 d later, were infected i.p. with WSN-OVA$_I$. To distinguish i.p.- and i.n.-primed CD8$^+$ T cells, CD90.1$^+$ or CD45.1$^+$ naive OT-1 cells were transferred into recipient mice 1 d before each infection, respectively. MLNs were harvested, embedded in OCT, and frozen at day 14 after i.p. challenge. (A) Frozen sections were stained with each congenic marker to visualize i.p.- or i.n.-primed antigen-specific CD8$^+$ T cells (green) and with antibodies to CD3, CD11c, or CD19 (red) to visualize total T cells, dendritic cells/macrophages, or B cells, respectively. Images are representative of three mice analyzed in two independent experiments (B).

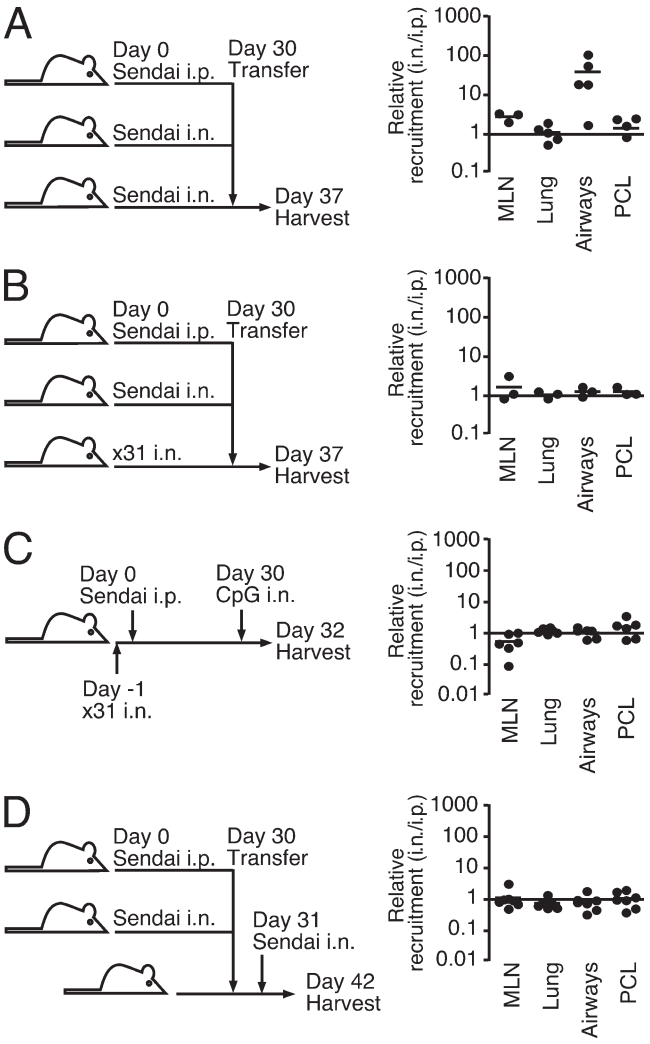

Figure 4. Site of initial priming is essential for antigen-dependent recruitment of memory CD8⁺ T cells to the airways under steady-state conditions. (A and B) Donor mice (CD45.1⁺CD90.2⁺ and CD45.2⁺CD90.2⁺) were infected either i.p. or i.n. with Sendai virus, respectively, and splenocytes were harvested and combined as described in Fig. 2. Recipient mice were infected with either Sendai virus (A) or influenza x31 virus (B) 30 d before donor cell transfer. 7 d after transfer, cells were isolated from the BAL, lung parenchyma, pleural cavity, MLN, and spleen and Sendai-specific donor CD8⁺ T cells were detected by flow cytometry. The data are shown as the relative recruitment to the indicated tissue comparing i.n.-primed donor cells versus i.p.-primed donor cells, and each symbol represents an individual mouse. (C) C57BL/6 mice were infected i.n. with influenza x31 1 d before i.p. infection with Sendai virus. After 30 d, mice were administered CpG i.n., and the relative recruitment of i.n.- and i.p.-primed cells to the indicated tissues was determined by staining with influenza- and Sendai-specific tetramers, respectively. (D) Donor memory T cells from i.n.- and i.p.-primed mice were prepared as described in A and B and injected into naive recipient mice. 1 d later, the recipient mice were challenged with Sendai virus and the relative recruitment of effector T cells from the donor populations was assessed at day 11 after infection. The data are representative of two independent experiments. Horizontal bars represent the mean.

reactivated by residual antigen impacts their ability to migrate specifically to the lung airways. Furthermore, the enhanced recruitment of i.n.-primed cells to the airways was dependent on antigen restimulation, as the relative recruitment between i.n.- and i.p.-primed cells was equivalent when transferred into mice primed with an irrelevant virus (Fig. 4 B). To investigate whether the enhanced recruitment to the airways was limited to steady-state conditions, we investigated the recruitment of i.n.- and i.p.-primed cells to the airways in response to localized inflammation. After administering CpG to the airways, both i.p.- and i.n.-primed memory CD8⁺ T cells were rapidly recruited to the airways with equal efficiency (Fig. 4 C). In addition, secondary effector CD8⁺ T cells generated from an equal number of i.n.- and i.p.-primed memory T cell precursors after transfer into a naive host were recruited

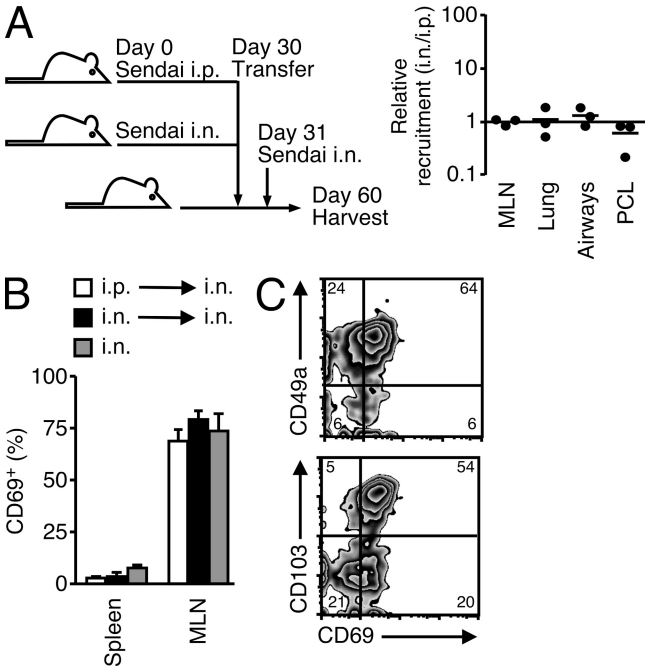

Figure 5. Memory CD8⁺ T cells initially primed i.p. acquire the characteristics of i.n.-primed cells after a secondary i.n. challenge. Donor mice (CD45.1⁺CD90.2⁺ and CD45.2⁺CD90.2⁺) were infected either i.p. or i.n. with Sendai virus, respectively. 30 d later, splenocytes were enriched for CD8⁺ cells using negative selection columns and total memory cells (CD44^{hi}) were purified by cell sorting. Cells were combined such that the number of Sendai $NP_{324-332}/K^{b+}$ cells derived from each donor was equivalent and intravenously transferred into recipient mice (CD45.2⁺CD90.1⁺). 1 d after transfer, the recipient mice were i.n. challenged with Sendai virus and analyzed 1 mo later. (A) Relative recruitment of i.n.-primed donor CD8⁺ T cells versus i.p.-primed donor CD8⁺ T cells at day 29 after challenge. Horizontal bars represent the mean. (B) Frequency of CD69⁺ cells among CD8⁺tetramer⁺ cells in the spleen and MLN for secondary memory T cells from each donor population and host primary memory T cells. Error bars indicate SD. (C) i.p.-primed CD8⁺tetramer⁺ donor cells in the MLN after secondary i.n. challenge with Sendai virus. The numbers indicate the percentage of cells in each quadrant gated on tetramer⁺ CD8⁺ cells. Samples were pooled from five mice. The data are representative of two independent experiments.

to the airways with equal efficiency at the peak of the secondary response (Fig. 4 D). Therefore, the inability of i.p.-primed memory CD8$^+$ T cells to be reactivated by residual antigen resulted in a dramatic impairment of their recruitment to the lung airways, but this impairment was manifest only during steady-state conditions.

Secondary i.n. challenge reprograms i.p.-primed memory CD8$^+$ T cells

Although the previous data have shown that the inability of i.p.-primed memory CD8$^+$ T cells to be efficiently recruited to the lung airways is a result of their inability to be reactivated by residual antigen in the MLN, and that a subsequent i.n. challenge could confer airway homing to secondary effector T cells generated from i.p.-primed precursors (Fig. 4 D), it was unclear whether this homing was maintained into the memory stage. To determine whether the defective homing of i.p.-primed memory T cells under steady-state conditions could be corrected by a subsequent i.n. infection, we used a dual adoptive transfer approach to compare the migratory efficacy of secondary memory T cells that had been generated from primary i.n.- and i.p.-primed memory CD8$^+$ T cells after secondary challenge. As shown in Fig. 5 A, the relative recruitment of secondary memory CD8$^+$ T cells to the airways 30 d after challenge was equal regardless of initial priming. In support of these data, cells initially primed i.p. were now able to be reactivated by residual antigen to a similar degree as i.n.-primed cells. As shown in Fig. 5 B, secondary memory CD8$^+$ T cells generated from i.p.- and i.n.-primed precursors (open bars and black bars, respectively) both expressed high levels of CD69 in the MLN, and this expression was comparable to primary host memory CD8$^+$ T cells (gray bars). In addition, the ability of secondary memory CD8$^+$ T cells initially primed i.p. to now recognize residual antigen correlated with increased expression of CD49a and CD103 (Fig. 5 C). Notably, the route of challenge is also important for the enhanced airway recruitment of secondary memory CD8$^+$ T cells, as an i.p. challenge resulted in minimal recruitment of secondary memory cells generated from both i.n.- and i.p.-primed precursors (Fig. S4). Thus, the inability of i.p.-primed memory CD8$^+$ to recognize residual antigen can be corrected by a subsequent i.n. virus challenge, and this correction fully restores the steady-state recruitment of memory CD8$^+$ T cells to the lung airways.

There is compelling evidence that memory T cells resident in peripheral tissues play a key role in mediating T cell–dependent protective immunity against microbial pathogens (Hansen et al., 2009; Woodland and Kohlmeier, 2009). Previous studies demonstrated that memory CD8$^+$ T cell numbers in the lung airways undergo a gradual decline in the first several months after a respiratory virus infection before finally stabilizing at a relatively low number that is maintained for the life of the host. Several related avenues of investigation have shown that both antigen-dependent and -independent processes can contribute to memory T cell recruitment to the airways, leading to the hypothesis that the gradual decline of airway-resident memory T cells is a consequence of residual antigen clearance (Kohlmeier and Woodland, 2009). Although the current study supports this hypothesis, we have extended these findings by showing that residual antigen by itself is insufficient. Rather, we provide conclusive evidence that the enhanced recruitment of memory CD8$^+$ T cells to the lung airways in the initial months after infection is mediated by two separate events: initial priming in the lung-draining LNs and recognition of residual antigen.

An important question raised by these studies is why i.p.-primed memory CD8$^+$ T cells fail to be activated by cognate residual antigen. First, this failure is not the result of an altered distribution of i.p.-primed memory CD8$^+$ T cells in the MLN (Fig. 3). Second, although it was possible that i.p. infection resulted in the generation of low-affinity memory CD8$^+$ T cells, this is also an unlikely explanation because we observed the same differences between i.p.- and i.n.-primed TCR transgenic memory CD8$^+$ T cells (Fig. S2 and not depicted). Third, there is no evidence that i.n.-primed memory CD8$^+$ T cells prevent the access of i.p. memory CD8$^+$ T cells to APCs because transient depletion of i.n.-primed memory CD8$^+$ T cells failed to enhance access of i.p.-primed memory CD8$^+$ T cells to residual antigen (Fig. S5). Nevertheless, despite all these similarities, only i.n.-primed memory CD8$^+$ T cells were selectively activated by residual antigen in the MLN.

Overall, the current findings show that initial priming of CD8$^+$ T cells in the lung-draining LNs during a respiratory infection enables these cells to access the secondary signals necessary for optimal recruitment to the lung airways under steady-state conditions. The requirement of both factors for the recruitment of memory CD8$^+$ T cells to the lung airways is unique to this site, suggesting that a specific imprinting program occurs after a respiratory virus infection. Clearly, these findings have to be considered for the design of vaccines intended to generate memory CD8$^+$ T cells capable of providing effective surveillance for respiratory pathogens.

MATERIALS AND METHODS

Viruses, mice, and infections. Sendai virus (Enders strain) and influenza virus A/HK-x31 (x31) were grown, stored, and titered as previously described (Hikono et al., 2007). The recombinant influenza virus A/WSN/33 (WSN)-OVA$_I$ and x31-OVA$_I$ containing the OVA$_{257–264}$ (SIINFEKL) peptide in the neuraminidase stalk were provided by R. Dutton (Trudeau Institute, Saranac Lake, NY). Female C57BL/6J, B6.PL-Thy1a/Cy (CD90.1$^+$), and B6.SJL-Ptprca Pep3/BoyJ (CD45.1$^+$) mice were purchased from The Jackson Laboratory. OT-I transgenic mice (CD45.1$^+$ and CD90.1$^+$ background) were provided by R. Dutton. Animals were housed under specific pathogen-free conditions. Mice (6–8 wk) were infected i.p. (10^4 EID$_{50}$ for Sendai, 1,333 PFU for WSN-OVA$_I$, and 5,667 PFU for x31-OVA$_I$) or i.n. (250 EID$_{50}$ for Sendai and 300 EID$_{50}$ for x31) with each virus in a variety of combinations as indicated. For i.n. infection, mice were anesthetized by i.p. injection of 2,2,2-tribromoethanol. Additionally, some groups of mice were challenged i.n. with 1 μg CpG ODN 1826 (InvivoGen). All animal studies were approved by the Trudeau Institute Animal Care and Use Committee.

Adoptive transfer model. For the dual adoptive transfer approach, congenic donor mice were either infected i.p. or i.n. with Sendai virus. 30 d later, CD8-enriched splenocytes were combined such that the number of Sendai NP$_{324-332}$/K^b–specific CD8$^+$ T cells in each donor population was equal (7×10^5 Sendai-specific cells from each donor). The combined cells were transferred

intravenously into recipient mice that had received x31 virus or Sendai virus by i.n. infection 30 d before transfer. Alternatively, if the recipient mice were to receive a challenge infection, a mixture of 5×10^3 Sendai-specific cells from each donor was transferred intravenously into naive recipient mice. 1 d later, recipient mice were challenged i.n. with Sendai virus.

Tissue harvest and staining. Tissues were harvested and stained, as previously described (Kohlmeier et al., 2008). In brief, single cell suspensions were stained with combinations of APC- or PE-conjugated Sendai $NP_{324-332}/K^b$ tetramer, APC-conjugated influenza $NP_{366-374}/D^b$ tetramer, and APC-conjugated $OVA_{257-264}/K^b$ tetramer. All tetramers were generated by the Trudeau Institute Molecular Biology Core. Tetramer-labeled cells were then stained with antibodies to CD8, CD27, CD43 (1B11), CD49a, CD45.1, CD44, CD62L, CD69, CD90.2, CD103, CD122, CD127, Ly6c, CXCR3, and KLRG1. Purified anti-CD49a mAb was labeled with R-Phycoerythrin (RPE) using the PhycoLink RPE Conjugation kit (ProZyme, Inc.). All anti–mouse mAbs used were purchased from BD, eBioscience, BioLegend, R&D Systems, or SouthernBiotech. Samples were run on FACSCalibur (BD) or CyAn (Dako) flow cytometers, and data were analyzed using FlowJo (Tree Star, Inc.).

Calculation of relative recruitment. The standardized number of tetramer$^+$ cells in each tissue as compared with spleen were calculated as follows: [(the number of tetramer$^+$ cells in each tissue/the number of tetramer$^+$ cells in spleen) × 100]. Then, the relative recruitment of $CD8^+$ T cells from the spleen to each tissue was calculated as follows: (the standardized number of tetramer$^+$ cells derived from the experimental group/the standardized number of tetramer$^+$ cells derived from the control group). Each symbol represents an individual mouse.

Immunofluorescence microscopy. Naive OT-1 cells ($CD90.1^+$) were sorted using a FACSVantage (BD) cell sorter with DIVA enhancement software and transferred i.v. into recipient (C57BL/6) mice. 1 d later, mice were infected i.n. with x31-OVA_I. 30 d after infection, naive OT-1 cells ($CD45.1^+$) sorted as previously described were also transferred i.v. into recipient mice, and mice were then challenged i.p. with WSN-OVA_I. MLNs were harvested from recipient mice, embedded in OCT (Sakura Finetek), and frozen over liquid nitrogen. Frozen sections (6 µm) were fixed in acetone/ethanol, air dried, and stained with combinations of anti-CD45.1-FITC, anti-CD90.1-APC, anti-CD11c-PE, anti-CD3-PE, and anti-CD19-PE. All frozen sections were viewed with a microscope (Axiovert 200M; Carl Zeiss, Inc.) and images were recorded with a digital camera (AxioCam; Carl Zeiss, Inc.).

Viral titers. Homogenized lungs were serially diluted and added to MDCK monolayers. After incubation at 35°C for 48 h, cells were fixed with acetone and stained with a combination of goat anti-parainfluenza mAb and biotin-labeled anti–goat IgG mAb (Abcam). Specific reaction was detected using alkaline phosphatase-conjugated streptavidin (Dako) and nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) as a chromogen. Foci were enumerated microscopically.

Online supplemental material. Fig. S1 shows that residual antigen is present in the MLN after i.p. infection. Fig. S2 shows that i.p.-primed memory $CD8^+$ T cells fail to express CD49a and CD103 in the MLN of i.n.-primed hosts. Fig. S3 shows representative staining and cell number data from the dual transfer experiments shown in Fig. 4. Fig. S4 shows that the route of secondary challenge can reprogram memory $CD8^+$ T cells regardless of the route of initial priming. Fig. S5 shows that depleting i.n.-primed memory $CD8^+$ T cells does not enable i.p.-primed cells to recognize residual antigen. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090283/DC1.

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REFERENCES

Bachmann, M.F., P. Wolint, K. Schwarz, P. Jäger, and A. Oxenius. 2005a. Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J. Immunol.* 175:4686–4696.

Bachmann, M.F., P. Wolint, K. Schwarz, and A. Oxenius. 2005b. Recall proliferation potential of memory CD8+ T cells and antiviral protection. *J. Immunol.* 175:4677–4685.

Belz, G.T., C.M. Smith, L. Kleinert, P. Reading, A. Brooks, K. Shortman, F.R. Carbone, and W.R. Heath. 2004. Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc. Natl. Acad. Sci. USA.* 101:8670–8675. doi:10.1073/pnas.0402644101

de Bree, G.J., E.M. van Leeuwen, T.A. Out, H.M. Jansen, R.E. Jonkers, and R.A. van Lier. 2005. Selective accumulation of differentiated $CD8^+$ T cells specific for respiratory viruses in the human lung. *J. Exp. Med.* 202:1433–1442. doi:10.1084/jem.20051365

Ely, K.H., T. Cookenham, A.D. Roberts, and D.L. Woodland. 2006. Memory T cell populations in the lung airways are maintained by continual recruitment. *J. Immunol.* 176:537–543.

Hansen, S.G., C. Vieville, N. Whizin, L. Coyne-Johnson, D.C. Siess, D.D. Drummond, A.W. Legasse, M.K. Axthelm, K. Oswald, C.M. Trubey, et al. 2009. Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat. Med.* 15:293–299. doi:10.1038/nm.1935

Hikono, H., J.E. Kohlmeier, S. Takamura, S.T. Wittmer, A.D. Roberts, and D.L. Woodland. 2007. Activation phenotype, rather than central– or effector–memory phenotype, predicts the recall efficacy of memory $CD8^+$ T cells. *J. Exp. Med.* 204:1625–1636. doi:10.1084/jem.20070322

Hogan, R.J., E.J. Usherwood, W. Zhong, A.A. Roberts, R.W. Dutton, A.G. Harmsen, and D.L. Woodland. 2001a. Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. *J. Immunol.* 166:1813–1822.

Hogan, R.J., W. Zhong, E.J. Usherwood, T. Cookenham, A.D. Roberts, and D.L. Woodland. 2001b. Protection from respiratory virus infections can be mediated by antigen-specific $CD4^+$ T cells that persist in the lungs. *J. Exp. Med.* 193:981–986. doi:10.1084/jem.193.8.981

Iwata, M., A. Hirakiyama, Y. Eshima, H. Kagechika, C. Kato, and S.Y. Song. 2004. Retinoic acid imprints gut-homing specificity on T cells. *Immunity.* 21:527–538. doi:10.1016/j.immuni.2004.08.011

Klonowski, K.D., K.J. Williams, A.L. Marzo, D.A. Blair, E.G. Lingenheld, and L. Lefrançois. 2004. Dynamics of blood-borne CD8 memory T cell migration in vivo. *Immunity.* 20:551–562. doi:10.1016/S1074-7613(04)00103-7

Kohlmeier, J.E., and D.L. Woodland. 2009. Immunity to respiratory viruses. *Annu. Rev. Immunol.* 27:61–82. doi:10.1146/annurev.immunol.021908.132625

Kohlmeier, J.E., S.C. Miller, and D.L. Woodland. 2007. Cutting edge: Antigen is not required for the activation and maintenance of virus-specific memory CD8+ T cells in the lung airways. *J. Immunol.* 178:4721–4725.

Kohlmeier, J.E., S.C. Miller, J. Smith, B. Lu, C. Gerard, T. Cookenham, A.D. Roberts, and D.L. Woodland. 2008. The chemokine receptor CCR5 plays a key role in the early memory CD8+ T cell response to respiratory virus infections. *Immunity.* 29:101–113. doi:10.1016/j.immuni.2008.05.011

Kündig, T.M., M.F. Bachmann, S. Oehen, U.W. Hoffmann, J.J. Simard, C.P. Kalberer, H. Pircher, P.S. Ohashi, H. Hengartner, and R.M. Zinkernagel. 1996. On the role of antigen in maintaining cytotoxic T-cell memory. *Proc. Natl. Acad. Sci. USA.* 93:9716–9723. doi:10.1073/pnas.93.18.9716

Laouar, A., V. Haridas, D. Vargas, X. Zhinan, D. Chaplin, R.A. van Lier, and N. Manjunath. 2005. CD70+ antigen-presenting cells control the proliferation and differentiation of T cells in the intestinal mucosa. *Nat. Immunol.* 6:698–706. doi:10.1038/ni1212

Laouar, A., M. Manocha, M. Wan, H. Yagita, R.A. van Lier, and N. Manjunath. 2007. Cutting edge: distinct NK receptor profiles are imprinted on CD8 T cells in the mucosa and periphery during the same antigen challenge: role of tissue-specific factors. *J. Immunol.* 178:652–656.

Legge, K.L., and T.J. Braciale. 2003. Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection. *Immunity.* 18:265–277. doi:10.1016/S1074-7613(03)00023-2

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.* 152:1653–1661.

Liu, L., R.C. Fuhlbrigge, K. Karibian, T. Tian, and T.S. Kupper. 2006. Dynamic programming of CD8+ T cell trafficking after live viral immunization. *Immunity.* 25:511–520. doi:10.1016/j.immuni.2006.06.019

Marco, A.J., M. Domingo, J. Ruberte, A. Carretero, V. Briones, and L. Dominguez. 1992. Lymphatic drainage of *Listeria monocytogenes* and Indian ink inoculated in the peritoneal cavity of the mouse. *Lab. Anim.* 26:200–205. doi:10.1258/002367792780740549

Marzo, A.L., H. Yagita, and L. Lefrançois. 2007. Cutting edge: migration to nonlymphoid tissues results in functional conversion of central to effector memory CD8 T cells. *J. Immunol.* 179:36–40.

Masopust, D., V. Vezys, E.J. Usherwood, L.S. Cauley, S. Olson, A.L. Marzo, R.L. Ward, D.L. Woodland, and L. Lefrançois. 2004. Activated primary and memory CD8 T cells migrate to nonlymphoid tissues regardless of site of activation or tissue of origin. *J. Immunol.* 172:4875–4882.

Mora, J.R., M.R. Bono, N. Manjunath, W. Weninger, L.L. Cavanagh, M. Rosemblatt, and U.H. Von Andrian. 2003. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature.* 424:88–93. doi:10.1038/nature01726

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.* 31:2574–2582. doi:10.1002/1521-4141(200109)31:9<2574::AID-IMMU2574>3.0.CO;2-V

Ray, S.J., S.N. Franki, R.H. Pierce, S. Dimitrova, V. Koteliansky, A.G. Sprague, P.C. Doherty, A.R. de Fougerolles, and D.J. Topham. 2004. The collagen binding alpha1beta1 integrin VLA-1 regulates CD8 T cell-mediated immune protection against heterologous influenza infection. *Immunity.* 20:167–179. doi:10.1016/S1074-7613(04)00021-4

Sigmundsdottir, H., J. Pan, G.F. Debes, C. Alt, A. Habtezion, D. Soler, and E.C. Butcher. 2007. DCs metabolize sunlight-induced vitamin D3 to 'program' T cell attraction to the epidermal chemokine CCL27. *Nat. Immunol.* 8:285–293. doi:10.1038/ni1433

Sung, S.S., S.M. Fu, C.E. Rose Jr., F. Gaskin, S.T. Ju, and S.R. Beaty. 2006. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. *J. Immunol.* 176:2161–2172.

van Panhuys, N., R. Perret, M. Prout, F. Ronchese, and G. Le Gros. 2005. Effector lymphoid tissue and its crucial role in protective immunity. *Trends Immunol.* 26:242–247. doi:10.1016/j.it.2005.03.005

Wiley, J.A., R.J. Hogan, D.L. Woodland, and A.G. Harmsen. 2001. Antigen-specific CD8(+) T cells persist in the upper respiratory tract following influenza virus infection. *J. Immunol.* 167:3293–3299.

Woodland, D.L., and J.E. Kohlmeier. 2009. Migration, maintenance and recall of memory T cells in peripheral tissues. *Nat. Rev. Immunol.* 9:153–161. doi:10.1038/nri2496

Zammit, D.J., D.L. Turner, K.D. Klonowski, L. Lefrançois, and L.S. Cauley. 2006. Residual antigen presentation after influenza virus infection affects CD8 T cell activation and migration. *Immunity.* 24:439–449. doi:10.1016/j.immuni.2006.01.015