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The Role of Antigen in the Localization of Naive, Acutely Activated, and Memory CD8$^+$ T Cells to the Lung During Influenza Pneumonia[1]

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The role of Ag in the recruitment and localization of naive, acutely activated, and memory CD8$^+$ T cells to the lung during influenza infection was explored using TCR-transgenic (Tg) mice. Naive, Thy1.2$^+$CD8$^+$ OT-I TCR-Tg cells were primed and recruited to the lung after transfer into congenic Thy1.1$^+$ recipients challenged with a genetically engineered influenza virus (influenza A/WSN/33 (WSN)-OVA$_I$) containing the K^b restricted OVA$_{257–264}$ epitope (siinfekl) in the viral neuraminidase stalk. However, if the transferred animals were infected with a similar influenza virus that expressed an irrelevant K^b epitope (WSN-PEPII), no TCR-Tg T cells were detectable in the lung, although they were easily visible in the lymphoid organs. Conversely, there were substantial numbers of OT-I cells found in the lungs of WSN-PEPII-infected mice when the animals had been previously, or were concurrently, infected with a recombinant vaccinia virus expressing OVA. Similar results were obtained with nontransgenic populations of memory CD8$^+$ T cells reactive to a murine γ-herpesvirus-68 Ag. Interestingly, the primary host response to the immunodominant influenza nucleoprotein epitope was not affected by the presence of memory or recently activated OT-I T cells. Thus, although Ag is required to activate the T cells, the subsequent localization of T cells to the lung during a virus infection is a property of recently activated and memory T cells and is not necessarily driven by Ag in the lung. ***The Journal of Immunology*****, 2001, 167: 6983–6990.**

During respiratory virus infections, CD8$^+$ T cells tend to dominate the lymphocyte component of the virus-induced inflammatory process (1, 2). The impression for many years was that most of these CD8$^+$ T cells were not specific for the particular pathogen but were passively recruited as a consequence of tissue damage and the production of various chemokines/cytokines that promote lymphocyte extravasation from the blood. Much has been learned recently about this latter aspect of inflammatory pathology (3–5). Our understanding of the virus-specific component of these cellular exudates has been greatly enhanced by the availability of tetrameric peptide/MHC class I complexes for the direct staining of Ag-reactive CD8$^+$ T cells (6). It is now obvious that the early experiments greatly underestimated the numbers of virus-specific CD8$^+$ T cells in, for example, a pneumonic lung (7, 8). Depending on the experimental model, 30–90% of the CD8$^+$ T cells recovered from the respiratory tract by bronchoalveolar lavage (BAL)[3] can be shown to be virus specific at the peak of the primary response (8–10).

Early studies comparing active and passive CD8$^+$ T cell recruitment to sites of virus-induced inflammatory pathology depended on cell transfer protocols using immune Thy-1 congenic donors and naive virus-infected recipients (1), and limiting dilution analysis (LDA) to determine the relative prevalence of acutely stimulated vs memory T cells (1, 11, 12). Although primed, donor Thy 1.1$^+$CD8$^+$ T cells were very much the minority population in naive Thy1.2$^+$ recipients (13), the frequencies of acutely stimulated and memory virus-specific T cells determined by LDA were similar between the host and donor T cells (12). Evidence was found for the non-Ag-specific recruitment of CD8 T cells during the infection (11, 12). Two limitations of these earlier studies were the inefficiency of the in vitro assays used to enumerate the Ag-specific cells and the inability to assess the effects on naive T cells with a defined specificity. Thus the experiments may have underestimated the actual numbers of T cells nonspecifically recruited, and the recruitment of naive T cells to the site of infection could not be studied. Both problems were solved by combining a more efficient method to detect the virus-specific T cells (MHC class I tetramers) and naive CD8 T cells with a defined Ag specificity (TCR-transgenic). The present experiments dissect the role of Ag in the localization profiles of naive, acutely activated, and memory Ag-specific CD8$^+$ T cells during respiratory infection with influenza virus.

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[3] Abbreviations used in this paper: BAL, bronchoalveolar lavage; i.n., intranasal(ly); LDA, limiting dilution analysis; NA, neuraminidase; NP, nucleoprotein; B/HK, B/Hong Kong/73; MLN, mediastinal lymph node; EID$_{50}$, the 50% infectious dose endpoint for eggs; γHV-68, γ-herpesvirus-68; Vacc-OVA, recombinant vaccinia virus expressing OVA; Vacc-p56, vaccinia recombinant expressing p56 epitope of γHV-68; VLA, very late Ag; WSN, influenza A/WSN/33.

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Materials and Methods

Mice

C57BL/6J (B6) Thy1.2$^+$ and congenic B6.PL Thy1.1$^+$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). A colony of the OT-I transgenic mouse strain that expresses a TCR specific for the OVA SIINFEKL ($OVA_{257–264}$) peptide presented in the context of H-2K^b (14) was established at St. Jude Children's Research Hospital (Memphis, TN) from breeding pairs provided by Dr. M. Bevan (University of Washington, Seattle, WA). All mice were first exposed to virus at 6–8 wk of age and were otherwise held under specific pathogen-free conditions.

Recombinant influenza viruses

The influenza A/WSN/33 (WSN)-OVA_I and WSN-PEPII influenza viruses containing the $OVA_{257–264}$ (siinfekl) peptide and a peptide PEPII (khylfrnl) derived from the EL4 tumor cell line (15) in the neuraminidase (NA) stalk were generated by reverse genetics (16) using the plasmids pT3WSN-OVA_I and pT3WSNPepII, with the NA gene flanked by an upstream T3 RNA polymerase promoter and a downstream Ksp6321 site. The in vitro transcripts were complexed with purified nucleoprotein (NP) and polymerase proteins to obtain reconstituted NA ribonucleoprotein complexes and transfected into 70–90% confluent Madin-Darby bovine kidney-cells infected 1 h before transfection with the helper virus WSN/HK (H1N2). Eighteen hours after transfection, virus recombinants in the supernatant were assayed for plaque formation on MDBK cells. The inserted sequence was confirmed by sequencing PCR-amplified cDNA. The WSN-p56 virus-expressing and H-2D^b-restricted epitope from the murine γ-herpesvirus-68 (γHV-68) was made in a similar manner and has been described previously (17).

Virus challenge

Mice were anesthetized with avertin (2,2,2-tribromoethanol) before intranasal (i.n.) challenge with 10^5 PFU of WSN-OVA_I or WSN-PEPII in 30 μl of PBS. Both influenza viruses were grown and titered in the Manin Darby canine kidney, MDCK, fibroblast cell line, obtained from the American Type Culture Collection (Manassas, VA). The WSN-p56 virus was grown and titered (by determining the 50% infectious dose endpoint for eggs, EID_{50}) in embryonated hen's eggs and mice were infected i.p. with 5.0×10^5 EID_{50}. Mice were also primed i.p. with 10^6 or 3×10^7 PFU, respectively, of recombinant vaccinia viruses expressing OVA (Vacc-OVA) (18) or the p56 epitope of γHV-68 (Vacc-p56) (17). The Vacc-OVA was supplied by Dr. J. Bennink (National Institute of Allergy and Infecctious Diseases, National Institutes of Health, Bethesda, MD). Mice that were primed and boosted with Vacc-p56 and WSN-p56 (17) were later challenged i.n. with 10^6 EID_{50} of the B/Hong Kong/73 (B/HK) influenza B virus, which does not generate a CD8$^+$ T cell response cross-reactive with that caused by the WSN influenza A virus.

Adoptive transfer experiments

Spleen cells from unprimed Thy1.2$^+$ TCR-transgenic donor females were transferred i.v. to female, unirradiated, B6.PL Thy1.1$^+$ recipients. The number of cells transferred is indicated in each figure. Recipient animals were challenged with virus(es) as indicated within 24–72 h of transfer.

Flow cytometric analysis

Spleen, lymph node, and BAL lymphocyte populations were stained as aliquots of 2×10^5 cells with various combinations of mAbs to CD8α (53-6.72 or CT8a), Thy1.1 (OX-7), Thy1.2 (30-H12), TCR-Vα_2 (B20.1), TCR-Vβ_5 (MR9-4), CD44 (IM7), and anti-CD62L (MEL-14) conjugated to FITC, PE, biotin, allophycocyanin, or PE-cyanine 5. The conjugated mAbs were purchased from BD PharMingen (San Diego, CA) or Caltag Laboratories (Burlingame, CA) and are referenced in their current catalogs. Biotinylated mAbs were developed with streptavidin-Red670 (Life Technologies, Bethesda, MD). Tetrameric complexes of H-2K^b/$OVA_{257–264}$ (K^bOVA_{257}), H-2D^b/influenza $NP_{366–374}$ (D^bNP_{366}), and H-2D^b/p56 epitope of γHV-68 (D^{b}p56) were prepared and used as described previously (8, 19). All cells were analyzed with CellQuest software (BD Biosciences, San Diego, CA), using either a BD Biosciences FACScan in three-color mode or a BD Biosciences FACSCalibur in four-color mode.

Single cell IFN-γ assay

Spleen, mediastinal lymph node (MLN), and macrophage-depleted BAL populations were cultured for 6 h in 96-well round-bottom plates at 5×10^5 to 8×10^5 cells per well in complete medium containing 10 μg/ml brefeldin A (Epicenter Technologies, Madison, WI), with or without 10 μM of the OVA (SIINFEKL) or influenza NP (ASNENMETM) peptides. After culture, the cells were placed on ice, washed in PBS/brefeldin A (10 μg/ml), stained with a mixture of anti-Thy1.2-FITC and anti-CD8α-tricolor, washed again, fixed with 1% formaldehyde, permeabilized in 0.5% saponin (Sigma-Aldrich, St. Louis, MO), and stained with anti-IFN-γ-PE (BD PharMingen) for 30 min on ice. The lymphocytes were then washed and analyzed on the FACScan in three-color mode with CellQuest software. The data were viewed as two-parameter plots of Thy1 vs IFN-γ staining using a combination lymphocyte and CD8$^+$ gate.

Results

Primary recruitment of OT-I CD8$^+$ T cells by the recombinant influenza viruses

Traditionally, it has been difficult to study the role of Ag in the recruitment of naive T cells. The limitation of conventional systems is that the prevalence of naive virus-specific CD8$^+$ T cells is too low for an accurate experimental analysis. To solve the problem of the low frequency of naive virus-specific T cells, we used TCR-transgenic T cells with a defined specificity. Adoptive transfer of OVA peptide ($OVA_{257–264}$)-specific, V$\alpha_2$$^+V\beta_5$$^+$ (Fig. 1*A*), naive (Fig. 1*B*), OT-I Thy1.2$^+$CD8$^+$ T cells (Fig. 1, *C* and *D*) into congenic Thy1.1$^+$ hosts resulted in a detectable population in the lymph nodes and spleen of even unimmunized mice (Fig. 1, *C* and *D*, and Fig. 2, *A* and *B*).

To examine recruitment of the naive OT-I T cells to the lung during influenza pneumonia, the recipient mice were infected with one of two otherwise identical WSN (H1N1) recombinant influenza viruses. WSN-OVA_I was engineered to express the K^b-restricted, OVA-derived peptide $OVA_{257–264}$, for which the OT-I T

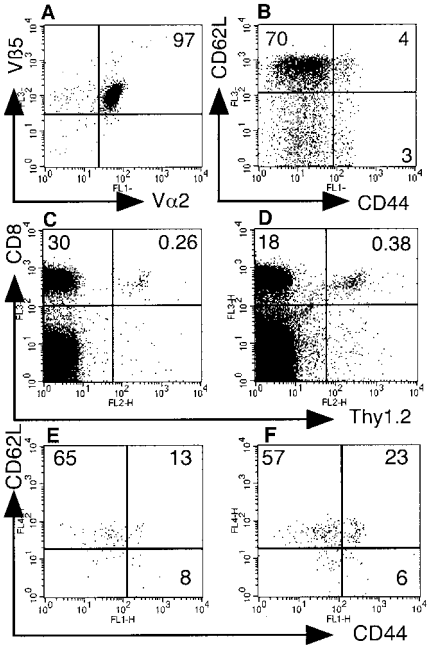

FIGURE 1. Adoptively transferred OT-I transgenic CD8$^+$ T cells persist in the recipients. *A*, Before transfer, pooled CD8$^+$ spleen cells from donor OT-I transgenic mice were analyzed for the expression of the transgenic Vα_2Vβ_5 TCR subsequent to staining with anti-CD8-PE, anti-Vα_2-FITC and anti-Vβ_5-biotin. The donor CD8$^+$Thy1.2$^+$ OT-I cells were also analyzed for the activation molecules CD44 and CD62L before transfer (*B*) subsequent to staining with anti-CD44-FITC and anti-CD62L-biotin. In the uninfected recipient Thy1.1$^+$ animals, following staining with anti-CD8-tricolor and anti-Thy1.2-PE, the Thy1.2$^+$ transgenic cells were easily detectable in the MLN (*C*) and spleen (*D*) 10 days after transfer of 1×10^7 OT-I transgenic spleen cells. The activation profiles of the detectable CD8$^+$Thy1.2$^+$ cells in the MLN (*E*) and spleen (*F*) were determined by anti-CD44-FITC and anti-CD62L-APC staining. The numbers in the quadrants indicate the percentage of positive cells in that region.

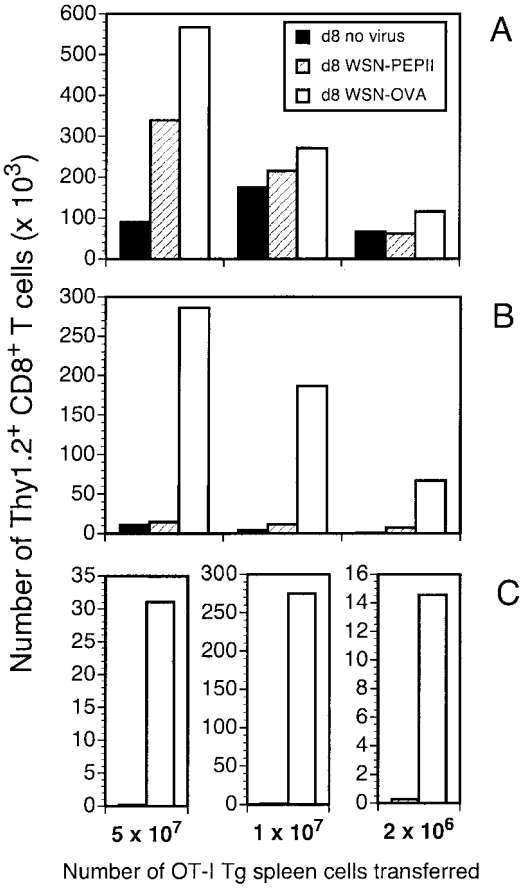

FIGURE 2. Naive T cells are not recruited to the lung during primary influenza infection. Two days after transfer of 5×10^7, 1×10^7, or 2×10^6 Thy1.2$^+$ OT-I spleen cells, Thy1.1$^+$ recipient animals were infected i.n. with 1000 PFU of WSN-OVA$_I$ or WSN-PEPII. Eight days after infection, a pool of five animals per group was sampled and analyzed for the prevalence of transgenic T cells by staining with anti-Thy1.2-FITC, anti-CD8-tricolor, and K^b/siinfekl tetramer-PE and anti-CD8-tricolor. The plots were made using a lymphocyte gate. The data are representative of repeat experiments. The total number of Thy1.2$^+$CD8$^+$ T cells detectable in the spleen (*A*), MLN (*B*), and BAL (*C*) of the mice was determined using the cell counts and percentages of CD8/Thy1.2 double positive cells. Filled bars, no virus; hatched bars, WSN-PEPII; open bars, WSN-OVA$_I$.

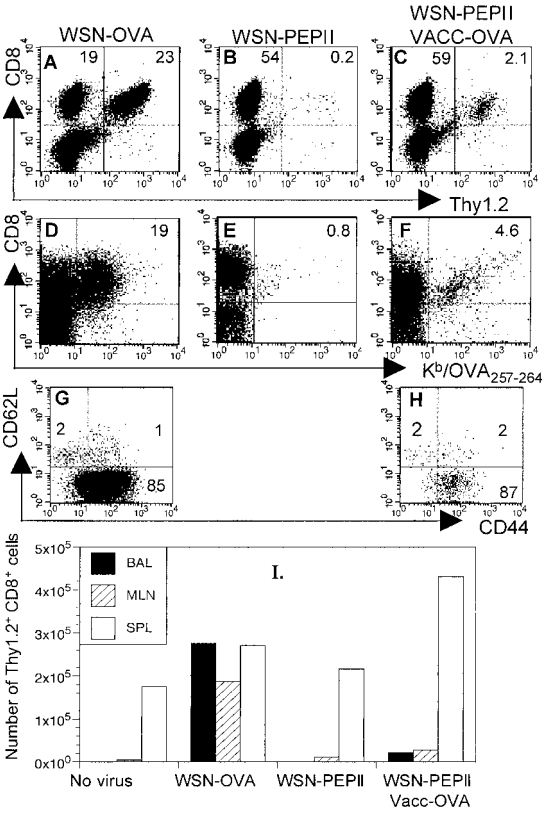

FIGURE 3. Detection of OT-I T cells in the BAL during primary influenza infection. As in Fig. 2, OT-I spleen cells were transferred into Thy1.1$^+$ hosts and the hosts were infected with WSN-OVA$_I$ or WSN-PEPII i.n. A third group of animals was coinfected with 10^6 PFU of Vacc-OVA i.p. The OVA-specific, Thy1.2$^+$ OT-I T cells infiltrating the lung are easily detected in the BAL 8 days after infection using either the Thy1.2 marker (*A–C*) or K^b/siinfekl tetramer (*D–F*). Less than 1% OVA-specific naive T cells are recruited to the lungs of the WSN-PEPII-infected mice, while activation of the OVA-specific T cells with Vacc-OVA in the WSN-PEPII-infected mice results in some non-Ag-specific recruitment to the lung. The OVA-specific cells in the BAL have an activated phenotype revealed by staining with CD44- and CD62L-specific Abs (*G–H*). *I*, The numbers of OT-I Thy1.2$^+$CD8$^+$ T cells in the BAL, MLN, and spleen were calculated from the FACS percentages and the cell counts for each organ. The data presented depict pooled organs from five mice and are representative of repeat experiments.

cells are specific, while WSN-PEPII expresses an unrelated K^b-restricted peptide derived from the EL4 tumor cell line (15). At the peak of the T cell response to influenza A virus (day 8) (1, 8, 12), the MLN, spleen, and BAL were sampled and stained with Ab to Thy1.2 to reveal the presence of the transgenic cells. In mice infected with the WSN-OVA$_I$ flu virus, there were substantially increased numbers of OT-I T cells in the spleen (Fig. 2*A*) and MLN (Fig. 2*B*), compared with uninfected mice. Also, WSN-OVA$_I$ infection resulted in a strong recruitment of the OT-I T cells to the lung (Fig. 2*C*) where up to 25% of the infiltrating CD8$^+$ T cells were Thy1.2$^+$ (Fig. 3*A*). In contrast, infection of the mice with the WSN-PEPII virus caused little change in the prevalence of the transgenic cells in the MLN and spleen (Fig. 2, *A* and *B*) and little or no evidence of the cells in the lung (Figs. 2*C* and 3*B*). Similar numbers were returned by K^{b}OVA$_{257}$ tetramer staining (Fig. 3, *D* and *E*), confirming the specificity of the Thy1.2 staining. Little of the OVA-specific response was generated by the host Thy1.1$^+$ T cells in the WSN-OVA$_I$-infected mice. This was supported by OVA$_{257–264}$-specific intracellular IFN-γ secretion, with a distribution of OVA-responsive cells similar to the Thy1.2$^+$ and tetramer$^+$ fractions (data not shown). Although these data suggest that T cell activation via Ag is a necessary prerequisite for trafficking of CD8$^+$ T cells to the infected lung, it was still not clear whether Ag is required in the tissue itself.

Recruitment of acutely activated CD8$^+$ T cells

To address the issue of whether Ag was required at the site of inflammation (in this case the lung) to draw the T cells in, or just to activate the T cells in the periphery, the following experiments were conducted. OT-I spleen cells were adoptively transferred into Thy1.1$^+$ congenic hosts as above, followed 24 h later by simultaneous challenge with the irrelevant WSN-PEPII influenza i.n. and Vacc-OVA i.p. Some animals received WSN-OVA$_I$ or WSN-PEPII alone i.n. The i.p. injection of vaccinia has previously been shown to produce infection in the peritoneum and in the ovaries of female mice (20). The animals coinfected with Vacc-OVA and the WSN-PEPII (Fig. 3, *B* and *E*) had a significant (3%) infiltration of Thy1.2$^+$K^b/OVA$^+$ OT-I T cells in the BAL compared with animals infected with WSN-PEPII alone (Fig. 3, *C*, *F*, and *I*). These

cells in the BAL had a profile characteristic of activated T cells (CD44^{high}CD62L^{low}) (Fig. 3, *G* and *H*). Thus the distal activation of CD8$^+$ T cells by Ag rapidly conveys the ability to enter the inflamed lung and does not require Ag at the site for the cells to get in. This suggests that the nonspecific recruitment of T cells into the pneumonic lung in the absence of Ag is a consequence of T cell activation.

Passive localization of CD8$^+$ memory T cells to the pneumonic lung

The question remained as to whether the nonspecific localization of T cells in the lung was a phenotype restricted to recently activated T cells, or if resting memory cells could also be found in the inflamed lung during an unrelated infection. A pool of naive Thy1.2$^+$ spleen cells from the transgenic mice were adoptively transferred (5×10^6 per mouse) into unirradiated congenic Thy1.1$^+$ B6 recipients. To establish a resting memory T cell population, the recipient mice were inoculated i.p. with 10^6 PFU of Vacc-OVA within 72 h of transfer. The animals were then rested for 6 wk to allow recovery from the vaccinia infection and to establish a pool of quiescent memory T cells. At the end of the 6-wk rest period, the animals were challenged with 10^3 PFU of either the WSN-PEPII or the WSN-OVA$_I$ i.n. As controls, some animals did not receive any influenza virus. The animals were subsequently sampled at 5 and 11 days after the flu inoculation for the presence and activation status of T cells with the transgenic phenotype (Fig. 4). Animals infected with the WSN-PEPII virus exhibited little change in the proportion (Fig. 4) or number (Fig. 5) of Thy1.2$^+$ OT-I T cells in the MLN or spleen, suggesting that little clonal expansion had occurred. In contrast, there were substantial increases in the number of OT-I T cells in mice receiving WSN-OVA$_I$ (Fig. 5). Despite the fact that the proportion of transgenic T cells did not appear to change with the WSN-PEPII infection, there was evidence of nonspecific recruitment of the OVA-specific cells to the lung at 5 days after infection, which subsequently subsided by day 11 (Figs. 4, 5*A*, and 6). The transient nature of this nonspecific recruitment was evident in the K^b/siinfekl tetramer staining as well as the intracellular IFN-γ response to the siinfekl peptide (Fig. 6), further indicating that these cells were OVA specific and responsive. For example, the IFN-γ response to the OVA$_{257–264}$ peptide in the WSN-PEPII-infected mice was higher at day 5 (4.5%) than at day 11 (<1%). Conversely, the OVA-specific IFN-γ response increased from 14 to 28% between days 5 and 11 in the WSN-OVA$_I$-infected mice (Fig. 6). As a positive control, the specific recruitment of the transgenic T cells by the WSN-OVA$_I$ virus infection was substantial and exhibited the early

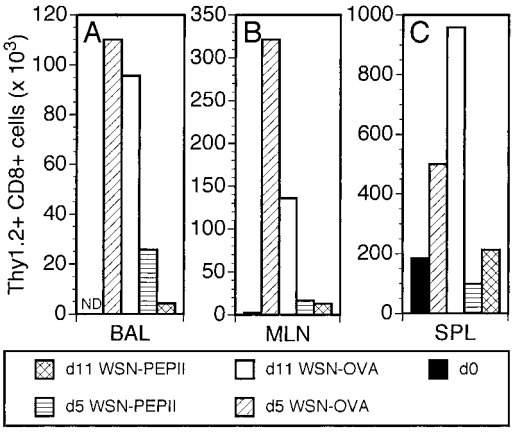

FIGURE 5. Recruitment and expansion of OT-I cells during secondary challenge with WSN-OVA$_I$ and WSN-PEPII influenza viruses. The total number of Thy1.2$^+$CD8$^+$ transgenic T cells present before (d0) and after (d5 and d11) influenza challenge was calculated from the percentages obtained by flow cytometry (Fig. 4) and the number of lymphocytes per organ, per mouse, using pooled organs from five mice. *A*, BAL. *B*, MLN. *C*, Spleen.

kinetics that are a hallmark of the recall response. Thus, without the Ag in the tissue, memory CD8$^+$ T cells are present in the lung early in the infection, as are recently activated cells. However, these early T cell immigrants are not maintained in the lung unless they encounter their specific ligand.

Passive recruitment of nontransgenic T cells

To be sure that the results with the OT-I T cells were not an artifact of the transgenic nature of the cells, the experiment was repeated in a nontransgenic system. Memory T cells specific for the D^{b}p56 epitope of the γHV-68 virus were generated by priming B6 mice i.p. with Vacc-p56. A second pool of traceable memory CD8 T cells specific for the D^{b}NP$_{366}$ epitope of the influenza A virus were generated by nonrespiratory i.p. challenge 1 mo later with recombinant influenza A/WSN-p56, also further stimulating the p56-specific T cells. These mice were subsequently infected i.n. with the unrelated B/HK influenza B virus after a further 7-wk interval, and the spleen, MLN, and BAL populations were analyzed. Data were gathered for the total CD8$^+$ T cell counts (Fig. 7, *A–C*) as well as the prevalence of γHV-68-p56-specific and influenza A virus NP-specific CD8$^+$ T cells using the D^{b}p56 and D^{b}NP$_{366}$ tetramers, respectively (Fig. 7, *D–F*).

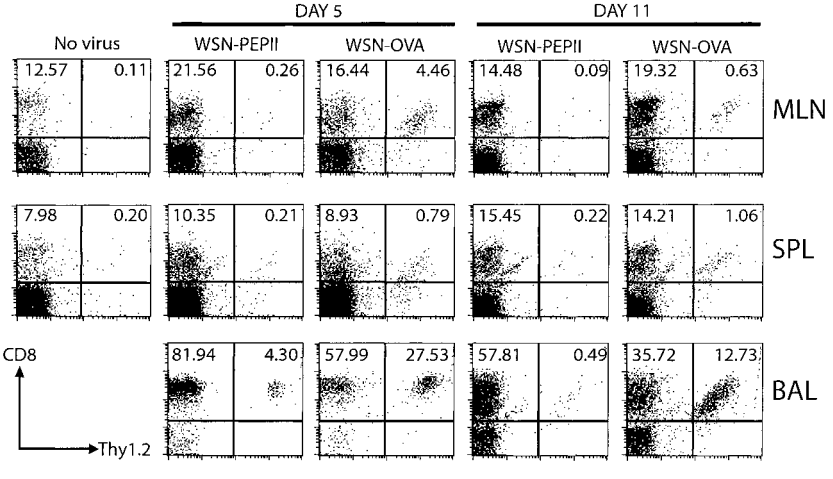

FIGURE 4. Memory CD8 T cells are passively recruited to the lung early in the influenza infection. Naive OT-I transgenic spleen cells (5×10^6) were transferred i.v. into Thy1.1$^+$ recipient mice. Seventy-two hours after transfer, the mice were inoculated with 10^6 PFU of Vacc-OVA i.p then rested for 6 wk to prime the OVA-specific T cells in vivo and create a resting memory population. At the end of the rest period the animals were infected i.n. with WSN-OVA$_I$ or WSN-PEPII, while some did not receive any i.n. infection (No virus). At 5 and 11 days after the influenza infection, the animals were sampled and analyzed for the presence of CD8$^+$Thy1.2$^+$ T cells in the MLN, spleen, and BAL. The percentages of CD8$^+$ and CD8$^+$Thy1.2$^+$ cells are indicated by the numbers in the quadrants.

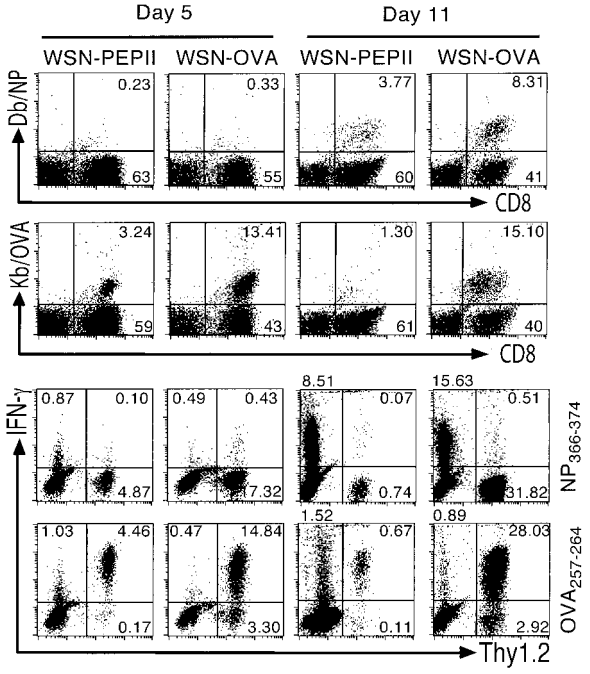

FIGURE 6. Analysis of Ag specificity and responsiveness of $CD8^+$ T cells from the BAL of Vacc-OVA-primed, influenza-infected mice by MHC tetramer and stimulation of intracellular IFN-γ. The BAL of Vacc-OVA-primed, influenza-infected mice was analyzed for the presence of influenza $NP_{366–374}$-specific and $OVA_{257–264}$ (siinfekl)-specific $CD8^+$ T cells by staining with $D^b/NP_{366–374}$ tetramer (*A*) or K^b/siinfekl tetramer (*B*), or stimulation with the $NP_{366–374}$ peptide (*C*) or siinfekl peptide (*D*). In *A* and *B* a lymphocyte gate was used for the analysis, while in *C* and *D* a combined lymphocyte and $CD8^+$ gate were used; the percentages are indicated in the quadrants.

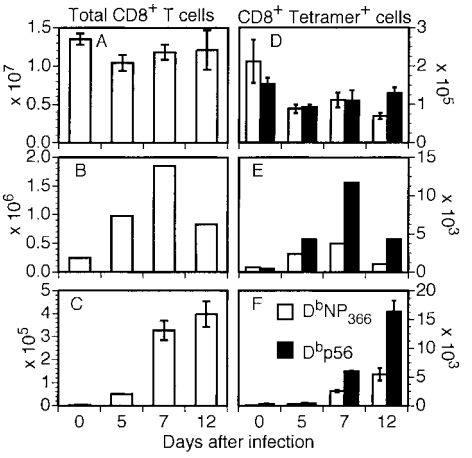

FIGURE 7. Two distinct nontransgenic populations of memory $CD8^+$ T cells are also passively recruited to the lung during influenza infection. B6 mice were primed first with i.p. inoculation with 10^6 PFU of recombinant vaccinia virus expressing γHV-68 p56 (17). One month later, the p56 response was boosted by i.p. inoculation with influenza WSN-p56 (17), which also stimulates the natural D^b-restricted CD8 response to influenza $NP_{366–374}$. These mice were subsequently infected i.n. with the unrelated B/HK influenza B virus after a further 7-wk interval, and the spleen (*A* and *D*), MLN (*B* and *E*), and BAL (*C* and *F*) populations were analyzed. Data were gathered for the total $CD8^+$ T cell counts (*A–C*) as well as the prevalence of γHV-68-specific and influenza A virus NP-specific $CD8^+$ T cells using the D^bp56 and D^bNP_{366} tetramers, respectively (*D–F*) (8, 17).

Immediately before challenge with the B/HK virus, ~1–2% of the $CD8^+$ T cells in the spleen were specific for either D^bp56 or D^bNP_{366} (day 0; Fig. 7, *A* and *D*). The MLN were small (day 0; Fig. 7, *B* and *E*), a characteristic feature of B6 mice that have not been challenged with a respiratory virus (1). Of the small number of $CD8^+$ lymphocytes recovered by BAL from what were essentially normal lungs, few were virus specific (day 0; Fig. 7, *D* and *F*). Within 5 days of the B/HK challenge, the number of memory $CD8^+$ T cells in the spleen specific for either unrelated virus had fallen (Fig. 7*D*) and the size of the MLN had shown some increase (Fig. 7, *B* and *E*). Peak cell numbers were recorded in the MLN on day 7 (Fig. 7, *B* and *E*) and in the lung on day 12 (Fig. 7, *C* and *F*). At this stage, ~5% of the $CD8^+$ T cells recovered from these B/HK-infected mouse lungs were specific for either D^bp56 or D^bNP_{366} (Fig. 7*F*). The kinetics of the nonspecific recruitment of the nontransgenic memory CD8 T cells differed from those of the OT-I transgenic cells. This may be due to differences in the nature of the inflammatory process between influenza A and influenza B infections. Alternatively, the diversity of the nontransgenic NP- and p56-specific T cells compared with the transgenic OT-I T cells may result in a higher degree of Ag cross-reactivity. In such a case, the observed recruitment could then be specific. In either case, primed memory T cells reactive to an apparently unrelated virus are consistently recruited to sites of virus-induced inflammatory pathology, as demonstrated by the earlier LDA experiments (11, 12).

Discussion

Using traditional methods of cellular immunology, it has been difficult to separate the issues of T cell activation by Ag and the requirement for Ag to be expressed in the tissue to attract specific T cells. One of the questions outstanding is whether any naive $CD8^+$ T cells would be recruited to the lung during an infection. Self-reactive T cells exposed to Ag in tissue could potentially undergo clonal expansion in situ, and the consequences of this for both the T cells and the tissue could be deleterious. For example, the activation of previously "ignorant" self-Ag-specific T cells in the tissue by inflammatory cytokines can lead to autoimmune disease (21). The alternative scenario is that the trafficking of naive T cells is restricted to the blood and secondary lymphoid organs to maximize the likelihood that their first encounter with Ag occurs in the highly regulated microenvironment of the lymph node or spleen. The observation that few T cells with a naive phenotype are ever observed in tissues including the lung does not satisfy the quandary, because it is difficult to know whether some element of the tissue microenvironment (besides Ag) causes the T cells to acquire an activated or memory phenotype. To address this question, it is necessary to know the Ag specificity of the T cells to accurately enumerate them. Unfortunately, because the frequency of naive T cells with any particular Ag specificity is too low to detect with conventional methods, this had not been done. Now, though, with the advent of TCR-transgenic mice and class I MHC tetramer technology, it has become possible to control the numbers of Ag-specific T cells present during an experimental immune response and also simultaneously determine the specificity of the cells and enumerate them (7, 8, 22, 23). The requirement for clonal expansion before detection is obviated.

Using the OT-I $CD8^+$ TCR-transgenic T cells and a combination of influenza and vaccinia viruses with and without the relevant $OVA_{257–264}$ epitope, we have shown that naive $CD8^+$ T cells are not recruited to the lung during a virus infection. Only when the T cells had been acutely activated by Ag, either distally via a nonrespiratory infection or when they were present as resting memory T cells, was there any evidence of T cells in the infected lung in the

absence of Ag. Thus the features that allow T cells to enter inflamed tissue are unrelated to Ag and are acquired by the T cells upon activation, presumably in the secondary lymphoid organs. Furthermore, these features are preserved on resting memory T cells, allowing the memory T cells to enter a site of possible infection quickly, where they can surveil for Ag-positive targets.

The trafficking of T cells from blood to tissue is regulated at several levels. It is well established that, upon activation by Ag, CD8$^+$ T cells down-modulate the expression of the lymph node homing receptor L-selectin (CD62L) (24–28) and up-regulate the integrins very late Ag (VLA)-4 and LFA-1 (27, 29–31). Consistent with this view is that influenza and parainfluenza virus-specific CD4 and CD8 T cells have been shown to express VLA-4 and have low levels of CD62L (24, 26, 28, 32–35). Both acutely activated and memory virus-specific T cells also express the tissue ligand CD44 and ICAM-1 (26, 36–39). Following activation by Ag in the secondary lymphoid organs, the T cells enter the circulation and roll along the vascular endothelium until they encounter the correct ligands (27, 30, 31, 40). Some tissue-specific ligands have been identified, such as L-selectin, E-selectin, and P-selectin for lymph node, skin, and gut homing T cells, respectively (24, 25, 27, 41–43). These tissue-specific ligands, or "addressins," tether the lymphocyte to the vasculature (27, 44, 45). Firm adhesion occurs next through interactions of LFA-1, ICAM-1, or VCAM (44, 46, 47). Engagement of VLA-4 (CD49d) partially activates the T cells, followed by diapedesis (31, 48, 49). For the lung, no lung tissue-specific ligands have yet been identified. Ag/MHC has been postulated to have a role in the activation of T cells at the vasculature (11, 12, 50–53) and in the process of extravasation, although this now seems not to be the case. Once in the tissue, if Ag is present, the T cells may further proliferate or become activated to effector status (7, 12). T cells that do not encounter Ag presumably pass through the tissue and back into the lymph nodes or circulation, though some memory T cells may become nonspecifically activated by the inflamed microenvironment. This nonspecific activation of memory T cells in the infected lung can also cause them to proliferate and/or become susceptible to apoptosis (54, 55), leading to a reduction in immune memory for unrelated Ags (55). So acute or previous encounter with viral Ags endows T cells with the essential constellation of adhesion molecules that allow entry into tissue.

Certainly, adhesion is only one level of regulation for tissue trafficking. The recent explosion of information concerning the roles of chemokines in directing lymphocytes to the correct tissue, or even within a given tissue, is only beginning to clarify this aspect of T cell migration. To date, although it has been attempted (4, 56), no lung-specific chemokines have been identified that can explain how T cells get to the lung in viral infections. Macrophage chemotactic protein-1 is only one factor known to be important for recruiting T cells to the lung (5, 57), although this is in a model of allergic sensitization. The specific chemokines induced during respiratory infection with influenza have not been identified. Ag activation of T cells could serve to increase the chemokine responsiveness of the cells through up-regulation of chemokine receptors. The evidence presented in this paper suggests that, assuming chemokines are critical for the recruitment of T cells to the infected lung, responsiveness to these factors may be a feature only of activated or memory T cells and not of naive T cells.

Interestingly, although not the focus of these studies, there was evidence that the host response to the OVA peptide was inhibited in the presence the OVA-specific OT-I T cells (Fig. 6). Yet the host response to the natural influenza epitope of the NP was not affected, either in the primary or secondary responses. In a previous report (58) similar OT-I T cells were shown to effectively suppress the host response to both the $OVA_{257–264}$ epitope and an unrelated epitope via competition for APCs. The differences between these studies are that firstly, the APC presenting influenza Ags may not be limiting, explaining why the NP response is intact even when the OT-I T cells had been primed (Fig. 6). Secondly, the numerical advantage of the transferred OT-I T cells in the primary challenge could explain the inhibition of the host (Thy1.1) response to OVA. This advantage may be partially overcome when the host T cells are primed with Vacc-OVA, because there is some evidence of a weak host response to OVA during a secondary response (Fig. 6). Interestingly, despite the fact that the OT-I donors for these studies were not Rag deficient (59, 60), there was little evidence of a donor (Thy1.2$^+$) T cell response to the influenza NP epitope, even though it is formally possible that some dual TCR OT-I cells would be NP specific.

Nonlymphoid tissues are home to a surprisingly large number of memory T cells (CD4 or CD8) (61, 62). Furthermore, these tissue-residing T cells have activation and effector phenotypes distinct from their counterparts in the lymph nodes and spleen (61, 62). The hypothesis is that some memory T cells enter tissue to provide secondary immunity regardless of where they have been primed or what Ag specificity they possess (61, 62). The possibility emerges that, in our experiments where the OT-I T cells were primed with Vacc-OVA i.p., the few OT-I T cells we observed early in the secondary influenza infection were T cells already present in the lung. However, mice that are maintained under specific pathogen-free conditions, without a known respiratory virus infection, have characteristically small MLNs and very few, if any, T cells recoverable in the BAL (63). In fact, almost no γ-HV68 p56- or flu NP-specific CD8 T cells were present in the BAL of mice given a nonrespiratory priming (Fig. 7, *C* and *F*). In contrast, virus-specific CD8 T cells are easily recovered from the BAL of mice long after they have been infected with, and cleared, a respiratory virus, even up to a year or more after the initial infection (64, 65). This suggests that respiratory infection is required to allow a significant number of T cells to enter the airways and that the infection may permanently alter the permeability of the lung to lymphocytes. This is supported by the observation that the MLN is also permanently altered after respiratory infections (63). The difference between these observations and those of Masopust et al. (66) may be in the way lymphocytes were isolated from the tissues. We sampled the lung by lavage, whereas the other study was performed by homogenization of perfused tissue. Thus, we cannot exclude the possibility that, after priming with vaccinia, some memory T cells were present in the lung tissue. Furthermore, in the present situation, the transient nature of the memory CD8 T cells during the secondary influenza challenge (Figs. 4 and 6) suggests that there may be significant turnover of lung-resident memory T cells during subsequent infections. The long-term effects of this turnover for memory T cells in different tissues and for immune protection are not known, but significant loss of memory has been described following sequential virus infections (67, 68). In any case, it is clear that naive T cells do not have the capacity to enter the lung tissue or airways, even during a respiratory infection, while activated and memory T cells do.

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