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Proliferative Expansion and Acquisition of Effector Activity by Memory CD4+ T Cells in the Lungs following Pulmonary Virus Infection

Erika L. Wissinger,*‡ Whitney W. Stevens,*‡ Steven M. Varga,* and Thomas J. Braciale2*†‡

The memory CD4+ T cell response to the respiratory syncytial virus (RSV) attachment (G) protein in the lungs of primed BALB/c mice undergoing intranasal challenge pulmonary RSV infection is dominated by effector T cells expressing a single Vβ-chain, Vβ14. We have used Vβ14 expression to examine the kinetics of the activation, accumulation, and acquisition of the effector activity of memory CD4+ T cells responding to pulmonary infection. This analysis revealed that proliferative expansion and effector CD4+ T cell differentiation preferentially occur in the respiratory tract following rapid activation within and egress from the lymph nodes draining the respiratory tract. These findings suggest that, in response to natural infection at a peripheral mucosal site such as the lungs, memory CD4+ T cell expansion and differentiation into activated effector T cells may occur predominantly in the peripheral site of infection rather than exclusively in the lymph nodes draining the site of infection. The Journal of Immunology, 2008, 180: 2957–2966.

In many viral model systems, memory CD4+ T lymphocytes play an important role in virus clearance and the resolution of infection through a variety of mechanisms, including the secretion of antiviral cytokines such as IFN-γ and TNF-α and the provision of help to B cells for the production of neutralizing Abs (1). In addition, this memory CD4+ T cell response may contribute to the development of immunopathology, as is the case for respiratory syncytial virus (RSV) infection (2–5). Although it is known that the development of these recall responses requires the activation and proliferation of resting memory lymphocytes and their subsequent maturation into fully armed effectors, the sites of induction and the precise kinetics of these responses are not well defined. In the present study, we examined the kinetics and the magnitude of the memory effector CD4+ T cell response in the lungs of infected mice using the well-characterized BALB/c mouse model of RSV infection. In this model system, BALB/c mice vaccinated with the RSV attachment (G) glycoprotein exhibit enhanced pulmonary injury after challenge RSV infection (4, 5).

As we have previously reported (6), in RSV G-primed BALB/c mice undergoing intranasal challenge infection with RSV, the memory T cell response in the respiratory tract is characterized by an accelerated accumulation of memory effector CD4+ T cells in the lungs that peaks at day 5 postinfection (p.i.). These CD4+ T cells predominantly recognize a single I-E d-restricted immunodominant epitope within the RSV G protein corresponding to aa 183–195 (6). In addition, a large percentage (60–70%) of the RSV G-specific effector CD4+ T cells that infiltrate the infected lungs is oligoclonal and uses a single TCR Vβ-chain, Vβ14, as part of the TCR (7). The presence of this uniquely restricted oligoclonal population of effector CD4+ T cells responding to infection in the lungs provided us an opportunity to examine the kinetics, the magnitude, and the development of effector activity of the memory CD4+ T cell response to challenge RSV infection in the lungs of wild-type mice by using Vβ14 expression on lung-infiltrating T cells as a marker of the responding memory CD4+ T cell population. Our analyses suggest that, following memory CD4+ T cell activation within and subsequent rapid egress from the draining lymph nodes (DLN), the accumulation of CD4+ Vβ14+ T cells in the lungs of G-primed mice following RSV challenge infection results largely from the extensive proliferation of responding memory CD4+ T cells and their subsequent differentiation into effector T cells within the infected lungs. This investigation into the rapid kinetics of memory CD4+ T cell activation and migration may have further implications for responses against other viral respiratory pathogens as well as memory T cell-mediated delayed type hyperreactivity (DTH) responses such as contact hypersensitivity or graft rejection.

Materials and Methods

Mice

Female BALB/cAnNTac(H-2b) mice were purchased from Taconic Farms and immunized (as described below) at 7–10 wk of age for all experiments. Mice were housed in a specific pathogen-free environment. All animal work was conducted according to protocols approved by the Animal Care and Use Committee of the University of Virginia (Charlottesville, VA).

Viruses and infection of mice

Recombinant vaccinia virus (vv) expressing the attachment (G) protein of RSV (vvG) (8) was a gift from J. L. Beeler (Food and Drug Administration, National Institutes of Health (NIH), Bethesda, MD). Recombinant vaccinia virus expressing β-galactosidase (vvβ-gal) was used as a negative control for immunization. The A2 strain of RSV was a gift from P. L.
Collins (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD) and was grown in Hep2-cells (American Type Culture Collection). Mice were primed with $3 \times 10^6$ PFU of vvG or vvβgal in a 10-μl volume by scarification at the base of the tail with a 25-gauge needle. Mice were typically rested for 2-3 mo after priming, then lightly anesthetized using a 2:1 mixture of ether (Malinckrodt Baker) and chloroform (Sigma-Aldrich), and intranasally infected with 1.2-5 $\times 10^6$ PFU of RSV in a 50-μl volume. In the representative experiments shown in Fig. 1, challenge infection of immune mice was conducted 3-4 wks after immunization. Identical results were obtained when the mice were rested for 2-3 mo after priming.

### Preparation of single-cell suspensions

**Lungs.** Blood was flushed from the lung vasculature with 4-5 ml of PBS containing 10 U/ml heparin (Sigma-Aldrich) administered via the right ventricle. The lungs were carefully removed and dissected away from the heart and peribronchial lymph nodes (PBLN). Lungs were placed into MLC medium consisting of RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FBS (Atlanta Biologicals), 10 U/ml penicillin G, 10 μg/ml streptomycin sulfate, 2 mM l-glutamine, 5 × 10^{-3} M 2-ME, 1 mM sodium pyruvate (Invitrogen Life Technologies), 0.1 mM nonessential amino acids (Invitrogen Life Technologies), and 10 μg/ml L-glutamine (Invitrogen Life Technologies). The tissues were minced finely and passed through a wire mesh screen.

**Spleens.** Spleens were carefully removed, placed into MLC medium, and passed through a fine wire mesh screen.

**PBLN and inguinal lymph nodes.** Lymph nodes dissected away from the lungs (PBLN) or isolated directly (inguinal lymph nodes) were placed into MLC medium as described above for the lungs and then gently disrupted between the frosted ends of two glass microscope slides. The slides were rinsed extensively to optimize cell recovery. For each of these tissues, particulate debris was removed by brief centrifugation at 1000 rpm.

### Surface staining and flow cytometric analysis

For multicolor FACS analysis, ~1-2 × 10^6 cells were blocked with purified anti-FcRIII/II mAb (clone 2.4G2) and then stained with allophycocyanin-conjugated anti-CD4 (clone RM4-5) and one of the following FITC-conjugated mAb: anti-Vβ14 (clone 14-2) or rat IgM (clone R4-22). All mAb used in FACS staining were purchased from BD Pharmingen. Stained cells were washed with a staining buffer consisting of PBS supplemented with 2% FBS and 0.02% NaN3, resuspended in staining buffer and fixed, and erythrocytes were lysed with FACS lysing solution (BD Biosciences). Cells were washed, resuspended in staining buffer, and analyzed in two-color mode using a BD Biosciences FACSCalibur flow cytometer. Background staining with the appropriate isotype-matched control mAb was subtracted from each individual value. Single-color controls were used in all multiparameter FACS analyses for electronic compensation, and between 60,000 and 100,000 events were acquired from each sample. Lymphocyte populations were first gated on forward and 90° side scatter properties and then analyzed using CellQuest software (BD Biosciences). Cells were washed, resuspended in staining buffer, and analyzed in two-color mode using a BD Biosciences FACSCalibur flow cytometer. Background staining with the appropriate isotype-matched control mAb was subtracted from each individual value. Single-color controls were used in all multiparameter FACS analyses for electronic compensation, and between 60,000 and 100,000 events were acquired from each sample. Lymphocyte populations were first gated on forward and 90° side scatter properties and then analyzed using CellQuest software (BD Biosciences).

### Propidium iodide staining and flow cytometric analysis

For DNA content analysis, ~2 × 10^6 cells from a pool of lung cells from two individual mice were blocked with purified anti-FcRIII/II mAb (clone 2.4G2), washed, resuspended in staining buffer, and then stained with one of the following FITC-conjugated mAb: anti-CD4 (clone RM4-5), anti-Vβ14 (clone 14-2), anti-CD11b (clone M170), rat IgG2b (clone A95-1), rat IgG2a (clone R35-95), or rat IgM (clone R4-22). Stained cells were washed and resuspended with staining buffer, erythrocytes were lysed, and cells were fixed with FACS lysing solution. Cells were washed and resuspended with permeabilization buffer (staining buffer containing 0.5% saponin (Sigma-Aldrich)) and then incubated at room temperature in permeabilization buffer and a 1.4 mg/ml final concentration of RNase A (Sigma) for 10 min. Propidium iodide was added to the cells to a final concentration of 0.4 mg/ml and incubated at room temperature in the dark for 30 min. Cells were then analyzed in two-color mode using a BD Biosciences FACSCalibur flow cytometer. Single-color controls were used for electronic compensation, and between 100,000 and 200,000 events were acquired using CellQuest software for each sample. Lung cell populations were analyzed for surface staining and DNA content/cell cycle status using ModFit LT software (Verity Software House).

### Intracellular cytokine staining and flow cytometric analysis

For intracellular cytokine staining, ~2 × 10^6 cells were stimulated for 5 h in the presence of 1 μg/ml brefeldin A (Sigma-Aldrich) and 1 μM RSV G183–195 peptide, or RSV M2 82–90 peptide (or the appropriate irrelevant control peptides). Cells were then washed in staining buffer, blocked with purified anti-FcRIII/II mAb (clone 2.4G2), and stained with allophycocyanin-conjugated anti-CD4, PerCP-Cy5.5-conjugated CD8α (clone 53-6.7), and FITC-conjugated anti-Vβ14 mAbs. Cells were again washed with staining buffer, fixed with FACS lysing solution, and washed and incubated in permeabilization buffer. Cells were then stained with either PE-conjugated anti-IFN-γ mAb (clone XMG1.2) or a PE-conjugated isotype control, rat IgG1 (clone R3-34). Cells were washed, resuspended in staining buffer, and analyzed in four-color mode using a BD Biosciences FACSCalibur flow cytometer. Electronic gating, compensation, and background staining controls were performed as described above.

## Results

### Kinetics of accumulation of CD4^+ Vβ14^+ T cells in the lungs of G-primed BALB/c mice following RSV infection

BALB/c mice were vaccinated with vvG, a recombinant vaccinia virus vector expressing the RSV G glycoprotein, or with vvβ-gal, a control vaccinia vector expressing β-galactosidase. Three or more weeks later the mice were infected intranasally with RSV. In the lungs of G-primed mice undergoing challenge RSV infection (Fig. 1a) there is a rapid increase in the total number of lung-infiltrating CD4^+ T cells within a narrow kinetic window between days 3 and 5 p.i., representing an ~5-fold increase (5.6 × 10^6 CD4^+ cells at day 5 p.i.) above the baseline level of ~1 × 10^6
CD4+ cells present between days 0–3 p.i. This is in contrast to the modest CD4+ T cell response in the lungs of RSV-infected control mice undergoing a primary response to RSV (Fig. 1b), in which there is only a slight (~3-fold) increase in the total number of CD4+ T cells infiltrating the lungs between days 3 and 7 p.i. (from ~2 × 10^6 cells at day 3 p.i. to ~6 × 10^6 cells at day 7 p.i.). Thus, the early and accelerated accumulation of activated CD4+ T cells observed in the lungs of G-primed mice undergoing challenge infection reflects the expected expansion and recruitment of the G-specific memory CD4+ T cell population present in G-primed mice.

Of note, lung-infiltrating CD4+ T cells that use the Vβ14 gene product in their TCR represent a large fraction of the total responding T cell population at day 5 p.i. This CD4+ T cell subset, which consists primarily of RSV G-specific memory effector T cells (6), represents ~30% of the total CD4+ T cell population detected in the lungs at day 5 p.i. and up to 60% of the day 5 lung CD4+ T cells secreting effector cytokines (e.g., IFN-γ) in response to RSV G (7). Importantly, the CD4+ Vβ14+ subset of T cells rapidly increases in total cell number in the infected lungs between days 3 and 5 p.i., which represents a 6-fold expansion of CD4+ Vβ14+ T cells in the lungs over this 48-h period (Fig. 1a).

By contrast, in control mice undergoing a primary pulmonary response to RSV infection, the responding CD4+ Vβ14+ population increased only modestly, from ~2 × 10^6 to ~5 × 10^6 cells between days 3 and 7 p.i. (Fig. 1b). This minimal expansion in CD4+ Vβ14+ cell number parallels the modest increase in overall CD4+ T cell number in the lungs during primary infection. Thus, these results illustrate that within a narrow kinetic window following challenge RSV infection of G-primed mice, there is significant accumulation of responding memory CD4+ T cells at the site of infection, the lung, with preferential accumulation of the G-specific memory CD4+ Vβ14+ T cell population.

Memory CD4+ T cell responses in central lymphoid tissues

The above results illustrated the rapid expansion in the total number of G-specific effector CD4+ Vβ14+ memory T cells in the lungs within the 48-h period between days 3 and 5 p.i. A likely explanation for the tempo of the response in the lungs was that G-specific memory cells, present in secondary lymphoid organs (such as the lymph nodes draining the lungs and the spleen), responded to viral Ags delivered to these sites by activating, proliferating, and differentiating into effector CD4+ T cells within these lymphoid compartments before efflux and recruitment of these G-specific CD4+ effector T cells to the site of infection, the lungs. We also expected that the expansion of these activated CD4+ Vβ14+ T cells in secondary lymphoid organs would temporally precede the subsequent trafficking to and accumulation of these cells in the lung, as has been observed for primary CD4+ T cell responses to respiratory virus infection (9).

When we examined the kinetics of accumulation of RSV G-specific memory CD4+ Vβ14+ T cells in the lungs (Fig. 2a), PBLN (Fig. 2b), and spleen (Fig. 2c), the anticipated initial expansion of this T cell population in central lymphoid compartments was not observed. In the PBLN, which drain the infected lungs (Fig. 2b), there was no detectable increase in the total number of CD4+ Vβ14+ T cells between days 2 and 5 p.i. (from 1.3 × 10^5 cells on day 3 to 1.3 × 10^5 cells on day 5 p.i.) and only a slight rise in the percentage of CD4+ T cells expressing Vβ14 (from 11 to 17%, respectively). Rather than preceding the accumulation of these G-specific CD4+ T cells in the lungs, the modest change in CD4+ Vβ14+ cell number in the PBLN paralleled the kinetics of the marked expansion in CD4+ Vβ14+ T cell number in the lungs during this time (Fig. 2a). Thus, the accumulation of CD4+ Vβ14+ T cells observed in the lungs between days 2 and 5 p.i. could not be accounted for by prior or concomitant proliferative expansion of these memory effector cells in the PBLN.

Along with the analysis of the draining PBLN, a parallel kinetic analysis of the frequency and total numbers of CD4+ Vβ14+ T cells present in the spleen after challenge infection was performed. This lymphoid compartment is also a potential site of induction of the recall response, including memory CD4+ T cell activation and proliferation in response to infection, and is a major reservoir of CD4+ Vβ14+ T cells in BALB/c mice. As demonstrated in Fig. 2c, between days 3 and 5 p.i. there was no significant change in the total number of splenic CD4+ Vβ14+ T cells. If the spleen were a prominent site of memory CD4+ T cell activation/proliferation and a primary donor of activated RSV-specific effector CD4+ T cells, proliferative expansion of CD4+ Vβ14+ T cells in the spleen would be expected before the accumulation of activated CD4+ T cells in the RSV-infected lungs. Sampling of other peripheral (non-draining) lymph nodes as well as peripheral blood over time after challenge RSV infection likewise revealed neither proliferative expansion of CD4+ Vβ14+ T cells in lymphoid tissues nor increased numbers of activated CD4+ Vβ14+ T cells in peripheral blood (CD4+ Vβ14+ T cells remained at 10–20% of total circulating CD4+ T cells in the blood between days 1 and 5 of challenge infection). Taken together, these data suggest that the marked expansion of Vβ14+ RSV G-specific CD4+ T cells in the lungs between days 3 and 5 p.i. cannot be easily accounted for by the activation and proliferation of memory CD4+ T cells in secondary lymphoid organs before the appearance of these T cells in the infected lungs.
Activation of resting G-specific memory CD4+ T cell precursors in lymphoid tissues

The observed accumulation of G-specific memory CD4+Vβ14+ T cells in the lungs following challenge RSV infection could reflect the proliferative expansion of memory CD4+Vβ14+ T cells present in the lungs at the time of challenge infection. As suggested by findings in recent publications (10, 11), this population of peripheral memory CD4+ T cells would localize to the lung parenchyma (and other peripheral sites) following primary immunization with the RSV G-expressing vaccinia vector and could potentially respond to challenge RSV infection in situ (i.e., in the infected lungs). Alternatively, these findings could also be explained by a mechanism whereby G-specific memory CD4+Vβ14+ T cells present in the recirculating T cell pool would enter the draining PBLN and activate in the PBLN in response to RSV infection. In this instance, the responding memory CD4+ T cells would have to rapidly egress (after Ag encounter) from the PBLN to the lungs, where the activated memory CD4+ T cells would then proliferate and give rise to G-specific effector CD4+ T cells.

The memory CD4+ T cell response to RSV G in BALB/c mice is largely directed to a single immunodominant epitope spanning residues 183–195 (7). Although G183–195-specific memory CD4+ T cells from G-primed mice did not secrete cytokines when stimulated with the 183–195 peptide epitope (directly ex vivo in short-term assay), we were, however, able to detect transient up-regulation of CD154 on CD4+ T cells from the G-primed mice after short-term in vitro exposure to the G183–195 epitope. Using this approach, we were able to estimate the frequency of G-specific memory CD4+ T cells in secondary lymphoid organs at ≤0.01% of total splenic CD4+ T cells from primed donors. This frequency was at the limits of detection for total CD4+ T cells isolated from the PBNL of G-primed mice. We were unable to detect G-specific memory CD4+ T cells by either effector cytokine (e.g., IFN-γ secretion) or CD154 up-regulation among CD4+ T cells isolated from the lungs of G-primed mice above the minimum background activity detected in the lungs of normal donors. Nevertheless either lung or PBNL resident memory CD4+ T cells (or both) could contribute to the recall response in the lungs.

To distinguish the relative contribution of lung and LN resident memory CD4+ T cells to the recall response to RSV G, we used the immunosuppressive agent FTY720, a sphingosine-1-phosphate receptor (SIPR) agonist, which acts by blocking the egress of circulating lymphocytes (in particular CD4+ T cells) from lymph nodes (12–14). Accordingly, if a lung-resident pool of CD4+ T memory cells gives rise to the expanded effector population present in the lungs at day 5 p.i. with RSV, the treatment of mice with FTY720 before RSV challenge infection should have no effect on the accumulation of effector CD4+Vβ14+ T cells in the lungs. However, if the pool of effector CD4+ T cells present at day 5 p.i. is derived from memory CD4+ T cells that must transit through the DLN, activate there, and migrate to the lungs (where proliferative expansion occurs), then treatment with FTY720 before infection should significantly abrogate the CD4+ T cell response in the lungs.

The results of the analysis of FTY720 treatment are shown in Fig. 3. Compared with untreated controls, G-primed mice administered FTY720 as little as 30 min before RSV infection exhibited an ~90% reduction in both total CD4+ T cells and CD4+Vβ14+ T cells in the lungs at day 5 p.i. (Fig. 3a). This inhibition of effector CD4+ T cell expansion and accumulation in the lungs was also reflected in the absence of G-specific, IFN-γ-secreting effector CD4+ T cells (enumerated by intracellular cytokine staining) in the lungs of FTY720-treated mice (Fig. 3a, inset).

Although FTY720 is believed to act preferentially on SIPRs displayed by lymphocytes (with the CD4+ T cell exhibiting the greatest sensitivity to the drug) (15, 16), other potential CD4+ T cell-independent effects of the drug such as suppression of RSV replication in the lungs and/or inhibition of viral Ag delivery to the PBLN through the inhibition of dendritic cell migration (17) might occur. This would also result in impaired viral Ag recognition by T cells (in the lung-draining PBLN), which could account for the observed inhibition of the memory CD4+ T cell response in the lungs. To evaluate viral Ag availability in the PBLN, we made use of the fact that murine CD8+ T cells are relatively resistant to the inhibitory effect of FTY720 and examined the effect of FTY720 pretreatment on the T cell response of mice primed with a chimeric G construct encoding a 10-aa immunodominant CD8+ T cell epitope from RSV M2 protein (18). As previously reported (5), mice primed with this chimeric RSV G/M2 vaccinia virus (vvG/M2) construct mount a memory CD4+ T cell response to RSV G and a memory CD8+ T cell response to the dominant M2 epitope upon challenge RSV infection. Pretreatment of vvG/M2-primed mice with FTY720 just before challenge RSV infection resulted in suppression of the G-specific memory CD4+ T cell response (comparable to the results shown in Fig. 3a). By contrast, the drug had no effect on the generation of an M2-specific memory CD8+ T cell response in the lungs, as demonstrated by the accumulation of M2-specific, IFN-γ-secreting effector CD8+ T cells at day 5 p.i. in pretreated vvG/M2-primed mice at levels comparable to those in primed, untreated controls (Fig. 3b). It should also be noted that the CD8+ T cell response in the lungs of BALB/c mice to respiratory virus infection is inhibitable by FTY720 administration but requires a drug dose ~10-fold higher than that used here to block the memory CD4+ T cell response. RSV M2-specific CD8+ T cells in the lungs of control M2 immune mice undergoing challenge infection ranged from 0.8–1.2 × 105 cells per lung set (days 1 and 2 p.i.) to 2.5 × 105 CD8+ M2 tetramer+ T cells (day 3 p.i.) and rapidly expanded to 2.5 × 106 cells (day 4 p.i.) and 6–8 × 106 cells (day 5 p.i.). The drug treatment regimen was not toxic for CD4+ T cells in general or for G-specific memory CD4+ T cells in particular, because a treatment of G-primed mice with FTY720 (a 5-day course) had no effect either on CD4+ T cell numbers or on the recall response to RSV-G in the lungs of primed mice when challenge RSV infection was conducted 15 days after the termination of drug administration (Fig. 3d). In drug treatment regimens conducted >15 days before challenge infection or >3 days postinfection, the fraction of CD4+Vβ14+ T cells capable of secreting IFN-γ in the intracellular cytokine staining assay ranged from 40 to 50%.

The above results supported the concept that the memory CD4+ T cells that traffic through the draining PBLN were the source of the G-specific effector CD4+ T cells accumulating in the lungs following challenge infection. Because these G-specific memory CD4+ T cells did not accumulate to detectable levels in the draining PBLN (Fig. 2b) during the recall response and presumably had rapidly migrated from the nodes, it was of interest to determine when after challenge infection the memory CD4+ T cells egress from the PBLN to populate the lungs. To determine this, we examined the impact of the FTY720 inhibition of CD4+ T cell egress from the PBLN on the lung CD4+Vβ14+ T cell response at day 5 p.i. by starting the administration of FTY720 to G-primed mice at 24 h intervals up to day 3 p.i. As Fig. 3c demonstrates, blockade of CD4+ T cell egress at 24 h p.i. significantly inhibited the effector CD4+ T cell response in the lungs at day 5 p.i. However, blockade of T
cell egress from the lymph nodes by FTY720 treatment initiated at 48–72 h p.i. had no significant effect on the magnitude of the effector CD4<sup>+</sup>V<sub>β</sub>14<sup>+</sup> T cell response in the lungs at day 5 p.i. These results suggest that memory CD4<sup>+</sup> T cells responding to pulmonary RSV infection rapidly transit through the draining PBLN (i.e., enter the nodes between 0 and 48 h p.i. and complete the process of activation and egress from the nodes by 48 h p.i.).

In conjunction with the above analysis of lung CD4<sup>+</sup> T cell accumulation, we also examined the number of CD4<sup>+</sup>V<sub>β</sub>14<sup>+</sup> T cells present at day 5 p.i. in the PBLN of G-primed RSV-challenged mice treated with FTY720 before or at the above time.
points p.i. We found that the number of CD4$^+$V$\beta$14$^+$ T cells was low and comparable to that of untreated mice. Thus, the inhibition of T cell egress from the nodes by FTY720 (i.e., cell trapping in the nodes) did not result in a substantial increase in G-specific CD4$^+$ T cell accumulation within the draining PBLN, and any retention of memory T cells in the node (i.e., as a result of FTY720 treatment at 24 h p.i. or beyond) was not associated with the significant proliferation of memory CD4$^+$ T cells within PBLN.

We also attempted to complement this analysis by the adoptive transfer of purified dye-labeled CD4$^+$ T cells from a G-primed donors into naive congenic recipient mice followed by challenge RSV infection of the recipients to directly track the migration of memory CD4$^+$V$\beta$14$^+$ T cells from the PBLN to the lungs. Unfortunately, the frequency of G-specific memory cells was too low to accurately quantitate/detect the migration of specific memory cells from the PBLN into the lungs, because these cells were only detected after proliferative expansion in the lungs.

**Proliferation of G-specific memory CD4$^+$ T cells in the lungs over time**

If, as the above results suggest, a small number of memory CD4$^+$ T cells trafficking through the draining PBLN of RSV-infected animals activate there and then rapidly egress (by 48 h p.i.) from the PBLN to the lungs without undergoing proliferative expansion within the draining nodes, the G-specific memory CD4$^+$V$\beta$14$^+$ T cells must actively proliferate in the lungs. Consequently, a substantial fraction of CD4$^+$ T cells, and specifically CD4$^+$V$\beta$14$^+$ T cells, resident in the lungs during the period of accelerated T cell accumulation in this site (i.e., day 4 p.i.) should be proliferating and have $>2$N DNA content (i.e., should be actively synthesizing DNA and be in the S or G$_2$/M phase of the cell cycle). To examine the cell cycle status of memory T lymphocytes in this organ, suspensions of total cells from infected lungs of G-primed and control mice were isolated at day 4 p.i. and stained with propidium iodide to determine the DNA content of the lung cells and obtain a snapshot of the percentages of lung cells of different lineages in the S/G$_2$/M phases of the cell cycle.

As anticipated, both in mice undergoing a primary RSV infection (Table I) and in mice mounting a memory response to the G protein after challenge RSV infection (Table I), the overall percentage of total lung cells in S phase of the cell cycle at day 4 p.i. was low (2 and 5%, respectively). When the DNA content of specific cell types within the lungs of G-primed mice undergoing challenge RSV infection was examined, only 2% of total lung cells expressing the myeloid cell (macrophage/dendritic cell) marker CD11b were in S or G$_2$/M phase (Table I). By contrast, ~12% of total lung CD4$^+$ T cells had $>2$ N DNA content (were in S or G$_2$/M) at day 4 p.i. (Table I). Strikingly, nearly 50% of the total lung CD4$^+$ T cells with $>2$ N DNA content expressed V$\beta$14, and ~40% of the lung CD4$^+$V$\beta$14$^+$ T cells were in the S/G$_2$/M phase of the cell cycle at day 4 p.i. in G-primed mice (Table I and Fig. 4).

Importantly, we detected negligible proliferation (cells with $>2$ N DNA content) of CD4$^+$V$\beta$14$^+$ T cells in the DLN during the first 4 days of challenge infection of G-primed mice (data not shown), consistent with the observed lack of accumulation of these cells in this site. We obtained comparable results when DNA content of the responding T cells was determined using the fluorescent cell-permeable DNA binding dye Draq5.

An examination of the kinetics of proliferating CD4$^+$V$\beta$14$^+$ T cell accumulation in the lungs of G-primed mice after RSV infection (Fig. 4) revealed that maximum proliferation occurred at day 4 p.i., when 42% of CD4$^+$V$\beta$14$^+$ T cells had $>2$N DNA content. This time point coincides with the period of accelerated accumulation of CD4$^+$V$\beta$14$^+$ T cells in the lungs during challenge infection (Figs. 1a and 2a), and precedes by 1 day the point of maximum accrual of memory effector CD4$^+$V$\beta$14$^+$ T cells in the lungs (at day 5 p.i.). It is also noteworthy that, at day 3 p.i. (when activated G-specific memory CD4$^+$ T cells have migrated to the lungs from the draining PBLN), only a small fraction of the total CD4$^+$V$\beta$14$^+$ T cells (~2%) are in cycle. Likewise, at day 5 p.i. active DNA synthesis among the lung CD4$^+$V$\beta$14$^+$ T cells is markedly reduced (~7%) relative to that at day 4 p.i. These data imply that the responding CD4$^+$ T cells that have entered the lungs are vigorously proliferating over a narrow kinetic window following egress of the activated G-specific memory CD4$^+$ T cell precursors from the PBLN.

**Expression of effector activity by activated memory CD4$^+$V$\beta$14$^+$ T cells**

Given the evidence for extensive proliferation of G-specific CD4$^+$V$\beta$14$^+$ T cells in the lungs as described above, it was of interest to examine the kinetics of the acquisition of effector function, specifically cytokine synthesis, by CD4$^+$V$\beta$14$^+$ T cells in the lungs. To address this issue, cells were isolated from the lungs, spleen, and PBLN of G-primed mice at various time points after RSV infection and the cells were examined for IFN-$\gamma$ production in response to acute antigenic stimulation in vitro. As Fig. 5a demonstrates, there is a significant (25-fold) increase in the total number of CD4$^+$V$\beta$14$^+$IFN-$\gamma$-secreting (IFN-$^+$ T cells in the lung within the 48 h period between days 3 and 5 p.i. (from 4 $\times$ 10$^4$ to 4 $\times$ 10$^5$ cells).
cells at day 3 p.i. to $1 \times 10^6$ cells at day 5 p.i.). As expected from our earlier results (Fig. 2), there is negligible accumulation of CD4$^+$ Vβ14$^+$ IFN-γ$^{-/−}$ T cells in either the PBLN or the spleen during this interval (Fig. 5a).

Memory CD4$^+$ T cells of the effector phenotype are believed to rapidly acquire the capacity to express effector activity (e.g., effector cytokine secretion) after encounter with Ag, while the expression of effector activity by central memory CD4$^+$ T cells may require proliferation and additional differentiation after antigenic stimulation. If, in the recall response to infection, G-specific memory CD4$^+$ Vβ14$^+$ T cells emigrate from the PBLN to the lungs as fully differentiated mature effector cells capable of Ag-dependent cytokine synthesis (IFN-γ production) during proliferative expansion in the lungs, then the fraction of the responding CD4$^+$ Vβ14$^+$ T cells in the lungs that are IFN-γ$^{+/+}$ would be constant even though the total number of CD4$^+$ Vβ14$^+$ IFN-γ$^{-/−}$ T cells increases in the lungs between days 3–5 p.i. (Fig. 5a). Alternatively, if these activated CD4$^+$ T cells acquire effector function (the capacity to secrete cytokines) as they proliferate in the lungs, then an increasing fraction of the Vβ14$^+$ T cells will be IFN-γ$^{-/−}$ over time as the T cells divide. As Fig. 5b demonstrates, the percentage of IFN-γ$^{+/+}$ CD4$^+$ Vβ14$^+$ T cells in the lungs increases substantially over time from ~7% cytokine positive at day 3 p.i. to nearly 50% cytokine positive at day 5 p.i. (Fig. 5b). By contrast, among the extremely small number of cytokine-secreting CD4$^+$ T cells in the PBLN and spleen, the frequency of IFN-γ$^{+/+}$ CD4$^+$ Vβ14$^+$ T cells in the draining PBLN remains relatively constant from day 3–5 p.i. and, similarly, the fraction of mature effector cells in the spleen only increases at day 5 p.i., when the frequency of these G-specific effector CD4$^+$ T cells has attained maximum level in the lungs (Fig. 5b). These data suggest that the maturation of these memory RSV G-specific CD4$^+$ T cells into cytokine-synthesizing effector cells may preferentially occur at the site of active cell division of these Vβ14$^+$ T cells in the infected lung.

Discussion

We have investigated the RSV G protein-specific memory CD4$^+$ T cell response in the lungs of G-primed wild-type BALB/c mice following intranasal challenge RSV infection using expression of the TCR Vβ14 gene product as a marker of the G-specific memory CD4$^+$ T cell population (6, 7). We found that RSV G-specific memory CD4$^+$ T cells rapidly accumulate in the lungs between days 3–5 p.i. in G-primed mice, with the most marked expansion in number among the dominant set of Vβ14-expressing G-specific effector CD4$^+$ T cells. The accumulation of G-specific memory CD4$^+$ T cells in the lung cannot be readily accounted for by the prior proliferation of memory T cell precursors and their maturation into effector CD4$^+$ T cells in lymphoid organs, such as the DLN and spleen, before migration to the lungs. However, memory CD4$^+$ T cell trafficking through and activation within lymph nodes draining the respiratory tract is apparently required, because FTY720-mediated blockade of CD4$^+$ T cell egress from lymph nodes before infection prevented subsequent effector cell accumulation in the lungs. Memory T cell activation and rapid migration from the DLN to the lungs is followed by a burst of proliferation by this responding T cell population, as demonstrated by an analysis of the DNA content of lung-infiltrating cells. A very high frequency (~50%) of G-specific CD4$^+$ Vβ14$^+$ T cells in the lung are in S/G$_1$/M phases of the cell cycle during the rapid accumulation of cells in the lung between days 3 and 5 p.i. The proliferative expansion of these T cells in the lungs is also associated with the acquisition of effector activity (anti-viral cytokine production) by the CD4$^+$ Vβ14$^+$ T cell pool infiltrating the lungs, suggesting that differentiation and cell division are concurrent for this population. Taken together, these data suggest (but do not formally prove) a model in which the recall response to viral infection at a mucosal surface (such as the lungs) is primarily mediated by quiescent, recirculating memory CD4$^+$ T cells that must first traffic through the DLN to respond to infection. These cells encounter Ag in the DLN, activate, rapidly egress from the nodes (before the onset of cell division), and migrate to the mucosal surface where the cells proliferate extensively and differentiate into mature effector cells at the major site of Ag deposition, in this instance, the respiratory tract.

This model implies an intrinsic difference in the responses of memory and naive T cells activated by antigenic stimulation in peripheral lymph nodes. The primary CD4$^+$ T cell response to viral respiratory infection has been well-documented and is characterized by the retention of Ag-specific T cells in DLN, during which multiple rounds of division occur over the first several days following Ag encounter and initial activation, followed by the migration of responding cells to the site of infection, (the lungs), beginning as late as day 6 p.i. In addition, these newly activated primary CD4$^+$ T cells acquire the capacity to secrete cytokines during proliferation and differentiation within the DLN (9). In contrast, our data suggest that memory CD4$^+$ T cells may only need to be retained in the DLN for a limited time (possibly much <24 h) to allow their activation and subsequent rapid egress from the nodes to the lungs without extensive proliferation in the nodes. A potential explanation for this observation has been suggested by Smith and colleagues, who propose that optimal clonal expansion and terminal differentiation of activated naive CD4$^+$ T cells requires interaction with B cells in lymph node follicles, whereas memory CD4$^+$ T cells may be capable of bypassing this requirement (19). These findings are in keeping with the extensive evidence that, when compared with naive T cells, memory T cells have a lower activation threshold, a lesser dependence on costimulation, a more rapid expression of effector activity, and altered responsiveness to certain cytokines (20–22). Furthermore, naive and memory T cells differ in the expression of cell surface adhesion molecules and chemokine receptors (23), which likely play an important role in regulating the transit time and localization of T cells (naive and memory) in the nodes. Our results suggest that, upon encounter with Ag and activation in the DLN, memory T cell.
cells have a markedly reduced retention time in the nodes compared with naive T cells, resulting in their rapid migration to the site of infection where T cell proliferation ensues. However, we cannot formally exclude the possibility that different (distinct) subsets of memory CD4+ T cells selectively respond preferentially in the DLN and the lung respectively.

Although not formally analyzed in this report, our results suggest that the memory G-specific CD4+ T cells mediating this recall response in the RSV-infected lungs most likely represent central memory CD4+ T cells, as this memory T cell subset transits through secondary lymphoid organs and has the capacity for extensive proliferation after Ag contact, while the effector memory CD4+ T cell subset can localize to peripheral tissues sites and rapidly produce cytokines in response to Ag (22–24). Using FTY720, a sphingosine-1-phosphate receptor agonist, we determined that the majority of the recall effector CD4+ T cell response to RSV in the lungs of G-primed mice is contributed by a G-specific memory CD4+ T cell component that requires activation in DLN to mediate the recall response (Fig. 3). In support of this interpretation, recent reports have demonstrated that the response of effector memory T cells is relatively short lived and subject to rapid turnover in vivo (24–26), with some even suggesting that effector memory cells may be derived from central memory precursors (27). These studies and others (28), taken in combination with our results, suggest that any G-specific memory T cell with the phenotype characteristic of effector memory T cells generated by vaccination is either short lived or represents a terminally differentiated pool of tissue-resident and/or circulating memory cells. If present, these cells may rapidly respond early in challenge infection (i.e., day 1–2), but they appear not to contribute substantially to the dominant effector T cell response producing enhanced injury in G-primed mice.

In addition, the FTY720 experiments further confirm the kinetic data within this study (Fig. 2) suggesting that G-specific memory CD4+ T cells present in the spleen do not contribute significantly to the subsequent recall response in the lungs. Because FTY720 strongly inhibits the egress of cells from the thymus, the peripheral and mesenteric lymph nodes, and Peyer’s patches, but inhibits less effectively those from the spleen or bone marrow (29, 30), any responding T cells of splenic origin would not be blocked by treatment with the drug either before or during RSV infection. As Fig. 3c demonstrates, there is inhibition of effector cell accumulation in the lungs of G-primed mice with FTY720 administration up to 24 h p.i. Although the effect of FTY720 administered on the recall CD4+ T cell response in the lungs is not due to selective drug toxicity for CD4+ T cells or an effect of the drug on viral Ag presentation in vivo, FTY720 administration has been reported to enhance regulatory T cell activation (31). Although we do not detect elevated numbers of CD4+CD25+FoxP3+ T cells in the DLN (or lungs) of FTY720-treated infected animals and only detect an effect of FTY720 when administered within the first 24 h of RSV infection, we cannot formally exclude the induction of a novel suppressor cell as the explanation for our findings. We did find that the effectiveness of FTY720 in inhibiting memory CD4+ T cell responses to challenge RSV infection increased as the interval between primary immunization and challenge infection increased. We found that an interval of 2–3 mo between immunization and challenge was optimal in our system. However the optimal time for drug administration would be likely to vary significantly depending on the type and route of vaccination and the agent used for a challenge infection and would likely need to be optimized for specific experimental conditions.

We have also examined the memory CD8+ T cell response to challenge RSV infection. (Ref. 32 and W. W, Stevens and T. J. Braciale, unpublished observations). Although the tempo of memory CD8+ T cell accumulation in the infected lungs following RSV challenge (32) directly parallels the tempo of the memory CD4+ T cell response (Fig. 1a), our preliminary analysis, using MHC class I tetramer staining, of memory CD8+ T cells activating in the DLN suggests that there is an initial burst of CD8+ T cell proliferation in the DLN (first evident at day 2 p.i.) associated with the detection of a small percentage (~10%) of activated RSV-specific CD8+ T cells in the lungs (also at day 2 p.i.). In the case of the memory response, CD8+ T cell proliferation in the lungs reaches maximum (by DNA content analysis) at day 3 p.i., whereas lung CD4+ T cell proliferation reaches maximum at day 4 p.i. Importantly, again as with the CD4+ T cell response, we detect no Ag-specific activated/proliferating memory CD8+ T cells in the spleen (or nondraining lymph nodes) of immune mice undergoing challenge infection before T cell accumulation and expansion in the lungs.

Our studies using FTY720 to inhibit CD4+ T cell egress from DLN implied that the time frame in which CD4+ T cells enter the DLN, activate, and then egress from these nodes must be brief (likely occurring over a 24 h period between 24 and 48 h p.i). This is in contrast to the prolonged retention time reported for naive CD4+ T cells responding in the DLN to respiratory virus infection in the mouse (9). This narrow window in which FTY720 inhibition occurs likely reflects, first, the time required for virus infection and replication in the respiratory tract to induce APC activation and the subsequent migration of Ag-bearing APC from the respiratory tract to the DLN during the first 24 h p.i. (33), followed then by the encounter of recirculating (and node-resident) memory CD4+ T cells with viral Ag in the DLN, and their subsequent activation and egress from the DLN in the ensuing 24 h.

It is of interest that we were unable to detect the accumulation of an expanded population of memory RSV G-specific V/β14+ CD4+ T cells in the DLN of FTY720-treated immune mice undergoing challenge infection. This result could be readily explained if the pool of circulating memory RSV G-specific CD4+ T cells is distributed throughout the blood and secondary lymphoid organs. If FTY720 administration is conducted immediately before challenge RSV infection, the memory T cells would be “trapped” (equally distributed and retained in) the secondary lymphoid organs. As a result, only a small fraction of the memory T cells would be localized to DLN at the time of challenge infection, and the proliferative expansion of the memory T cells in the DLN in response to infection would therefore be limited. This finding could also imply that activated memory CD4+ T cells, which fail to egress from the DLN, undergo accelerated apoptosis in the nodes. Although we are not able to detect preferential or increased apoptosis of the CD4+V/β14+ T cells in the DLN of infected FTY720-treated recipients (E. L. Wissinger, unpublished observations), we cannot exclude this explanation for our results. To further address the fate of activated memory CD4+ T cells “trapped” in the DLN of FTY720-treated animals, it will be necessary to develop reagents (e.g., RSV-specific MHC class II multimers) capable of identifying and quantifying the potentially small number of activated (and possibly apoptotic) memory CD4+ T cells retained in the DLN of treated animals.

Another potential implication of our findings relates to the potential contribution of recirculating memory CD4+ T cells to the recall CD4+ T cell response to Ag exposure at sites other than the respiratory tract, such as contact sensitivity and other forms of peripheral DTH responses. These responses are generally considered to be mediated by tissue-resident effector/effector-memory T cells elicited by Ag contact at the peripheral site (i.e., the skin).
Ag-specific responding memory CD4⁺ T cells are, in classic contact sensitivity, typically first detected at the site of Ag exposure 24 h after the elicitation of the response by Ag in the skin (34), with proliferative expansion of responding CD4⁺ T cells potentially occurring at this site. Our findings suggest an alternative explanation for the classic DTH response, i.e., that this rapid recall response is largely mediated by memory T cells that encounter Ag in the DLN within hours of Ag exposure, activate, and rapidly migrate from the nodes to the peripheral site over the subsequent 24–48 h. Accordingly, the contribution of long-lived circulating memory CD4⁺ T cells to the recall response (in the respiratory tract or other peripheral sites) will be particularly prominent if the time between initial Ag contact (sensitization) and the elicitation of the recall response is prolonged (i.e., weeks to months), because the frequency of cells of the effector memory subset, both in the tissues and the circulation, markedly decreases over time (35–37). Consistent with this concept, we have found that the efficiency of FTY720-mediated inhibition of the memory CD4⁺ T cell response to challenge RSV infection in the lungs increases with greater time between RSV G-specific and challenge virus infection (E. L. Wissinger and T. J. Braciale, unpublished observations).

Several studies have suggested that, for both CD4⁺ and CD8⁺ T cells, a relatively brief encounter with processed peptide/MHC complexes (<24–30 h) is sufficient to both activate these cells and render them competent to undergo multiple rounds of programmed division. In this instance, cell division occurs without continued antigenic stimulation (38–40). However, in the case of primary CD4⁺ T cells, it has also been reported that continued exposure to Ag further increases the extent of proliferation and promotes the viability of activated CD4⁺ T cells (39). For viruses producing localized infection in the respiratory tract, including RSV, the bulk of the viral Ag (in the form of virions and virus-infected cells) is present in the lungs. Thus, for memory CD4⁺ T cells, additional exposure of activated CD4⁺ T cells to Ag at the site of infection in the periphery may play a more important role in regulating the magnitude of the effector T cell response than has been observed for the induction of the primary response by naive T cells. If, as we believe, memory CD4⁺ T cells may reside in the DLN for a limited time (<24 h), then additional contact with Ag at the site of infection could be an essential step in the development of the effector phase of the memory CD4⁺ T cell response in the periphery.

A number of groups have demonstrated a correlation between the number of divisions of activated T cells and the acquisition of effector activity for both CD4⁺ and CD8⁺ T lymphocytes (9, 41–44), and our results are consistent with these earlier findings. In this report, we detected minimal effector activity (IFN-γ-secretion) by the CD4⁺ VB14⁺ T cell subset in the lungs at day 3 p.i., maximum proliferation (as detected by propidium iodide staining) at day 4 p.i., and finally maximum effector T cell activity (i.e., cytokine production) in the lungs at day 5 p.i. (Fig. 5). This finding, and the absence of significant IFN-γ production by the CD4⁺ VB14⁺ T cells in the draining PBLN and spleen over this period, suggests that the proliferation of these RSV G-specific T cells in the infected lungs is linked to the development and acquisition of effector activity by these responding CD4⁺ T cells. It remains to be determined whether this division-linked effector T cell differentiation is dependent upon antigenic stimulation in the infected lungs or is preprogrammed by T cell/APC interaction in the DLN.

Several lines of evidence suggest that the effector cytokine responses of memory CD4⁺ T cells are programmed during the initial Ag encounter of naive CD4⁺ T cells (45–49). In experimental RSV infection the effector cytokine response of memory CD4⁺ T cells directed to RSV G appears not to be fixed by the priming of naive T cell precursors because, upon challenge RSV infection, the balance between Th1 and Th2 effector cytokine production by the memory CD4⁺ T cells can be influenced by other cell types (i.e., memory CD8⁺ T cells) (2, 5, 50) and cytokines (51, 52) elicited in the lungs during challenge infection. This raises the interesting possibility that the environment of the infected lung may influence the differentiation of the memory CD4⁺ T cells entering the lung during their conversion to effector cells. Indeed, such plasticity of memory cell phenotype has been suggested previously, notably by Sallusto and colleagues, who found that polarized Th1 or Th2 human memory CD4⁺ T cells restimulated in vitro under the converse polarizing conditions could initiate gene expression with the cytokine profile opposite to the one at priming (53).

If, as our results suggest, CD4⁺ T cells complete their differentiation into effector cells at the primary site of infection, then the differentiating T cells responding to RSV may be susceptible to the immunoregulatory effects of viral gene products expressed at high levels at the site of maximum virus replication (i.e., the lungs) (32). Such a mechanism may, in part, help explain the lack of durable immunologic memory in the respiratory tract associated with RSV infection and the susceptibility to repeated infections with this virus observed in humans (54).

Disclosures
The authors have no financial conflict of interest.

References


