Cutting Edge: Virus-Specific CD4⁺ Memory T Cells in Nonlymphoid Tissues Express a Highly Activated Phenotype

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Recent studies have shown that CD4\(^+\) memory T cells persist in nonlymphoid organs following infections. However, the development and phenotype of these peripheral memory cells are poorly defined. In this study, multimerized MHC-Ig fusion proteins, with a covalently attached peptide sequence from the Sendai virus hemagglutinin/neuraminidase gene, have been used to identify virus-specific CD4\(^+\) T cells during Sendai virus infection and the establishment of peripheral CD4\(^+\) memory populations in the lungs. We show declining frequencies of virus-specific CD4\(^+\) T cells in the lungs over the course of \(\sim 3\) mo after infection. Like peripheral CD8\(^+\) T cells, the CD4\(^+\) have an acutely activated phenotype, suggesting that a high level of differentiation is required to reach the airways and persist as memory cells. Differences in CD25 and CD11a expression indicate that the CD4\(^+\) cells from the lung airways and parenchyma are distinct memory populations. The Journal of Immunology, 2002, 169: 6655–6658.

**Cutting Edge: Virus-Specific CD4\(^+\) Memory T Cells in Nonlymphoid Tissues Express a Highly Activated Phenotype\(^1\)**

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D\(^4\)\(^+\) memory T cells play an important role in the cellular response to secondary viral infections (1, 2). In addition to providing B and T cell help, peptide-primed CD4\(^+\) T cells can enhance viral clearance by an Ab-independent mechanism that is mediated in part by IFN-\(\gamma\) (3, 4). Interestingly, a recent study has shown that CD4\(^+\) memory cells in lymphoid tissues are relatively unstable and decline progressively following the resolution of a viral infection (5). It has recently emerged that virus-specific CD4\(^+\) and CD8\(^+\) memory T cells persist, not only in secondary lymphoid organs (lymphoid memory cells) but also in a variety of peripheral tissues (peripheral memory cells) including the lungs (2, 6–9). In the case of CD8\(^+\) T cells, it was found that the numbers of memory cells in the lungs decreased gradually over a 6-mo period, whereas the numbers of lymphoid memory cells remained relatively stable for the life of the animal (10). This decline in peripheral CD8\(^+\) memory cells correlated approximately with the decline in protective cellular immunity described in other studies (1). Several phenotypic and functional differences distinguished the peripheral CD8\(^+\) memory cells from their lymphoid counterparts, including the expression of several acute activation Ags and some constitutive effector functions (6, 10, 11).

Although peripheral CD4\(^+\) memory cells have been described in several models (9, 12–14), including mice that have recovered from Sendai virus infection (2), their life span and phenotype in the peripheral tissues have not been investigated.

In this study, multimerized MHC-Ig fusion proteins, containing I-A\(^b\) class II MHC molecules with a covalently attached peptide sequence from the Sendai virus hemagglutinin/neuraminidase (HN)\(^3\) gene (HN\(_{419–433}/A\(^b\) multimers), have been used to identify virus-specific CD4\(^+\) T cells during Sendai virus infection and the establishment of peripheral CD4\(^+\) memory populations in the lungs. The data show declining frequencies of virus-specific CD4\(^+\) T cells in the lungs after viral infection. Like peripheral CD8\(^+\) memory T cells, the virus-specific CD4\(^+\) T cells in the lungs remained highly activated \(> 1\) mo after viral clearance.

**Materials and Methods**

**Mice and viral stocks**

Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Taconic Farms (Germantown, NY) and housed under specific pathogen-free conditions. At 8–12 wk of age, 2,2,2-tribromoethanol-anesthetized mice were intranasally infected with 125 50\% egg infectious doses (EID\(_{50}\)) of Sendai virus (Enders), or 300 EID\(_{50}\) A/HK-x31 (x31, H3N2) influenza virus (15, 16).

**Priming mice with peptide Ags**

The Sendai virus and influenza virus epitopes have been described previously (17–19). The dominant HN\(_{421–436}\) epitope was originally defined by overlapping peptides (18). Subsequent studies (20) identified the core sequence (HN\(_{419–433}\)) used to construct the HN\(_{419–433}/A\(^b\) multimer. Peptides for vaccination and intracellular cytokine staining were purchased from New England Peptide (Fitchburg, MA). Mice were vaccinated with HN\(_{421–436}\) peptides as previously described (3) and infected 30 days after boosting.

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\(^3\)Abbreviations used in this paper: HN, hemagglutinin/neuraminidase; EID\(_{50}\), 50% egg infectious dose; NP, nucleoprotein; MLN, mediastinal lymph node; BAL, bronchoalveolar lavage.
Sample preparation for flow cytometry

Lung airway cells were collected by bronchoalveolar lavage (BAL) five times in HBSS. Single-cell suspensions were prepared from the lung tissues, spleens, and mediastinal lymph nodes (MLN) by passage through cell strainers. Dissociated lung cells were centrifuged over 40% isotonic Percoll gradients at 400 × g for 25 min. Washed cells from the Percoll interface were further purified by centrifugation over lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada). E were depleted from BAL and spleen suspensions by treatment with buffered ammonium chloride solution, and adherent cells were removed by plastic adherence. BAL cells were spun through 40% Percoll to remove low density surfactant globules.

Assembly of multimerized MHC-Ig fusion proteins for flow cytometry

MHC-Ig fusion proteins were generated using the extracellular domains of the αβ-chains from I-Ab MHC class II molecules, paired via a fos:jun leucine zipper, and attached to the hinge-Fc fragment of mouse IgG2a (20). The HN419 αβ epitope was attached to the I-Ab β-chain via a flexible linker. HN419-αβ/I-Aβ multimers were produced in S2 insect cells and purified by the Molecular Biology Core Facility at the Trudeau Institute. Fusion proteins were further multimerized using protein A conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR), for 2 h at room temperature. Cells (1 × 10^6) were stained with 1 μg of HN419-αβ/I-Aβ multimer in 100 μl of staining buffer for 1 h at room temperature, and PerCP-conjugated anti-CD4 Abs (BD PharMingen, San Diego, CA) for 30 min on ice. CD8+ T cells were stained with APC-conjugated nucleoprotein (NP)324–332/Kb tetramers and PE-conjugated anti-CD8 Abs (11). Fixed samples were analyzed using FACS caliber and CellQuest software (BD Biosciences, Mountain View, CA).

Intracellular cytokine staining

Nonadherent cells were cultured for 4 h in the presence of CFSE-labeled syngeneic spleen cells, peptides (1 μg/ml), and 20 μg/ml brefeldin A, and stained for IFN-γ as previously described (2). An influenza virus-specific peptide (hemagglutinin peptide 192–207) was used as a specificity control. CFSE+ feeder cells were excluded during analysis.

Results

Detection of virus-specific CD4+ T cells with multimerized MHC-Ig fusion proteins

The primary response to Sendai virus infection in C57BL/6 mice is dominated by CD4+ T cells specific for the HN421-436/I-Aβ epitope (18). To analyze the response to this epitope in more detail, we generated MHC-Ig fusion proteins, containing Aβ heterodimers with the covalently attached peptide sequence HN419–433, as Ag-specific staining reagents (20). As shown in Fig. 1, there were large numbers of HN419–433/I-Aβ-specific CD4+ T cells in the BAL of mice infected with Sendai virus 10 days earlier. Very little background staining was detected on BAL cells from influenza virus-infected control animals or BAL cells stained with an equivalent concentration of the protein A-conjugate alone or an irrelevant fusion protein moth cytochrome c 96–108/Eκ (20) (data not shown). These studies confirmed highly specific staining of Sendai virus-specific CD4+ T cells using the HN419–433/I-Aβ multimers.

Declining numbers of virus-specific CD4+ T cells in the lungs after Sendai virus infection

Staining with NP324–332/Kb class I tetramers or HN419–433/I-Aβ multimers (Fig. 2) and intracellular cytokine analysis (Fig. 3) were used to compare the frequency of virus-specific CD4+ and CD8+ T cells in the lung airways and parenchyma after infection. As in previous studies, the percentage of NP324–332/Kb-specific CD8+ T cells reached a maximum 9 days after viral infection and decreased only slightly over the following month (10). In contrast, the percentage of HN419–433/I-Aβ-specific CD4+ T cells peaked 1 day earlier than the CD8+ T cells and decreased substantially during the next 2 wk (Fig. 2). The frequencies of virus-specific CD4+ T cells in the lymphoid organs were below the level of detection. Very similar results were obtained by intracellular cytokine staining of IFN-γ-producing CD4+ T cells in peptide-stimulated cultures (Fig. 3). IFN-γ-producing CD4+ cells were not detected in the lungs by ELISPOT analysis beyond 100–150 days after infection (data not shown). Together, these data indicated that virus-specific CD4+ T cells in the lungs were less durable than CD8+ memory cells. This decline in peripheral CD4+ memory cells was analogous to the loss of CD4+ memory cells from lymphoid tissues (5).

Phenotypic analysis of virus-specific CD4+ memory T cells

The low frequencies of Ag-specific CD4+ T cells in the lungs beyond day 25 after infection prevented direct phenotypic analysis of the established memory population. To enhance the numbers of Ag-specific CD4+ T cells in the lungs, C57BL/6 mice were primed
CD11a was also expressed at reduced levels, as has previously been reported for CD8<sup>+</sup> T cells in the lung airways (our unpublished data and Ref. 21). Expression of OX40, CD11c, CD95, and CD154 was not detected (data not shown). A notably different pattern of surface Ags was detected on CD4<sup>+</sup> T cells from the parenchymal tissues of the lungs. Like the BAL cells, the HN<sub>419-433</sub>/Ab-specific CD4<sup>+</sup> T cells from the lung parenchyma expressed CD69 and CD44 at high levels, with reduced CD62L and CD45RB expression. However, these cells were distinct from the BAL cells in that CD25 was absent, and CD11a was expressed at high levels as in the other tissues. Many of the tetramer-negative CD4<sup>+</sup> cells in the lung parenchyma were less activated and expressed lower levels of CD44 and high levels of CD62L and CD45RB. Heterogeneous CD43 expression was found on all the lung-derived CD4<sup>+</sup> T cell populations. A substantial population of HN<sub>419-433</sub>/Ab-specific CD4<sup>+</sup> T cells in the MLNs also expressed CD25 and CD69, as has previously been reported for Sendai virus-specific CD8<sup>+</sup> memory cells (10).

Together, these markers indicate that distinct populations of virus-specific CD4<sup>+</sup> memory cells are left in the lung airways and parenchymal tissues after Sendai virus infection. The acute activation status of the CD4<sup>+</sup> cells in the BAL suggests that only the most differentiated cells reach the lung airways and persist as memory cells.

**Discussion**

In this study, we have used HN<sub>419-433</sub>/Ab multimers (20) to monitor virus-specific CD4<sup>+</sup> T cells during respiratory virus infection and to identify surface markers on peripheral CD4<sup>+</sup> memory T cells in the lungs after viral clearance. These studies show a maximum frequency of HN<sub>419-433</sub>/Ab-specific CD4<sup>+</sup> T cells in the lungs 8 days after viral infection, which declined substantially over the course of the following 2 wk. Analysis of IFN-γ-producing CD4<sup>+</sup> T cells by intracellular cytokine staining gave very similar results. These data indicate a substantial difference in the dynamics of the response by CD4<sup>+</sup> and CD8<sup>+</sup> memory T cells in the lungs, and suggest that CD4<sup>+</sup> memory populations are less stable in both lymphoid (5) and nonlymphoid tissues than are CD8<sup>+</sup> memory
The numbers of virus-specific CD4+ cells in the lungs dropped below the level of detection by ELISPOT analysis between 100 and 150 days postinfection (data not shown), whereas the frequencies and numbers of Ag-specific CD8+ cells in earlier studies remained high for several months (10). These data have important implications for vaccination, because priming CD4+ T cells with peptides can reduce the numbers of CD8+ memory cells generated during viral infection (3, 4), in essence replacing relatively stable CD8+ memory T cells with less stable CD4+ memory cells.

Analysis of surface Ags indicated that a large majority of the virus-specific CD4+ T cells in the lung airways retained a highly activated phenotype and expressed high levels of the acute activation markers CD69 and CD25 on day 41 after infection. This was similar to the activated phenotype of virus-specific CD8+ T cells in the lungs described in earlier studies (10). One possible explanation for the activated phenotype of the virus-specific memory cells was persistent presentation of Ag after infection. However, previous studies using lacZ-expressing T cell hybrids, have shown that neither NP324–332/Kb nor HN421–436/Aβ epitopes can be detected beyond day 10 postinfection (22). Therefore, it seems unlikely that the difference in numbers of virus-specific CD4+ and CD8+ T cells in the lungs after viral infection (Fig. 2) was the result of different kinetics of Ag presentation by the two epitopes. It is unclear whether peptide immunization had any lasting impact on the phenotype of the CD4+ T cells from the lungs. However, identical markers on HN419–433/Aβ-specific and multimer-negative CD4+ T cells in the BAL indicated that CD4+ cells from other viral epitopes shared the same phenotype. Mice primed with HN421–436 peptides in CFA also cleared Sendai virus with accelerated kinetics (3), and there was no indication of prolonged Ag presentation by 5-bromo-2'-deoxyuridine incorporation (data not shown). A particularly interesting finding of these studies is that CD11a expression was lower on BAL-derived CD4+ memory cells than naive CD4+ cells. Memory T cells in other peripheral tissues are CD11a+high. An identical observation has recently been reported for CD8+ memory T cells in the lung airways (our unpublished data and Ref. 21). Because CD11a has adhesion functions, it is possible that this molecule helps to control T cell migration in the lungs.

Together our data suggest that distinct populations of virus-specific CD4+ memory T cells remain in the lung airways and parenchymal tissues after Sendai virus infection. This difference is illustrated by the levels of CD25 and CD11a expression on the two cell populations and suggests that terminal differentiation may be required for T cells to reach the lung airways and persist as peripheral memory cells. Although we find different population kinetics by virus-specific CD4+ and CD8+ memory T cells in the lungs after viral clearance, both cell types give rise to distinct populations of lymphoid memory cells and peripheral memory cells that are characterized by an acutely activated phenotype. These peripheral memory cells have been shown to play an important role in protection against secondary viral infections in earlier studies (2, 10).

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References