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Respiratory Syncytial Virus–Specific CD8+ Memory T Cell Responses in Elderly Persons

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Background. We investigated respiratory syncytial virus (RSV)–specific CD8+ memory T cell responses in healthy control participants (n = 31) and in patients with chronic obstructive pulmonary disease (COPD) (n = 9), with respect to frequency, memory phenotype, and proliferative requirements.

Methods. The properties of RSV-specific CD8+ T cells were analyzed by use of RSV tetramers. The proliferative requirements of RSV-specific CD8+ T cells were analyzed by culture of peripheral-blood mononuclear cells with RSV peptide in combination with distinct cytokines.

Results. RSV-specific CD8+ memory T cells showed a high level of expression of CD27 and interleukin-7Rα and a low level of expression of CCR7. In the healthy participants, the frequency of RSV tetramer+ CD8+ T cells was significantly lower than the frequency of influenza virus A (FLU) tetramer+ CD8+ T cells (P < .0001). In contrast to FLU tetramer+ CD8+ T cells, we could detect RSV tetramer+ CD8+ T cells in the subgroup of elderly healthy participants (age, ≥55 years) and in the patients with COPD only after in vitro expansion. Expanded RSV-specific T cells produced interferon-γ and granzyme B.

Conclusion. We provide evidence that a pool of functional RSV-specific CD8+ memory T cells persists in the peripheral blood of healthy individuals and patients with COPD. Low numbers of RSV-specific memory T cells in the elderly and in patients with COPD may explain the increased susceptibility to RSV infection in these populations.

Respiratory syncytial virus (RSV) circulates through communities during annual epidemics and causes serious infections in susceptible individuals. In young children, RSV can cause severe bronchiolitis, resulting in high morbidity. Although RSV infection is widespread and virtually everyone encounters RSV from an early age on, reinfections occur frequently during life [1]. In healthy adults, reinfections lead to mild symptoms and often remain unrecognized. Recent studies have shown, however, that immunocompromised individuals, elderly individuals, and adults with underlying cardiopulmonary conditions (e.g., chronic obstructive pulmonary disease [COPD]) are at risk for severe RSV infection [2–5]. These clinically severe manifestations of RSV infection indicate that immunity to RSV is not complete in these individuals.

Presently, most data on cellular RSV immunity are obtained from animal studies. Studies in mice have shown that cytotoxic T lymphocytes (CTLs) contribute to the clearance of RSV infection [6]. In addition, it has been shown that patients with defects in T cell immune responses do not effectively clear the virus, indicating that T cells do indeed play an important role in the control of RSV infection [7]. Therefore, it may well be supposed that, analogous with other viral infections, CTL responses to RSV contribute to the control of infection and to the prevention of reinfection in humans. The establishment of a virus-specific CD8+ memory T cell pool is essential to achieve long-term immunological protection. Advances in the characterization of virus-specific CD8+ T cells have provided insight into the development of immunological memory for latent and cleared viral infections [8, 9]. The development of virus-specific CD8+ memory T cells in
RSV-Specific Memory T Cell Responses

**PARTICIPANTS, MATERIALS, AND METHODS**

**Study population.** The healthy participants (total \( n = 31 \); median age, 54 years; age range, 30–86 years), who were recruited via local advertisements, had no history of pulmonary disease and had normal lung function. The patients with COPD (\( n = 9 \); median age, 69 years; age range, 50–76 years) were recruited from the outpatient clinic of the Academic Medical Center, Amsterdam. Inclusion criteria for patients with COPD were a smoking history of at least 15 pack-years and a forced expiratory volume in 1 s (FEV\(_1\)) vital capacity ratio <0.7. The reversibility of FEV\(_1\), was ≤12% of baseline after inhalation of 400 mg of salbutamol (Airomir; Pharma). Spirometric evaluation of the healthy participants and the patients with COPD was performed as recommended elsewhere [14]. All of the patients with COPD continued their medication, which consisted of bronchodilator therapy and inhaled glucocorticosteroids.

The healthy participants and the patients with COPD had no current symptoms of FLU or RSV infection. All of the study participants provided written, informed consent, and the study was approved by the Medical Ethics Committee of the Academic Medical Center of the University of Amsterdam.

**Peptides.** The HLA-A1 RSV-M\(_{229-237}\) peptide YLEKESIYY, the HLA-B7 RSV-NL\(_{4}\) peptide NPKASLLSL [13], the HLA-A1 FLU-NP\(_{44-52}\) peptide CTELKLSDY [15], and the HLA-A2 FLU-M1\(_{18-26}\) peptide GILGFVFTL [16] were purchased from the Immunohematology and Blood Transfusion–Leiden University Medical Center peptide synthesis library facility (Leiden, The Netherlands). The peptides were dissolved in dimethylsulfoxide (5 mg/mL; Merck).

**Peptide-major histocompatibility complex class (MHC) I tetramers.** The following RSV and FLU tetramers were used: allophyocyanin (APC)–conjugated HLA-A1 tetramer loaded with the FLU-NP\(_{44-52}\) CTELKLSDY peptide (Proimmune); APC-
conjugated HLA-A1 tetramer loaded with the RSV-M\textsubscript{229–237} YLEKESIYY peptide; HLA-A2 tetramer loaded with the FLU-M\textsubscript{158–66} GILGFVFTL peptide; and HLA-B7 tetramer loaded with the RSV-NL\textsubscript{9} NPKASLLSL peptide. The last 3 tetramers were provided by Drs. D. van Baarle and K. Tesselaar (Sanquin Research at the Central Laboratory for Blood Transfusion Service, Amsterdam, The Netherlands).

**Isolation of peripheral-blood mononuclear cells (PBMCs).** Heparinized venous-blood samples were obtained from HLA-A1−, HLA-A2−, and HLA-B7−positive healthy individuals and patients with COPD. PBMCs were isolated by standard density gradient techniques and were cryopreserved until analysis.

**Flow-cytometric analyses.** PBMCs (1 × 10\textsuperscript{6}) were incubated with tetrameric complexes and different combinations of the following antibodies: CD27−fluorescein isothiocyanate (FITC; homemade clone 3A12), CD28−FITC (BD Biosciences), HLA-DR−FITC (BD Biosciences), CD45RA−phosphatidylethanolamine (PE; Sanquin Research at the Central Laboratory for Blood Transfusion Service), CCR7−PE (BD Biosciences), IL-7Ra−PE (Immunotech), CD38−PE (BD Biosciences), and CD8−PerCP-Cy5.5 (BD Biosciences). PBMCs were labeled in accordance with the manufacturers’ instructions, washed with PBS containing 0.01% (wt/vol) NaN\textsubscript{3} and 0.5% (wt/vol) bovine serum albumin (PBA), and analyzed by use of a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences).

**Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling.** PBMCs were labeled with 0.5 μmol/L CFSE (Molecular Probes) in PBS by shaking for 10 min at 37°C. Cells were then washed and resuspended in the Iscove modification of Dulbecco’s medium (IMDM) (10% human pool serum [HPS]) with antibiotics.

**Culture and stimulation of cells.** CFSE-labeled cells were cultured in culture medium for 7 days in 24-well plates at a concentration of 1 × 10\textsuperscript{6} cells/mL/well. RSV-A1, RSV-B7, or FLU-A1 peptides were added at a final concentration of 1.25 μg/mL. RSV and FLU antigens (10 μg/mL; Microbix Biosystems) were used to stimulate cells. The FLU antigen contained total viral lysate, and the RSV antigen contained lysates of in-
RESULTS

Decreasing frequency of RSV-specific CD8+ T cells with increasing age. The frequency of RSV (HLA-B7/NL and HLA-A1/M229–237) tetramer+ CD8+ T cells in the peripheral blood of the healthy participants and the patients with COPD was analyzed. In parallel, the frequency of FLU tetramer+ CD8+ T cells was determined. In the healthy participants, there was a gradual decrease in the frequency of RSV tetramer+ CD8+ T cells with increasing age ($r = -0.6; P = .003$) (figure 1), whereas there was no decrease in the frequency of FLU tetramer+ CD8+ T cells with increasing age ($r = -0.2; P = .3$). In the patients with COPD, we could not detect RSV tetramer+ CD8+ T cells, whereas we could detect FLU tetramer+ CD8+ T cells (median, 0.07%; range, 0.07%–0.80%; expressed as a percentage of total CD8+ T cells). In those healthy participants in whom we could detect both RSV tetramer+ and FLU tetramer+ CD8+ T cells, the frequency of RSV tetramer+ CD8+ T cells (median, 0.015%; range, 0.003%–0.070%) was significantly lower than the frequency of FLU tetramer+ CD8+ T cells (median, 0.08%; range, 0.03–0.46) ($P = .0001$) (figure 2).

To detect the presence of RSV-specific CD8+ T cells in a more sensitive assay, we labeled total PBMCs from the elderly healthy participants and the patients with COPD and cultured the cells for 7 days with RSV-A1 or RSV-B7 peptides in combination with IL-2. The fraction of tetramer+ CFSElow cells, gated on total CD8+ T cells, was used as a measure of the frequency of RSV-specific CD8+ memory T cells. Culture in medium alone or in the presence of IL-2 alone did not result in the proliferation of tetramer+ T cells. After culture with RSV peptide in the presence of IL-2, RSV tetramer+ CFSElow
cells, gated on CD8+ T cells, in the elderly healthy participants and the patients with COPD were 0.4% (range, 0.07%–4.0%) and 0.3% (range, 0.1%–3.0%), respectively (figure 3). The frequency of RSV tetramer+ CD8+ T cells tended to be lower than the frequency of FLU tetramer+ CD8+ T cells in both the elderly healthy participants and the patients with COPD, although the differences were not statistically significant (P = .08 and P = .2, respectively) (figure 3).

**Memory phenotype of circulating RSV-specific CD8+ T cells.**
To characterize the memory phenotype of RSV-specific CD8+ T cells, we analyzed the level of expression of CD28, CD27, CCR7, IL-7Rα, HLA-DR, and CD38 on RSV tetramer+ CD8+ T cells in 5 of the young healthy participants (median age, 34 years; age range, 30–54 years). RSV tetramer+ CD8+ T cells were uniformly characterized by a high level of expression of CD28 and CD27 and a low level of expression of CCR7. All RSV tetramer+ CD8+ T cells showed high level of expression of IL-7Rα (figure 4A–4C). These findings demonstrate that RSV tetramer+ CD8+ T cells have a memory phenotype. In addition, most RSV tetramer+ CD8+ T cells were negative for HLA-DR and CD38, indicating that they were resting memory T cells (figure 4C and table 1).

**Functional capacity of RSV-specific CD8+ memory T cells and need for antigen-induced factors to proliferate.** An im-
Table 1. Memory phenotype of respiratory syncytial virus (RSV)– and influenza virus A (FLU)–specific CD8+ T cells.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Total CD8+ T cellsa</th>
<th>RSV tetramer+a</th>
<th>FLU tetramer+a</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD27</td>
<td>92 (60–99)</td>
<td>99 (87–100)</td>
<td>94 (80–100)</td>
</tr>
<tr>
<td>IL-7Ra</td>
<td>85 (23–93)</td>
<td>91 (79–100)</td>
<td>84 (67–99)</td>
</tr>
<tr>
<td>CD28</td>
<td>86 (62–97)</td>
<td>93 (75–100)</td>
<td>93 (85–100)</td>
</tr>
<tr>
<td>CCR7</td>
<td>47 (27–77)</td>
<td>8 (0–71)</td>
<td>44 (0–57)</td>
</tr>
<tr>
<td>CD38</td>
<td>6 (3–49)</td>
<td>22 (0–44)</td>
<td>4 (0–8)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>5 (1–15)</td>
<td>12 (0–44)</td>
<td>8 (4–9)</td>
</tr>
</tbody>
</table>

NOTE. IL, interleukin.

a Values expressed are median (range) percentages of cells gated on total CD8+ T cells.

b Values expressed are median (range) percentages of cells gated on tetramer+ CD8+ T cells.

DISCUSSION

In the present study, we characterized RSV tetramer+ CD8+ T cells as resting memory T cells that circulated in low frequency in the peripheral blood of the young healthy participants and in even lower frequencies in the peripheral blood of the elderly healthy participants and the patients with COPD. These cells displayed the characteristics of fully functional memory T cells with respect to proliferative capacity, cytokine production, and expression of cytolytic mediators after reactivation in vitro.

RSV is a ubiquitous virus that infects virtually everyone annually from an early age on. RSV infection causes severe bronchiolitis in young children. After primary infection, children generate a CTL response to RSV during the first year [18, 19]. Once primary infection has been overcome, older children and healthy adults remain sufficiently protected against reinfection with RSV, which generally causes only mild symptoms. Several studies have shown that, in healthy adults, CTL responses to RSV are maintained and may contribute to the control of RSV infection. In these studies, the RSV nuclear protein and a fusion protein have been identified as targets for CTL responses [12, 20, 21]. Recent studies have shown, however, that elderly individuals and patients with underlying cardiopulmonary disease are at risk for the development of severe disease after reinfection with RSV. That RSV is associated with a high reinfection rate with accompanying high morbidity in susceptible individuals poses a challenge to the analysis of the development and maintenance of the CD8+ T cell memory response to RSV.

To achieve enduring immunological protection, it is essential that an RSV-specific CD8+ memory T cell pool persist after primary infection. After clearance of the primary infection, a memory pool is established that is composed of different patterns of expression of cell-surface markers, depending on the viral specificity of the memory T cells [8, 22]. In the present study, we analyzed the persistence of RSV-specific CD8+ memory T cells by tetramer staining for 2 immunodominant RSV...
Figure 5. Proliferative requirements of respiratory syncytial virus (RSV)-specific CD8+ T cells. Dot plot of cells stimulated for 7 days with medium alone (A), RSV-A1 peptide (B), RSV-A1 peptide plus RSV antigen (C), RSV-A1 peptide plus RSV antigen in the presence of a blocking antibody against the interleukin (IL)-2R α-chain (D), RSV-A1 peptide plus IL-2 (E), RSV-A1 peptide plus IL-7 (F), RSV-A1 peptide plus IL-15 (G), and RSV-A1 peptide plus IL-21 (H). I, Stimulation with IL-15 alone. The dot plot on the left is gated on total lymphocytes. The dot plot on the right is gated on total CD8+ T cells. Flow cytometry shown is representative of 3 experiments. CFSE, carboxyfluorescein diacetate succinimidyl ester.

epitopes [12, 13]. In line with the findings of a study by Heidema et al. [12], we found a high level of expression of CD27 and CD28 on RSV tetramer+ cells. We also performed a more extensive characterization of RSV-specific memory T cells and studied whether changes in these characteristics could be induced on in vitro reactivation of these cells. We observed a low level of expression of CD38 and HLA-DR on RSV tetramer+ T cells; these cells can be characterized as resting memory T cells. This phenotype resembles that of FLU-specific CD8+ T cells [10]. On antigen-specific reactivation in vivo or after prolonged stimulation in vitro, CD27 and CD28 expression is downregulated [23, 24]. The decrease in the level of CD27 expression is associated with the acquisition of stable effector functions of CD8+ T cells specific for persistent viruses, such as human cytomegalovirus (CMV) [24]. In contrast to CMV-specific CD8+ T cells, all RSV tetramer+ T cells expressed high levels of CD27. This indicates that, at least in the circulation, RSV infection does not persist. A recent study by Kaech et al. [11] showed

Figure 6. Production of granzyme B (GrB) by respiratory syncytial virus (RSV) tetramer+ CD8+ T cells after restimulation. Shown are dot plots of an RSV tetramer+ CD8+ T cell line (A) and isotype control (B). PE, phosphatidylethanolamine.
that, in mice, virus-specific CD8⁺ T cells that express IL-7Rα have the properties of long-lived memory T cells. RSV-specific CD8⁺ T cells uniformly expressed high levels of IL-7Rα, which were down-modulated after stimulation in vitro. The high level of expression of IL-7Rα correlated with the expression of CD27 on RSV tetramer⁺ T cells. The present data suggest that the expression of IL-7Rα on virus-specific CD8⁺ T cells is related to functionally competent long-lived memory T cells in humans as well. It remains to be investigated, however, whether the kinetics of IL-7Rα expression in the formation of memory after primary infection in humans are the same as those that have been described in mice [11]. In addition, we showed that expanded RSV tetramer⁺ CD8⁺ T cells produced IFN-γ and granzyme B when restimulated with their cognate peptide. These data indicate that RSV-specific CD8⁺ T cells are able to regain effector functions on restimulation.

The maintenance of a stable memory T cell pool is a dynamic process based on homeostatic proliferation of memory T cells and expansion on antigenic boosting. In this process, cytokines that use the common cytokine-receptor γ chain are involved. We showed that RSV-specific memory T cells adequately proliferate on stimulation with IL-2 and IL-15, and to a lesser extent on stimulation with IL-7 and IL-21, when combined with RSV peptide. These data indicate that RSV-specific memory T cells are able to reexpand in response to antigenic boosting and are responsive to stimulation with common γ-chain cytokines. In our experimental setup, we were not able to induce proliferation of RSV tetramer⁺ CD8⁺ T cells with RSV peptide alone, indicating that expansion of RSV-specific memory T cells is dependent on factors produced by helper cells.

In contrast to the findings for the younger healthy participants, RSV-specific CD8⁺ T cells were not directly detectable by tetramer staining in the peripheral blood of the elderly healthy participants and patients with COPD but were detectable only after culture. This was in contrast to FLU-specific CD8⁺ T cells, which were detectable in all participants directly ex vivo. The use of tetramers containing immunodominant epitopes of RSV or FLU provides a valuable tool for the characterization of virus-specific T cells, although it must be keep in mind that a portion of virus-specific T cells may be missed because they do not recognize the epitopes contained in the tetramer. In the present study, we related the decrease in the frequency of RSV-specific T cells in the elderly healthy participants to the frequency of FLU-specific T cells in the same participants (figure 2). Our findings indicate that the circulating RSV-specific CD8⁺ memory T cell pool is considerably smaller than the FLU-specific CD8⁺ memory T cell pool and that the RSV-specific CD8⁺ memory T cell pool decreases in size with increasing age. These findings are consistent with those of the study by Looney et al. [25] showing, in elderly individuals, hampered RSV-induced IFN-γ production in total PBMCs, a result that could be explained by a decrease in RSV memory response. We showed here that the frequency of RSV-specific memory T cells in the patients with COPD was comparable to the frequency of RSV-specific memory T cells in the age-matched healthy participants. Our data indicate that the susceptibility to develop severe symptoms on infection with RSV in patients with COPD may be due to an age-related decrease in the size of the RSV-specific CD8⁺ memory T cell pool. This susceptibility may be further increased by disease-associated local factors in the airways.

All of the healthy participants >65 years old and all of the patients with COPD in the present study received their annual influenza vaccination 4–6 months before inclusion. Recent studies have shown that influenza vaccination to some extent boosts the FLU-specific CD8⁺ T cell compartment [26, 27]. In addition, the natural reinfection rate for RSV is, apparently, lower than that for FLU [28]. This frequent antigen-specific boosting may, at least in part, explain the higher frequency of FLU-specific CD8⁺ T cells than of RSV-specific CD8⁺ T cells observed here. We showed that RSV-specific T cells are fully competent memory T cells that are able to respond to antigen-specific stimulation in vitro but need help from CD4-derived factors. These findings provide starting points for the development of strategies for vaccination (and for the use of adjuvants) that may be useful in the prevention of severe RSV infection in vulnerable populations, such as the elderly.

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