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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, $\geqslant$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

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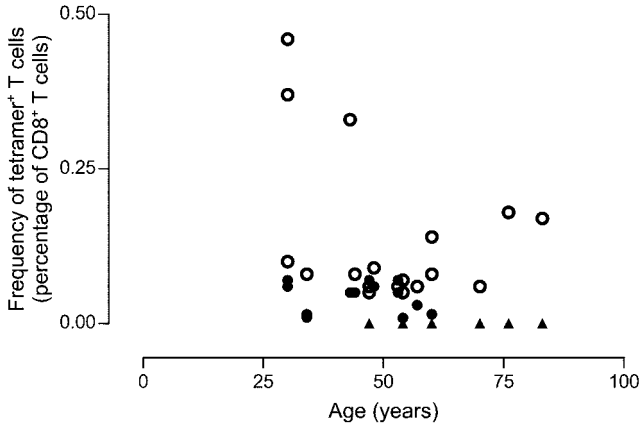

Figure 1. Correlation between age (*X*-axis) and the frequencies of respiratory syncytial virus (RSV) and influenza virus A (FLU) tetramer$^+$ CD8$^+$ T cells (*Y*-axis), in a subset of HLA-A1–positive healthy control participants ($n = 21$; median age, 53 years; age range, 30–86 years). Black circles represent RSV-A1 tetramer$^+$ CD8$^+$ T cells, black triangles represent data for the healthy participants in whom RSV-A1 tetramer$^+$ CD8$^+$ T cells were not detectable before culture (Spearman rank correlation coefficient for total RSV tetramer$^+$ CD8$^+$ T cells and age, $r = -0.6$; $P = .003$), and white circles represent FLU-A1 tetramer$^+$ CD8$^+$ T cells (Spearman rank correlation coefficient for total FLU tetramer$^+$ CD8$^+$ T cells and age, $r = -0.2$; $P = .3$). The frequencies of tetramer$^+$ T cells are expressed as percentages of total CD8$^+$ T cells.

humans has been shown to be related to the expression of different cell-surface molecules, such as CD27, CD28, and CCR7. On primary infection, virus-specific CD8$^+$ T cells are activated and differentiate into effector T cells (CD27$^+$CD28$^+$CCR7$^-$) that fight the virus. After clearance of the virus, the virus-specific T cell pool contracts, and a small memory T cell pool that can undergo rapid reactivation on reinfection persists. Virus-specific memory T cells specific for latent viruses may have distinct phenotypes, ranging from CD27$^+$CD28$^+$CCR7$^-$ to CD27$^-$CD28$^-$CCR7$^-$ [8]. With respect to cleared viruses, He et al. have demonstrated that influenza virus A (FLU)–specific T cells have a CD27$^+$ memory phenotype [10]. Recently, in a murine model of lymphocytic choriomeningitis virus infection, it was found that memory T cells also express interleukin (IL)–7Rα [11]. The ability to respond to the homeostatic cytokine IL-7 may provide memory T cells with the ability to persist for prolonged periods in the absence of antigen. Until the present study, no data have been available on the expression of this cytokine receptor on virus-specific human CD8$^+$ T cells.

Little is known about the formation and characteristics of CD8$^+$ memory T cell responses to RSV in humans. Insight into the long-term maintenance of RSV-specific memory T cells in humans after natural RSV infection and into the characteristics of RSV-specific memory CD8$^+$ T cells is essential to the understanding of memory formation and may explain the susceptibility patterns for reinfection in certain vulnerable groups, such as the elderly and patients with COPD. In the present study, we analyzed the size of the RSV-specific CD8$^+$ memory T cell pool in the peripheral blood of young healthy control participants, elderly healthy control participants, and patients with COPD. We enumerated RSV-specific CD8$^+$ T cells by use of tetramers containing 2 recently discovered immunodominant HLA class I epitopes for RSV [12, 13]. The frequency of RSV-specific CD8$^+$ T cells was determined in relation to the frequency of FLU-specific CD8$^+$ T cells. In a subgroup of the healthy participants, RSV tetramer$^+$ CD8$^+$ T cells were characterized with respect to the expression of CD28, CD27, CCR7, IL-7Rα, and activation markers CD38 and HLA-DR. Furthermore, we determined the functional characteristics of RSV tetramer$^+$ CD8$^+$ T cells with respect to proliferative requirements.

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 bronchodilatator therapy and inhaled glucocorticosteroids. The healthy participants and the patients with COPD had no current symptoms of FLU or RSV infection. All of the healthy participants >65 years old and all of the patients with COPD ($n = 12$ and $n = 9$, respectively) had been immunized with influenza vaccine 4–6 months before inclusion. 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_9 peptide NPKASLLSL [13], the HLA-A1 FLU-$NP_{44–52}$ peptide CTELKLSDY [15], and the HLA-A2 FLU-$M1_{58–66}$ 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: allophycocanin (APC)–conjugated HLA-A1 tetramer loaded with the FLU-$NP_{44–52}$ CTELKLSDY peptide (Proimmune); APC-

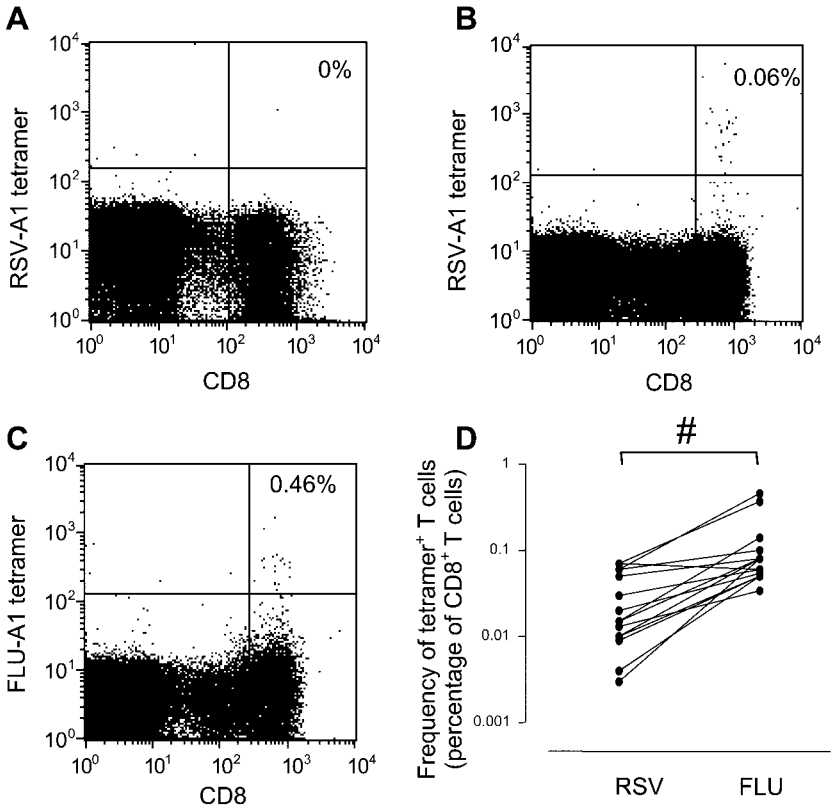

Figure 2. Detection of low nos. of respiratory syncytial virus (RSV) tetramer$^+$ CD8$^+$ T cells in peripheral blood. Shown are representative dot plots for an HLA-A1–negative healthy participant and an HLA-A1–positive healthy participant. *A,* RSV-A1 tetramer staining for an HLA-A1–negative participant. Shown is a dot plot of anti–CD8-PerCP-Cy5.5 fluorescence vs. RSV-A1 tetramer–allophycocanin (APC) of T cells gated on forward-scatter and side-scatter parameters. *B* and *C,* Dot plots showing anti–CD8-PerCP-Cy5.5 fluorescence vs. RSV-A1 tetramer–APC and influenza virus A (FLU)–A1 tetramer-APC, respectively, of T cells gated on forward-scatter and side-scatter parameters. The dot plot showing the FLU-A1 tetramer staining contains fewer events than does the dot plot showing the RSV-A1 tetramer staining. *D,* Paired measurement of FLU HLA-A1 tetramer$^+$ CD8$^+$ T cells and RSV HLA-A1 tetramer$^+$ CD8$^+$ T cells in the peripheral blood of a subset of the healthy participants ($n = 15$; median age, 44 years; age range, 30–60 years). The frequencies of tetramer$^+$ T cells are expressed as percentages of total CD8$^+$ T cells. $^{\#}P < .05$ (Wilcoxon signed rank test).

conjugated HLA-A1 tetramer loaded with the RSV-$M_{229–237}$ YLEKESIYY peptide; HLA-A2 tetramer loaded with the FLU-$M1_{58–66}$ GILGFVFTL peptide; and HLA-B7 tetramer loaded with the RSV-NL_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 use of standard density gradient techniques and were cryopreserved until analysis.

Flow-cytometric analyses. PBMCs (1×10^6) 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-7Rα–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_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 1×10^6 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-

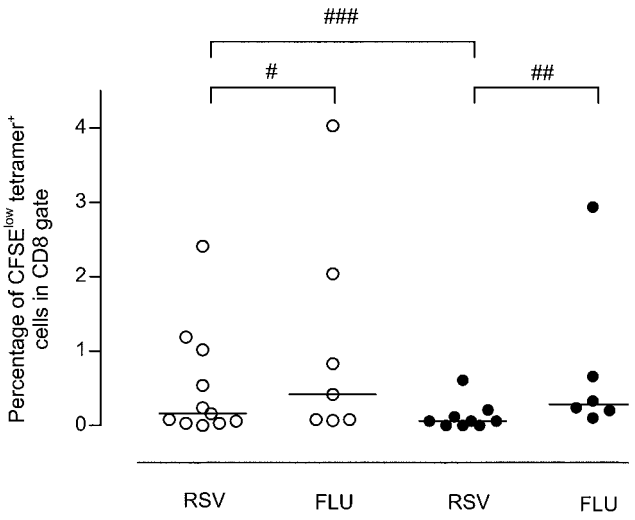

Figure 3. Frequencies of respiratory syncytial virus (RSV) and influenza virus A (FLU) tetramer$^+$ CD8$^+$ T cells after culture in the presence of RSV or FLU peptide and IL-2 for 7 days. Cells are gated on CD8$^+$ lymphocytes. White symbols represent a subset of the healthy participants ($n = 11$; median age, 72 years; age range, 54–86 years), and black symbols represent the patients with chronic obstructive pulmonary disease (COPD) ($n = 9$; median age, 69 years; age range, 50–76 years). Cells from 5 healthy participants were stained with RSV HLA-A1 tetramer, and cells from 6 healthy participants were stained with HLA-B7 tetramer. For the patients with COPD, these nos. were 5 and 4, respectively. CFSE, carboxyfluorescein diacetate succinimidyl ester. $^{\#}P = .08$ (Wilcoxon signed rank test); $^{\#\#}P = .2$ (Wilcoxon signed rank test); $^{\#\#\#}P = .2$ (Mann-Whitney U test).

fected cells. For stimulation, IL-2 (50 U/mL; Biotest), IL-7 (10 ng/mL; Strahtmann), IL-15 (10 ng/mL; R&D Systems), or IL-21 (25 ng/mL; gift from Zymogenetics) was added. For blocking experiments, anti–IL-2R α-chain (clone BG5; Sanquin Research at the Central Laboratory for Blood Transfusion Service) was used. Flow-cytometric analysis was performed before culture and after 7 days of culture.

Cytokine-producing capacity was measured by restimulation of cells after 7 days of culture by adding RSV-A1 or RSV-B7 peptides to the culture medium for 1 h at 37°C. Brefeldin A (1 μmol/L; Sigma Chemical) was added after 1 h, and cells were incubated for another 5 h. Cells were treated with FACS Lysing Solution and FACS Permeabilizing Solution (BD Biosciences). The permeabilized cells were incubated with anti–interferon (IFN)–γ–PE (BD Biosciences), anti–granzyme B–PE (BD Biosciences), or IgG1-PE (as isotype control) plus CD8-PerCP-Cy5.5 (BD Biosciences). Cells were washed with PBA and were analyzed by use of a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences).

Generation of an RSV-specific CD8$^+$ T cell line. RSV tetramer$^+$ CD8$^+$ T cells were sorted by use of FACSAria (BD Biosciences). Cells were resuspended in HPS, washed, and resuspended in IMDM (10% HPS). Autologous Epstein-Barr virus (EBV)–transformed B cells, which were UV irradiated and loaded with RSV-A1 peptide, were used as antigen-presenting cells. RSV tetramer$^+$ CD8$^+$ T cells were plated in a 96-well plate at a concentration of 1, 3, and 10 cells/well and were incubated with the RSV-A1 peptide–loaded EBV-transformed B cells (1×10^4/well), heterologous UV-irradiated PBMCs from 3 different healthy participants (1×10^4/well), and recombinant IL-2 (25 U/mL). Cells were cultured at 37°C and were restimulated weekly by exchanging medium with RSV-A1 peptide (1.25 μg/mL) and recombinant IL-2 (25 U/mL).

Statistical analysis. Between-group analysis was performed with the nonparametric Mann-Whitney U test. Within-group analysis was performed with the Wilcoxon signed rank test. For correlations, the Spearman nonparametric correlation test was used. Two-sided testing was performed. $P < .05$ was considered to be statistically significant.

RESULTS

Decreasing frequency of RSV-specific CD8$^+$ T cells with increasing age. The frequency of RSV (HLA-B7/NL_9 and HLA-A1/$M_{229–237}$) 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 (HLA-A1/$NP_{44–52}$ and HLA-A2/$M1_{58–66}$) 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$^+$ CFSE^{low} 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$^+$ CFSE^{low} cells, gated on CD8$^+$ T cells, were detected in the elderly healthy participants and in the patients with COPD at median frequencies of 0.2% (range, 0%–2.4%) and 0.06% (range, 0%–0.6%) ($P = .1$), respectively. The median frequencies of FLU tetramer$^+$ CFSE^{low}

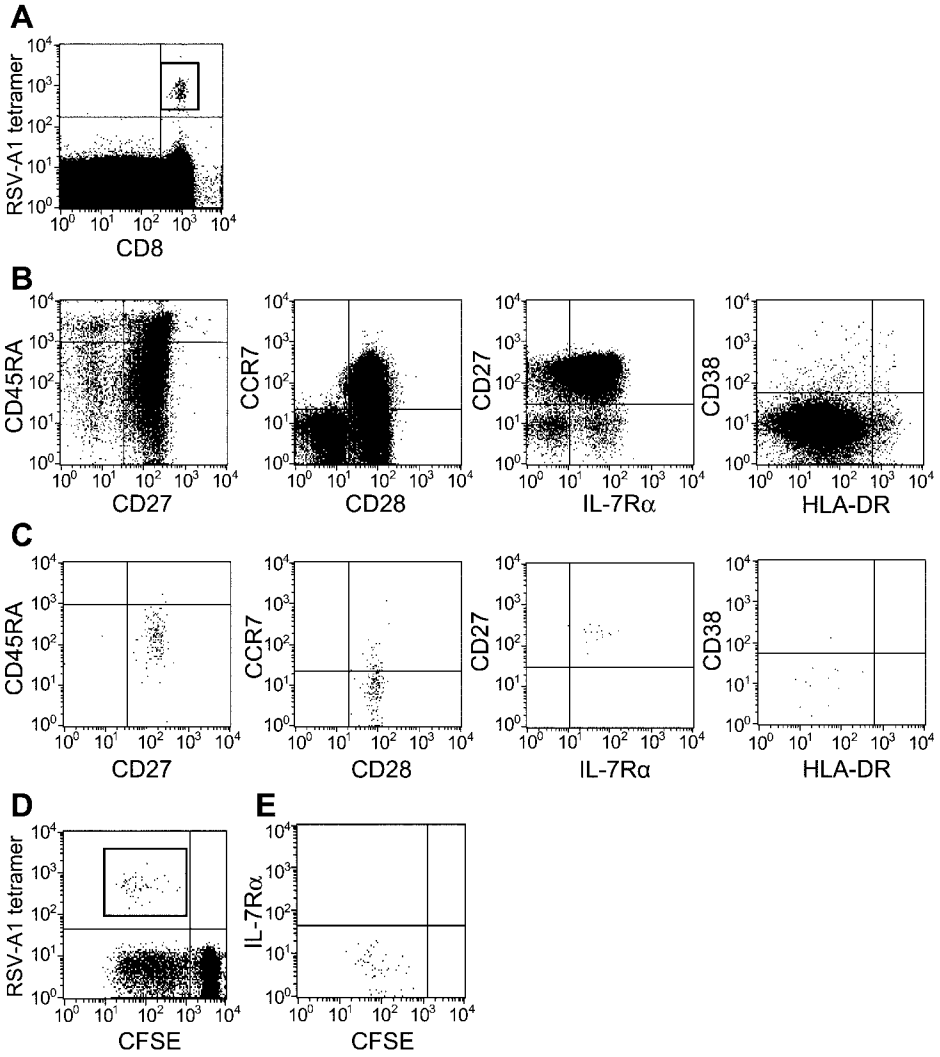

Figure 4. Memory phenotype of respiratory syncytial virus (RSV)–specific $CD8^+$ T cells. Shown is a representative dot plot from a healthy participant. *A,* Dot plot of anti–CD8-PerCP-Cy5.5 fluorescence vs. RSV-A1-tetramer–allophycocanin of T cells gated on forward-scatter and side-scatter parameters. *B,* Dot plots gated on $CD8^{bright}$ T cells. *C,* Dot plots gated on RSV $tetramer^+$ $CD8^+$ T cells. The rectangle in panel *A* represents the gate for RSV $tetramer^+$ $CD8^+$ T cells. The stainings for CD27, interleukin (IL)–7Rα, HLA-DR, and CD38 in panels *B* and *C* are obtained from a different experiment with the same peripheral-blood mononuclear cell sample from the same healthy participant. *D,* Dot plot of cells from the same participant cultured for 7 days in the presence of RSV-A1 peptide and IL-2. The rectangle in the dot plot in panel *D* represents the gate for proliferated RSV $tetramer^+$ carboxyfluorescein diacetate succinimidyl ester $(CFSE)^{low}$ T cells. *E,* Dot plot gated on proliferated RSV $tetramer^+$ $CFSE^{low}$ T cells.

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 CD27 and CD28 and a low level of expression of CCR7. All RSV $tetramer^+$ $CD8^+$ T cells showed high level of expression of IL-7Rα (figure 4*A*–4*C*). 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 4*C* 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.

Marker	Total CD8+ T cells[a]	RSV tetramer+ CD8+ T cells[b]	FLU tetramer+ CD8+ T cells[b]
CD27	92 (60–98)	99 (87–100)	94 (80–100)
IL-7Rα	85 (23–93)	91 (79–100)	84 (67–99)
CD28	86 (62–97)	93 (75–100)	93 (85–100)
CCR7	47 (27–77)	8 (0–71)	44 (0–57)
CD38	6 (3–49)	22 (0–44)	4 (0–8)
HLA-DR	5 (1–15)	12 (0–44)	8 (4–9)

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.

portant feature of CD8+ memory T cells is the capacity to expand in response to reinfection. To study whether RSV tetramer+ CD8+ T cells have this potential in vitro, we labeled, with CFSE, total PBMCs from 3 HLA-A1–positive healthy participants and cultured the cells for 7 days either in the presence of RSV-A1 peptide alone or in combination with RSV antigen. After 7 days, the frequency of RSV tetramer+ T cells was analyzed in the CFSE^{low}CD8+ fraction. Culture with RSV peptide alone did not induce antigen-specific proliferation. Culture in combination with RSV antigen, however, did result in antigen-specific proliferation of RSV tetramer+ CD8+ T cells. This proliferation could be reduced to a large extent by addition of a blocking anti–IL-2R antibody to the culture medium (figure 5*A*–5*D*). In summary, these data suggest that stimulation with RSV peptide alone is not sufficient to induce antigen-specific proliferation and that antigen-induced factors are needed to efficiently expand the RSV memory compartment.

Once a stable memory T cell pool is established in vivo, common γ-chain cytokines (IL-2, IL-7, IL-15, and IL-21) play a central role in the maintenance of memory T cells and can drive them to rapid expansion when the virus is reencountered [17]. To investigate whether common γ-chain cytokines can induce RSV-specific memory T cells to proliferate, we incubated total PBMCs in combination with peptide and either IL-2, IL-7, IL-15, or IL-21. As a control, we cultured total PBMCs with cytokines alone. Coculture of peptide in combination with IL-15 and IL-2, and to a lesser extent with IL-7 and IL-21, induced a strong proliferation of RSV tetramer+ CD8+ T cells (figure 5*E*–5*H*). When cytokines alone were cultured with PBMCs, only a small percentage of RSV tetramer+ CD8+ T cells was detected (figure 5*I*). After culture with RSV peptide in combination with IL-2 or RSV antigen, all RSV tetramer+ CD8+ T cells downregulated IL-7Rα (figure 4*D* and 4*E*).

After restimulation, memory T cells have to develop into effector cells. Therefore, we tested, in 3 of the healthy participants, whether the expanded RSV tetramer+ fraction was able to produce IFN-γ. We restimulated total PBMCs, after culture in the presence of RSV peptide and IL-2, with either RSV peptide or an HLA-mismatched peptide (FLU-HLA-A2/GILGFVFTL) as a negative control. After 6 h, we analyzed the intracellular expression of IFN-γ in the CFSE^{low}CD8+ fraction. Stimulation with RSV peptide induced a subset of IFN-γ+ cells (median, 0.8%; range, 0.4%–1.5%), gated on CFSE^{low}CD8+ T cells, which correlated well with the number of tetramer+ T cells after culture of PBMCs from the same healthy participants (data not shown). Next, we generated an RSV-specific CD8+ T cell line. After culture for 4 weeks and weekly restimulation with RSV peptide, all cells gained a high level of granzyme B expression (figure 6). These findings indicated that the expanded RSV tetramer+ T cells were indeed functionally competent memory 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, 13, 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

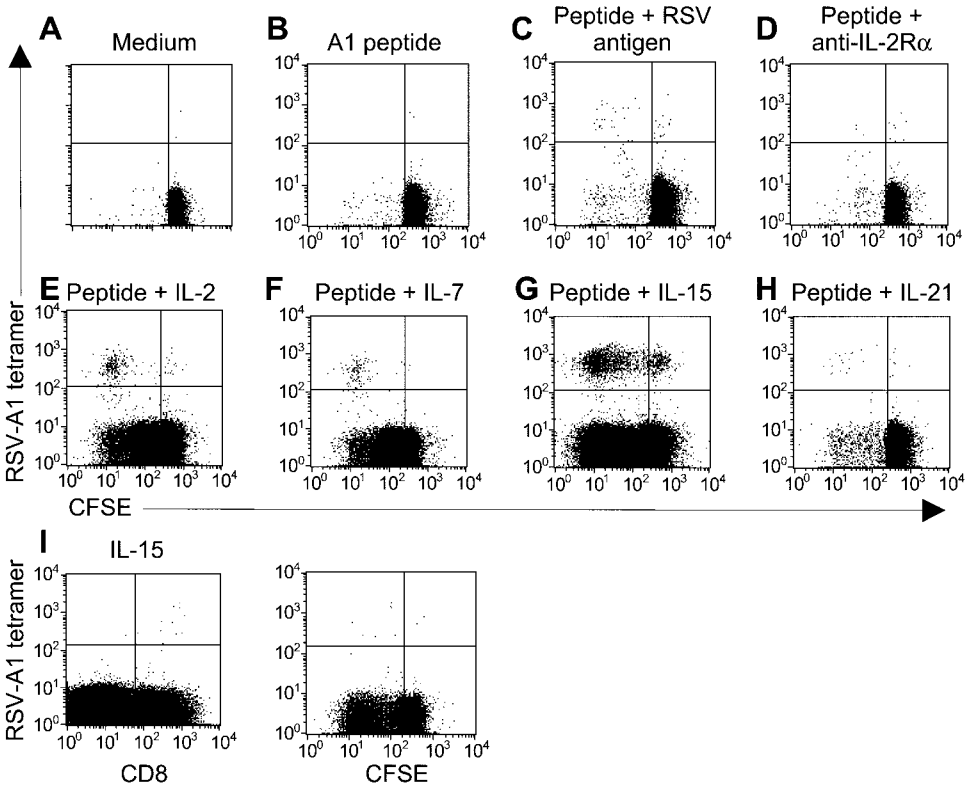

Figure 5. Proliferative requirements of respiratory syncytial virus (RSV)–specific $CD8^+$ T cells. Dot plot of cells stimulated for 7 days with medium alone *(A),* RSV-A1 peptide *(B),* RSV-A1 peptide plus RSV antigen *(C),* RSV-A1 peptide plus RSV antigen in the presence of a blocking antibody against 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

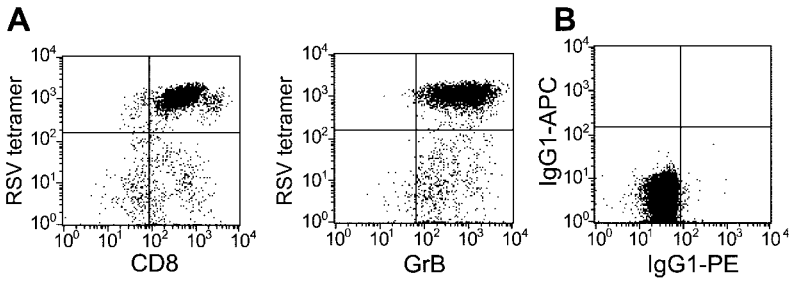

Figure 6. Production of granzyme B (GrB) by respiratory syncytial virus (RSV) $tetramer^+$ $CD8^+$ T cells after restimulation. Shown are dot plots of an RSV $tetramer^+$ $CD8^+$ T cell line *(A)* and isotype control *(B).* PE, phosphatidylethanolamine.

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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