Characterization of CD4+ Memory T Cell Responses Directed against Common Respiratory Pathogens in Peripheral Blood and Lung

Godelieve J. de Bree,1,2 Hans Daniels,3 Muriel van Schilfgaarde,4 Henk M. Jansen,1 Theo A. Out,2 René A. W. van Lier,2 and René E. Jonkers1

Departments of 1Pulmonology and 2Experimental Immunology, Academic Medical Center, Amsterdam, 3Department of Pulmonology, Medical Center Alkmaar, Alkmaar, and 4Laboratory for Vaccine Research, National Institute of Public Health and the Environment, Bilthoven, The Netherlands

Background. We investigated CD4+ memory T cell responses to influenza virus (FLU), respiratory syncytial virus (RSV), and nontypeable Haemophilus influenzae (NTHi).

Methods. The precursor frequencies of antigen-specific CD4+ cells were determined by in vitro expansion of peripheral blood mononuclear cells from healthy individuals (n = 9) and patients with chronic obstructive pulmonary disease (COPD; n = 16). The expression of CD27 and CCR7 and the production of interferon (IFN)–γ and interleukin-2 was measured directly ex vivo. Furthermore, the phenotypic and functional properties of CD4+ T cells residing in the lung were analyzed and compared with those of circulating CD4+ memory cells from the same donors (n = 8).

Results. FLU-, RSV-, and NTHi-specific CD4+ memory T cells circulated at low frequencies in the peripheral blood of healthy individuals and patients. RSV- and NTHi-specific CD4+ T cells had a memory phenotype with moderate to high CD27 and CCR7 expression. In contrast to the low frequencies of circulating FLU-specific CD4+ T cells, we found an enrichment of differentiated CD4+ FLU-specific cells and high IFN-γ expression in CD4+ memory cells in lung tissue.

Conclusion. No gross defects were found in circulating CD4+ memory cells specific for pathogens associated with COPD. However, the large differentiated CD4+ memory T cell pool residing in the lung may contribute to a large extent to local antiviral immunological protection.

Infection with common respiratory pathogens, such as influenza virus (FLU) and respiratory syncytial virus (RSV), causes serious clinical problems in susceptible individuals—the elderly, patients with underlying cardiopulmonary disease (e.g., chronic obstructive pulmonary disease [COPD]), and immunocompromised individuals [1–4]. In healthy individuals, however, re-infection with FLU or RSV causes only mild clinical symptoms. In addition to viral pathogens, persistent bacterial colonization of the lower airways—with nontypeable Haemophilus influenzae (NTHi) being one of the most frequently isolated species—has also been associated with more-severe airway inflammation and the development of clinical symptoms in patients with COPD and those with bronchiectasis [5–7]. The occurrence of clinical symptoms on reinfection suggests that the immunological defence against these common respiratory pathogens is hampered in susceptible individuals.

Immunity to respiratory viral and bacterial pathogens depends on host defense by antigen-specific CD4+ T cells. CD4+ T cells are central players in the adaptive immune response, on one hand by providing help for B cells in the production of antibodies [8] and on the other hand by supporting virus-specific CD8+ T cells [9]. Recent studies have shed light on functionally different types of antigen-specific CD4+ T cells [10]. Two subsets of CD4+ T cells with different effector functions

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Reprints or correspondence: Godelieve J. de Bree, Dept. of Experimental Immunology, Academic Medical Center, 1105 AZ Amsterdam, The Netherlands (g.j.debree@amc.uva.nl).

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can be discerned with respect to the expression of CCR7. CD4+ T lymphocytes expressing CCR7 are considered to be central memory cells that have the capacity to home to lymphoid organs. Effector-type CD4+ T cells have lost CCR7 expression and are localized in target tissues [11]. Furthermore, the expression of CD28 and CD27 on virus-specific CD4+ T cells has been shown to be related to the type and persistence of the pathogen [12, 13]. With respect to respiratory pathogens, FLU-specific CD4+ memory T cells have recently been characterized as central memory CD4+ T cells, with a high expression of CCR7, CD28, and CD27 [14–16]. At present, however, little is known about the characteristics of circulating CD4+ memory T cells specific for RSV or NTHi. Insight into the constellation and functional properties of the virus-specific memory T cell pool may provide insight into the susceptibility pattern of re-infections in vulnerable patient groups.

In the present study, we characterized the circulating CD4+ memory T cell compartment specific for FLU, RSV, and NTHi with respect to the frequency, phenotype, and functional properties in healthy individuals and, as a group of susceptible patients, in patients with COPD. We also compared the distribution and properties of FLU-specific CD4+ T cells in the lung and peripheral blood.

**SUBJECTS, MATERIALS, AND METHODS**

**Study population.** Healthy donors (n = 9; median age, 57 years; range, 50–71 years) were recruited by local advertisements, had no history of pulmonary disease, and had normal lung function. For the paired analysis of peripheral blood mononuclear cells (PBMCs) and CD4+ T cells in lungs, 8 patients (median age, 71 years; range, 69–74 years) undergoing lobectomy for a localized solitary peripheral lung carcinoma were included. All patients were free of symptoms of upper respiratory tract infection and were not receiving corticosteroids, chemotherapy, or irradiation. Furthermore, they had not received antibiotics for 2 weeks preceding inclusion in the study. Patients with COPD (n = 16; median age, 59 years; range, 48–74 years) were recruited from the outpatient clinic of our institute and from the Medical Center Alkmaar. Inclusion criteria were smoking history of at least 15 pack-years and forced expiratory volume in 1 s (FEV1):vital capacity ratio <0.7. The reversibility of FEV1 was ≤12% of baseline after the inhalation of 400 μg of salbutamol (Airomir; Pharma) [17]. Spirometric analysis was performed as recommended elsewhere [18]. All patients continued their medication, which consisted of bronchodilator therapy and inhaled glucocorticosteroids. A subgroup of patients (n = 4) had a history of recurrent infections of the lower respiratory tract with NTHi, with at least 3 positive sputum cultures during the year preceding inclusion in the study. All subjects gave written, informed consent, and the study was approved by the Medical Ethics Committee, Academic Medical Center, University of Amsterdam.

**Isolation of PBMCs.** Heparinized venous blood samples were obtained from healthy individuals and patients. PBMCs were isolated by standard density-gradient techniques and were cryopreserved until analysis.

**Isolation of lung mononuclear cells (LMCs).** LMCs were isolated from lung tissue as described by Holt et al. [19]. In brief, tissue specimens (1 × 1 cm) were sliced to 1-mm thickness and incubated for 20 min in RPMI 1640 that contained 20 mmol/L HEPES, 15% (wt/vol) fetal calf serum (FCS), and 50 U/mL DNAse (Sigma Chemical) with shaking at 37°C. Next, a second incubation step for 60 min with shaking at 37°C was performed in the same medium supplemented with collagenase (300 U/mL). A cell suspension of lung tissue was obtained by grinding the tissue through a flow-through chamber. Erythrocyte counts were confirmed to be <5% of erythrocyte counts in the paired blood sample. LMCs were isolated from the lung homogenate by standard density-gradient techniques and were cryopreserved until analysis. The yield of 1 cm3 of lung tissue was generally 10 × 106 LMCs.

**Purification of NTHi outer membrane protein P6.** P6 purification was adapted from the protocol described by Karalus and Murphy [20]. A 4-L overnight culture of NTHi strain d1 [21] was prepared in brain-heart infusion broth. Bacteria were harvested by centrifugation at 3000 g for 20 min at 4°C and suspended in 250 mL of buffer B (1% SDS, 0.1 mol/L Tris, 0.5 mol/L NaCl, and 10 μg/mL RNAse A [pH 8]; Sigma). The suspension was sonicated for 3–4 min with a Branson Sonifier at 4°C, incubated for 30 min at 37°C, and centrifuged at 60,000 g for 30 min at 25°C. The supernatant was discarded. This sequence was repeated 3 times with buffer B and 2 times with buffer B without RNAse.

The pellet was resuspended in 45 mL of Tris-buffered saline (TBS; 0.01 mol/L Tris and 0.15 mol/L NaCl [pH 7.4]) with 0.2% SDS and incubated for 45 min at 65°C. The suspension was centrifuged at 100,000 g for 1 h at 25°C. The supernatant was cooled on ice and centrifuged an additional time for 10 min at 4200 g at 4°C, to pellet excess SDS. The P6 protein was concentrated from the supernatant using an Amicon Ultra-15 centrifugal filter with a 10-kDa membrane, followed by buffer exchange with TBS. The concentration of P6 was determined using the bicinchoninic acid protein assay reagent (Pierce Chemical) with bovine serum albumin as a standard. The purity of the protein was assessed by SDS-PAGE, which revealed a single 16-kDa band. The P6 preparation was confirmed to be lipo-oligosaccharide free by tricine-SDS-PAGE, followed by silver staining [22].

**Determination of antigen-specific T cell frequency using carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution and calculation of the precursor frequency.** PBMCs were
Figure 1. No proliferation of cord blood–derived CD4+ cells after stimulation with respiratory syncytial virus (RSV) or influenza virus (FLU). Cord blood mononuclear cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured for 7 days in the presence of medium alone (red), FLU antigen (blue), RSV antigen (yellow), or phytohemagglutinin (green). Each histogram is gated on total CD4+ cells and shows the CFSE staining.

Figure 2. A, A representative example of a 7-day proliferation experiment. Total peripheral blood mononuclear cells (PBMCs) from a patient were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured in the presence of respiratory syncytial virus (RSV) antigen, influenza virus (FLU) antigen, or nontypeable Haemophilus influenzae outer membrane protein P6. Dot plots, gated on total lymphocytes, show CFSE vs. CD4–peridinin chlorophyll protein–Cy5.5 staining on day 7. Nos. in quadrants represent the precursor frequencies, calculated as described in Subjects, Materials, and Methods, for the different antigens, corrected for background cell division. 

B, Patients and healthy control subjects have similar frequencies of FLU-, P6-, and RSV-specific CD4+ T cells in the peripheral blood. Black circles represent patients, and white circles represent healthy individuals. Precursor frequencies were determined after culturing total PBMCs for 7 days in the presence of RSV antigen, FLU antigen, or P6. The precursor frequencies for the different antigens are corrected for background cell division. The precursor frequencies of RSV-specific CD4+ T cells were less abundant in both control subjects (*P = .06, Wilcoxon signed rank test) and patients (**P = .002).

labeled with 0.5 μmol/L CFSE (Molecular Probes) in PBS for 10 min with shaking at 37°C. Cells were washed and resuspended in culture medium (Iscove’s modification of Dulbecco’s medium with 10% human pool serum and antibiotics). RSV antigen (10 μg/mL; Microbix Biosystems), FLU antigen (10 μg/mL; Microbix Biosystems), purified P6 (30 μg/mL), or Staphylococcus aureus enterotoxin B (SEB; 2 μg/mL; ICN/Fluka) was used to stimulate the cells. The FLU antigen contained total viral lysate. The RSV antigen contained lysates of infected cells. After culture for 7 days, the precursor frequency (the percentage of cells in the initial population that underwent ≥1 division after culturing) was calculated as follows. First, on the basis of the CFSE histogram gated on total CD4+ T cells after culture in the presence of SEB, cell markers were set on each cell division. Next, the marker settings were copied to the CFSE histogram of cells from the same donor cultured with antigen, and the number of cells in each division was determined and used for calculation in the following formula: \[ \left\{ \frac{\Sigma_{n=1}^{n} (P_{n}/2^n)}{[\Sigma_{n=0}^{n-1} (P_{n}/2^n)]} \right\} \], where \( n \) is the number of divisions that cells have gone through and \( P_{n} \) is the number of cells in division \( n \) [23].

**Determination of antigen-specific CD4+ T cells by intracellular cytokine staining.** RSV-, FLU-, and NTHi-specific CD4+ T cell frequencies were determined as described elsewhere [10, 24, 25]. In brief, PBMCs were stimulated for 17 h with RSV antigen (10 μg/mL), FLU antigen (10 μg/mL), P6 (30 μg/mL), or SEB (2 μg/mL) at a final volume of 1 mL of RPMI 1640 (Life Technologies) that contained 10% heat-inactivated FCS, 1 μg/mL (final concentration) VLA-4 monoclonal antibody (CD49d; BD Biosciences), and CD28 (2 μg/mL, Sanquin CLB-CD28/1). For the final 5 h of culture, brefeldin A (Sigma) was added to the culture in a final concentration of 10 μg/mL. Next the cells were stained with CD4–peridinin chlorophyll protein–Cy5.5 (BD Biosciences); CD69-allophycocyanin (Caltag Laboratories); and either CD27–phycoerythrin (PE), CD28-PE, or CCR7-PE (all BD Biosciences). Subsequently, the intracellular staining procedure was performed as described above, using anti–interferon (IFN)–γ–fluorescein isothiocyanate or interleukin (IL)–2–PE (both BD Biosciences). To quantify RSV-, FLU-, or P6-specific CD4+ T cells, background levels of staining were subtracted from the antigen-stimulated samples.

**Statistical analysis.** Between-group analysis was performed...
using the nonparametric Mann-Whitney U test. Comparisons within patient groups were made using the Kruskal-Wallis test. If this yielded a significant result, further pairwise comparisons within the group were made using the Wilcoxon signed rank test. The control group was analyzed similarly. Two-sided testing was done; P<.05 was considered to be statistically significant.

RESULTS

Lower frequency of circulating RSV-specific CD4+ T cells than of FLU-specific CD4+ T cells in the peripheral blood of patients with COPD. In the immunological control and clearance of FLU infection, antigen-specific CD4+ T cells are indispensable in providing B cells help for secretion of pathogen-specific immunoglobulins [8] and in maintaining the virus-specific CD8+ T cell pool [9]. To test CD4+ T cell responses to a variety of respiratory pathogens, we first set out to analyze the frequency of FLU-, RSV-, and NTHi-specific cells in peripheral blood from healthy control subjects and patients with COPD. Purified P6 was used as NTHi-specific immunodominant epitope [26] for the analysis of P6-specific CD4+ T cell memory responses. Cell lysates of RSV- and FLU-infected cells were used as antigenic stimuli. To check for contamination, by, for instance, cytokines in the lysate, CFSE-labeled cord blood mononuclear cells were cultured in the presence of antigen (phytohemagglutinin was used as positive control). The FLU and RSV antigen preparations did not induce proliferation (figure 1).

Total PBMCs from healthy control subjects (n = 9) and patients (n = 16) were labeled with CFSE and cultured for 7 days

<table>
<thead>
<tr>
<th>Group</th>
<th>Medium (0.7–6.7)</th>
<th>RSV (0.1–6.2)</th>
<th>FLU (0.4–10.2)</th>
<th>P6 (0.2–8.1)</th>
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<tr>
<td>Patients</td>
<td>0.7 (0.7–6.7)</td>
<td>1.1 (0.1–6.2)</td>
<td>2.5 (0.4–10.2)</td>
<td>1.5 (0.2–8.1)</td>
</tr>
<tr>
<td>Control subjects</td>
<td>0.3 (0.06–3.6)</td>
<td>0.9 (0.2–3.2)</td>
<td>1.5 (0.4–6.9)</td>
<td>0.7 (0.3–4.1)</td>
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NOTE: Data are median (range) frequency.
in the presence of FLU antigen, RSV antigen, or P6. After 7 days, the precursor frequency of FLU-, RSV-, and P6-specific CD4+ cells were calculated as described in Subjects, Materials, and Methods (an example is shown in figure 2A). The antigen-stimulated samples were corrected for background cell division (uncorrected frequencies are shown in table 1). The frequencies of FLU-, RSV-, and P6-specific CD4+ T cells between healthy control subjects and patients were similar (figure 2B). However, within-group analysis showed that, compared with FLU, RSV-specific cells appear to be less abundant in control subjects (P = .06, Wilcoxon signed rank test; not statistically significant) and significantly lower in patients with COPD (P = .002, Wilcoxon signed rank test).

Central memory phenotype of circulating RSV- and P6-specific CD4+ T cells. The expression of CD27 and CCR7 on RSV- and P6-specific CD4+ T cells in a subgroup of healthy control subjects (n = 3) with high precursor frequencies for RSV and P6 was determined directly ex vivo. RSV- and P6-specific CD4+ T cells were identified on the basis of the production of IFN-γ in CD4+CD69+ T cells after in vitro stimulation with RSV antigen or P6 for 17 h (figure 3). The majority of RSV-specific CD4+ T cells coexpressed CD27 and CCR7 (figure 3; table 2), whereas the P6-specific CD4+ T cell population had a slightly lower expression of these markers (figure 3; table 2).

To determine whether presumed continuous antigen exposure is reflected by a change in the phenotypic characteristics of P6-specific memory cells, CD27 expression on P6-specific CD4+ T cells was analyzed in 4 patients with recurrent NTHi infections of the lower respiratory tract. However, both frequencies and CD27 expression were similar between colonized and noncolonized patients (data not shown).

Accumulation of differentiated FLU-specific CD4+ T cells in the lung. On the basis of the expression of IL-2 and IFN-γ by CD4+ T cells, a subdivision has been made between central memory cells, which preferentially express IL-2, and effector memory CD4+ cells, which produce mostly IFN-γ [27]. We previously found that the human lung contains a large pool of activated (HLA-DR+CD45R0+) and differentiated (CD27+CD28+) CD4+ cells, which suggests that the local CD4+ T cell pool contains primed effector-type CD4+ T cells [28]. To analyze this further, we stimulated paired samples of PBMCs and LMCs (n = 6) with SEB and measured intracellular levels of IL-2 and IFN-γ (an example is shown in figure 4A and 4B). We found a lower fraction of CD4+ cells producing IL-2 in the lung (P = .09; Wilcoxon signed rank test) and a significantly higher fraction of IFN-γ cells in the lung (P = .03) (figure 4C). The percentage of cells that expressed both IL-2 and IFN-γ was similar between the peripheral blood and lung (P = 1.0) (figure 4C). These findings indicate that the lung memory CD4+ T cell pool is skewed toward an effector phenotype.

In an earlier study, we found that, for FLU and RSV, substantial compartmentalization of virus-specific CD8+ T cells takes place in the human lung [28]. Furthermore, it has been shown that, in general, the lung contains a marked enrichment of activated CD4+CD45R0+ memory cells [18, 28, 29]. Because we found very low frequencies of circulating FLU-specific CD4+ cells, we hypothesized that these may reside locally. Therefore, we determined the percentage and phenotype of FLU-specific CD4+ T cells in paired samples of LMCs and PBMCs from 8

<table>
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<th>Cell expression</th>
<th>RSV</th>
<th>P6</th>
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<tr>
<td>CD27+</td>
<td>82</td>
<td>72</td>
</tr>
<tr>
<td>(71–93)</td>
<td>(56–100)</td>
<td></td>
</tr>
<tr>
<td>CCR7+</td>
<td>80</td>
<td>67</td>
</tr>
<tr>
<td>(71–83)</td>
<td>(63–68)</td>
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NOTE. Data are median (range) expression of CD27 and CCR7 on CD4+CD69+ interferon-γ cells (n = 3).
individuals. FLU-specific CD4+ T cells were identified on the basis of the production of IFN-γ in the CD4+CD69+ T cell fraction after in vitro stimulation with FLU antigen for 17 h (figure 5A and 5B). In this group, the percentage of IFN-γ-producing FLU-specific CD4+ T cells appeared to be higher in LMCs than in PBMCs, although this was not statistically significant (for LMCs, median, 0.25% [range, 0%–1.3%]; for PBMCs, median, 0.1% [range, 0%–0.38%]; P = .07, Wilcoxon signed rank test) (figure 5C). The CD27 expression on IFN-γ+CD69+ cells was lower on LMC-derived CD4+ T cells than on PBMC-derived CD4+ T cells (for CD27+ cells gated on CD4+CD69+IFN-γ- cells in LMCs, median, 57.9 [range, 34.9–68.8]; in PBMCs, median, 87.1 [range, 66.7–87.5]). Because of the limited availability of material, we were unfortunately unable to perform the same experiments for RSV and P6.

**DISCUSSION**

A large variety of common respiratory pathogens that circulate through the community renders groups of susceptible individuals—such as immunocompromised patients, elderly persons, and patients with pulmonary diseases, including COPD—at risk of developing clinical symptoms on reinfection [4]. Several studies have shown that CD4+ memory cells contribute to the defense against RSV [30] and FLU [31]. A central property of memory cells is their potential to proliferate in response to antigenic stimulation. In our study, we found that, on antigen-specific in vitro expansion, low frequencies of CD4+ T cells specific for FLU, RSV, and P6 were detectable, which indicated that all donors could potentially mount a CD4+ memory cell response. The precursor frequencies of antigen-specific CD4+ T cells in patients were similar in age-matched healthy control subjects. This implies that the size of the circulating CD4+ memory T cell pool in patients cannot explain the increased susceptibility to infection.

In the present study, we chose to analyze antigen-specific CD4+ T cells by using cell lysates of RSV- and FLU-infected cells. Use of this assay requires, however, that we take into account that the measured responding T cell fraction is limited to those directed against the epitopes processed and presented in the culture. It must be realized that a proportion of antigen-specific cells may be missed. Although we have to take into account that the antigen preparation differs, it seems that the frequencies of RSV-specific CD4+ memory cells were significantly lower than the frequencies of FLU-specific cells in patients with COPD. This finding is in parallel with the earlier observation that the size of the circulating FLU-specific CD8+ memory T cell pool was significantly smaller than the RSV-specific memory T cell pool in healthy elderly individuals and in patients with COPD [32]. This considerably smaller memory T cell compartment specific for RSV might be due to a lower contact frequency with RSV than with FLU. Furthermore, studies in mice have indicated that RSV has the ability to hamper adequate T cell receptor activation [33], thereby impairing the induction of an adequate T cell activation. Although it remains to be investigated whether RSV in humans makes use of similar mechanisms to evade T cell activation, it cannot be excluded that this might lead to a reduced RSV-specific memory pool, resulting in a less effective defense against RSV infection in patients.

During acute infection, naive CD4+ T cells are primed, undergo clonal expansion, and differentiate into effector cells. After clearance of the primary infection, antigen-specific memory cells persist that can be subdivided into functionally different subsets on the basis of the expression of CD28, CD7, and CCR7 [34]. On one hand, circulating CD4+ memory cells directed against FLU, which is an acute virus, show high expression of CD28 and CD7 [16] and have high proliferative potential and a good ability to give B cell help [35]. On the other hand, in situations where the antigen is not cleared but persists, as in latent cytomegalovirus infection, CD27 and CCR7 [34]. On one hand, circulating CD4+ memory cells directed against FLU, which is an acute virus, show high expression of CD28 and CD7 [16] and have high proliferative potential and a good ability to give B cell help [35]. On the other hand, in situations where the antigen is not cleared but persists, as in latent cytomegalovirus infection, CD27 [16] and CCR7 [34]. On one hand, circulating CD4+ memory cells directed against FLU, which is an acute virus, show high expression of CD28 and CD7 [16] and have high proliferative potential and a good ability to give B cell help [35]. On the other hand, in situations where the antigen is not cleared but persists, as in latent cytomegalovirus infection, CD27 and CCR7 [34].
that the CD4+ T cell compartment in the lung contains a significantly higher fraction of IFN-γ cells, compared with their circulating counterparts. Furthermore, in the lung, there is a marked enrichment of FLU-specific CD4+ T cells that show a further differentiated phenotype characterized by lower CD27 expression. This is, to our knowledge, the first study of FLU-specific CD4+ T cells residing in human lungs. Most data about the properties and distribution of virus-specific CD4+ T cells have been from animal models. Concerning respiratory viruses, memory cells have been shown to persist in both secondary organs and the lung. Reinfection induced a rapid expansion of local, lung-residing T cells [39, 40]. The data presented here are in accordance with the findings of animal studies and show that, in addition to the memory CD8+ T cell compartment, the CD4+ memory T cell fraction is also compartmentalized in the lung. These findings may provide a starting point for studying the local antigen-specific immune response in susceptible individuals and for the development of local vaccination strategies.

References


