Human CD8\(^{+}\) T cell responses against five newly identified respiratory syncytial virus-derived epitopes

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CD8\(^{+}\) T lymphocytes play a major role in the clearance of respiratory syncytial virus (RSV) infections. To be able to study the primary CTL response in RSV-infected children, epitopes presented by a set of commonly used HLA alleles (HLA-A1, -A3, -B44 and -B51) were searched for. Five epitopes were characterized derived from the matrix (M), non-structural (NS2) and second matrix (M2) proteins of RSV. All epitopes were shown to be processed and presented by RSV-infected antigen-presenting cells. HLA-A1 tetramers for one of these epitopes derived from the M protein were constructed and used to quantify and phenotype the memory CD8\(^{+}\) T cell pool in a panel of healthy adult donors. In about 60 % of the donors, CD8\(^{+}\) T cells specific for the M protein could be identified. These cells belonged to the memory T cell subset characterized by expression of CD27 and CD28, and down-regulation of CCR7 and CD45RA. The frequency of tetramer-positive cells varied between 0\(^{-}\)4 and 3 per 10\(^{4}\) CD8\(^{+}\) T cells in PBMC of healthy asymptomatic adult donors.

INTRODUCTION

Respiratory syncytial virus (RSV) is a pneumovirus in the family Paramyxoviridae. Infections with RSV occur in yearly epidemics (Brandt et al., 1973) causing mild colds in immunocompetent adults and in most children. However, about 3 % of infants under the age of 1 year develop severe bronchiolitis or pneumonia requiring hospitalization (Shay et al., 1999). The immunological basis for the different susceptibility to RSV infection, especially in infants, remains unclear. About 50 % of children who had RSV bronchiolitis during infancy have subsequent episodes of wheezing in the first decade of life (Sigurs et al., 2000; Stein et al., 1999; Bont et al., 1999).

CD8\(^{+}\) T cells play an important role in host defence during most viral infections. Indeed, it was shown that in RSV infections CD8\(^{+}\) T cells are necessary to clear the virus. While healthy children clear RSV in 7–21 days, children with disorders of T cell-mediated immunity shed the virus for months (Hall et al., 1986). Also, in murine models, depletion of CD8\(^{+}\) T cells either using monoclonal antibodies (mAbs) or by removing the thymus causes persistent shedding of virus, while the transfer of small numbers of CD8\(^{+}\) T cells results in virus clearance within 10 days (Cannon et al., 1987). However, CD8\(^{+}\) T cells can also augment disease in a dose-dependent manner (Alwan et al., 1992, 1994; Cannon et al., 1988; Graham et al., 1991). Thus, a delicate balance exists between effective virus eradication and immune pathology.

Several studies have demonstrated an RSV-specific CD8\(^{+}\) T cell response in infants after primary RSV infection (Isaacs et al., 1987; Mbawuike et al., 2001). In these studies, the specific CTL responses were mainly detected in children with mild clinical disease. It was also found that the CD8\(^{+}\) T cell memory response after primary RSV infection might be short lived, as the memory CD8\(^{+}\) T cell frequency in the pre-season of the second year was low. Other groups showed a higher frequency of RSV-specific CD8\(^{+}\) T cells in infants with severe RSV infections compared with those experiencing mild disease (de Waal et al., 2003). As there was a significant difference in age and timing of sampling between the studies, this may account for the differences found.

To study the role of CD8\(^{+}\) T cells in pathogenesis of and protection against RSV disease, the identification of CTL epitopes might be of great value. This would allow monitoring of the acute and memory CTL response in
RSV-infected patients and during vaccine trials. So far, a few RSV epitopes recognized by human CD8+ T cells have been identified (Brandenburg et al., 2000; Gould et al., 2000; Rock & Crowe, 2003; Venter et al., 2003). However, it would be useful to characterize a broader repertoire of epitopes for highly prevalent HLA alleles. In the present paper, we have identified five new RSV-specific CTL epitopes. Potential HLA class I-binding peptides, as predicted by the computer selection program SYFPEITHI, were tested by stimulating peripheral blood mononuclear cells (PBMC) from healthy adult blood donors and measurement of IFN-γ production by CD8+ T cells using intracellular staining assays. Most donors of different age groups responded to one or more of the RSV epitopes depending on the HLA alleles expressed by each individual. We further showed that all epitopes are also presented on the cell surface of RSV-infected cells. The memory T cells found in these adult donors had down-regulated CD45RA, expressed CD28 and CD27, and were CCR7low. The frequency of memory T cells varied between 0.4 and 3 per 10^6 CD8+ T cells.

**METHODS**

**PBMC.** Buffy coats were obtained from HLA-typed healthy adult blood donors who gave informed consent. As all children have been infected with RSV by the age of 3 and will be reinfected every 2–3 years thereafter, all adult donors have been primed with RSV. PBMC were isolated by Ficoll-Paque gradient centrifugation (Pharmacia Biotech) and used immediately or stored in liquid nitrogen.

**Virus and peptides.** The human RSV A2 strain stock was grown on Hep-2 cells, PEG purified and titrated by plaque assay. Candidate peptides were synthesized by standard solid-phase 9-fluorenylmethoxy-carbonyl (Fmoc) chemistry. The purity of the peptides varied from 50 to 90%, as determined by analytical reverse-phase HPLC.

**Tetramers.** Labelled HLA-B7 tetramers containing the published peptide NPKASLLSL (NP306-314) derived from the nucleoprotein (NP) of RSV were purchased from Proimmune (Goulder et al., 2000). The HLA-A1 tetramer containing peptide YLEKESIYY (M229–237) derived from the M protein was constructed by the CLB (Amsterdam, The Netherlands).

**Intracellular staining of peptide-specific T cells for flow cytometry.** To identify dominant epitopes presented by MHC class I molecules, we sequenced candidate peptides for their ability to induce IFN-γ production in CD8+ T cells. PBMC were cultured in 24-well plates (1 x 10^6 cells per well) in AIM-V medium (Gibco) supplemented with 2% human pooled serum (HPS), penicillin and streptomycin (P/S) and 40 U recombinant human IL-2 (rhIL-2) ml^-1. Peptides were added to a final concentration of 1 μM. After 10 days of culture, cells were plated in a 96-well round-bottomed plate (Costar) (0.5 x 10^6 cells per well) in AIM-V medium supplemented with P/S, 2% HPS, 40 U rhIL-2 ml^-1 and co-stimulatory antibodies (anti-CD28 and anti-CD49d). Cells were restimulated with either 1 μM of the same peptide or 1 μg staphylococcal enterotoxin B ml^-1 or not restimulated (negative control). After 1 h of incubation at 37°C, 10 μg Brefeldin A (BFA) ml^-1 (BD-PharMingen) were added to accumulate cytokines in the cell. After 5 h, 2 mM EDTA was added to arrest activation and to remove adherent cells from the well. After incubating for 20 min at room temperature, cells were washed twice in FACS buffer (PBS containing 0.02% azide, 2% FCS and 2 mM EDTA). Phycoerythrin (PE)-labelled anti-CD3 mAb (BD-PharMingen) and Cy5-labelled anti-CD8 mAb (BD-PharMingen) were added for surface staining. After 30 min of incubation on ice, cells were washed twice in ice-cold FACS buffer. Cells were permeabilized and fixed using FACS permeabilizing/fixation solution (BD-PharMingen). Cells were stained intracellularly after an additional wash in Perm-wash (BD-PharMingen) with fluorescein isothiocyanate (FITC)-labelled IFN-γ mAb (clone 304049; BD-PharMingen) for 30 min on ice. Cells were washed three times in Perm-wash and fixed in 1% paraformaldehyde in PBS for 20 min. Cells were resuspended in FACS buffer and kept at 4°C until analysis. Cell staining was analysed on a FACS Calibur using CellQuest software (BD Bioscience). A response was considered to be positive when the number of CD8+ T cells producing IFN-γ following antigen stimulation was at least three times greater than the number of cells producing IFN-γ without restimulation with antigen.

We confirmed that the epitopes that were identified were indeed presented in the context of the HLA alleles used in the epitope prediction program SYFPEITHI. To this end, we stimulated cells from two donors who were, respectively, HLA-A3, -A11, -B51, -B35 (donor 8) and HLA-A2, -B44, -C5 (donor 9) with the peptides M235–139 (predicted to be HLA-A3 restricted), M236–72 (B44 restricted), NS241–49 (BS1 restricted) and M195–203 (B51 restricted). Ten days after peptide stimulation, the responder T cell cultures were restimulated with antigen-presenting cells (APC) that only shared the HLA allele used for epitope prediction or with APC lacking this HLA allele but instead sharing some other class I molecules with the responder cells. The APC used were PBMC that had been depleted of CD3+ cells using magnetic activated cell sorting (MACS) separation (Miltenyi Biotech). The APC were loaded with the peptide epitopes for 1 h in medium without serum and unbound peptides were removed by extensive washing. Unloaded APC served as negative controls. IFN-γ production by responder T cells was measured after 5 h by intracellular staining.

**Presentation of the dominant epitopes during RSV infection.** PBMC were infected with RSV at an m.o.i. of 5 and cultured in 24-well plates (1 x 10^6 cells per well) in AIM-V medium with 2% HPS, P/S and 40 U rhIL-2 ml^-1. After 10 days of proliferation, cells were either stimulated with the appropriate peptide or not restimulated (negative control). Cells stimulated twice at day 0 and 10 with the same peptide served as a positive control. The HLA-A1 tetramer was used to identify M229–237-specific T cells 10 days after infection of PBMC with RSV. Extracellular staining of M229–237 tetramer-positive cells with anti-CD3 and anti-CD8 was performed as described below.

To identify the response against whole RSV virions in previously stimulated PBMC, we infected PBMC with RSV at an m.o.i. of 5. Cells were cultured as described above. On day 8, PBMC from the same donor were depleted for CD8+ T cells using negative-selection MACS separation columns (Miltenyi Biotech). Two days prior to use, the remaining cells were infected with RSV at an m.o.i. of 5, thus allowing the APC to present RSV epitopes. On day 10, these APC were added to the PBMC that had been stimulated with RSV on day 0. Stimulation was allowed to occur for 5 h in the presence of BFA. Intra- and extracellular staining was performed as described below.

**Phenotyping of tetramer-positive T cells.** PBMC of HLA-A1-positive donors were washed in FACS buffer and blocked in blocking buffer (FACS buffer containing 10% HPS) for 20 min on ice. Cells were stained with 5 μl aliphophocyanin-labelled HLA-A1 tetramer containing the M229–237 peptide. After 20 min of incubation at
room temperature, cells were stained with different extracellular mAbs conjugated as indicated: anti-CD8–pteridin chlorophyll a protein, -CD45RA–PE, -CD27–FITC, -CD28–FITC, -CCR7–PE (BD-PharMingen). After a further 20 min, cells were washed twice in FACS buffer and used immediately for FACS analysis.

RESULTS

Selection of peptides containing HLA-binding motifs using the SYFPEITHI prediction program

We used the SYFPEITHI prediction program (Rammensee et al., 1999; http://www.uni-tuebingen.de/uni/kki) to select possible RSV-derived CTL epitopes for five different HLA alleles: HLA-A1, -A3, -B8, -B44 and -B51. This program predicts the probability of peptides being presented by certain HLA class I molecules, based on the presence of HLA-binding motifs. HLA alleles were selected based on a high prevalence in the Caucasian population. The eight highest-scoring peptides for each HLA allele were tested for their capability to stimulate IFN-γ production by CD8+ T cells in PBMC from healthy adult blood donors. We found that the frequency of RSV-specific CD8+ T cells was too low in most donors to measure a significant direct response following short (5 h) peptide stimulation when using intracellular staining for IFN-γ (Fig. 1b). Therefore, we cultured the PBMC for different time periods with the peptides and determined that the best response was found after allowing the peptide-specific T cells to proliferate for 10 days in the presence of rHuIL2 (Fig. 1d and e; Table 1). As the IFN-γ production of CD8+ T cells would have ceased 10 days after initial peptide stimulation, cells were restimulated at this time with the same peptide for 5 h before measuring IFN-γ production. PBMC that were stimulated on day 0 but not restimulated on day 10 served as the negative control (Fig. 1c). Except for HLA-B8, at least one epitope was identified per HLA class I allele using this procedure (Fig. 1; Table 1). For the HLA-B51 allele, a second, less-dominant epitope was also identified.

All the functional epitopes were screened in at least three donors with different HLA types. The magnitude of responses between different donors varied, as well as the dominance of the response against the different epitopes. However, for all peptides it was found that all donors responding to a peptide expressed the HLA molecules used for epitope predictions, while other HLA alleles were not shared in all responding donors (Table 1). These experiments strongly suggested that the dominant epitope was specifically presented by the predicted MHC allele.

HLA restriction of RSV-derived epitopes

We next set out to affirm that the HLA molecules used for epitope predictions were indeed the restriction elements for the five newly identified epitopes. To address this issue, we measured T cell responses when the peptides were presented by APC that only shared the HLA allele used for epitope prediction with the T cells. APC that had other HLA molecules in common with the responder T cells, but lacked the expression of the HLA molecules used for epitope predictions, were used as a control. We only found a positive response when the HLA molecule that was used for epitope prediction was present on the APC (Fig. 2).

Fig. 1. (a–d) Frequency of IFN-γ-producing CD8+ T cells after stimulation of PBMC with the M264–72 peptide for different time periods. Intracellular IFN-γ production was measured directly after 5 h with no peptide added (a) and with 1 μM peptide M264–72 added (b), or in cultures grown for 10 days with peptide M264–72 and IL2, after a short restimulation (5 h) with the same peptide (d). The negative control for the day 10 time point was stimulated on the first day with the M264–72 peptide, but not restimulated on day 10 (c). (e) Frequency of IFN-γ-producing CD8+ T cells after stimulation with different peptides. PBMC were stimulated with the selected peptides on day 0 and further cultured in IL2 for 10 days. Cells were either restimulated on day 10 (right column) or not (left column). In both experiments, CD3+ cells were gated in a lymphocyte/lymphoblast gate. The percentage of IFN-γ-producing CD8+ T cells in this gated population is given in the upper right quadrant of the figures.
This procedure proved unequivocally that peptide M2151–159 was presented by HLA-A3, peptide M264–72 was presented by HLA-B44 and that peptides NS241–49 and M195–203 were presented by HLA-B51. For HLA-A1 we prepared a tetramer containing M229–237. This tetramer recognized an expanded population of CD8\(^+\) T cells in PBMC that had been cultured with the M229–237 peptide and rHuIL2 for 10 days (Fig. 3a). This tetramer-positive population was not present in PBMC that were not stimulated with the peptide or in peptide-stimulated PBMC from a donor that lacked the HLA-A1 molecule. Thus, this result confirmed that HLA-A1 was the molecule that presented M229–237.

All epitopes identified are presented on cells infected with RSV

When synthetic peptides are used to activate T cells, results should be treated with caution because a positive response does not prove indisputably that the particular peptide of the virus was originally responsible for the induction of the memory T cells that are found in the donor. Similar results may have been obtained if the peptide happened to elicit a cross-reactive T cell population that originated from a response against a different (viral) antigen. Therefore, we checked whether the dominant epitopes were processed and presented by APC after infection with RSV. In PBMC grown for 10 days with live RSV, CD8\(^+\) T cells recognized by the HLA-A1/M229–237 tetramer were expanded (Fig. 3a). Thus, the HLA-A1-restricted peptide was indeed processed and presented by APC exposed to the virus. The specificity of the response measured by tetramer staining was confirmed by the lack of staining in PBMC of an HLA-A1-negative donor cultured with RSV.

To prove that the other peptides were also presented on virus-infected cells, PBMC cultured for 10 days with RSV were restimulated with the dominant peptides or left unstimulated at this time point. Intracellular IFN-\(\gamma\) production was measured after 5 h of incubation with peptides. In RSV-infected cultures, a clear response could be detected following restimulation with peptide, although the magnitude of the response was lower than in cultures that were stimulated both at day 0 and day 10 with the peptide (Fig. 3b and c).

### Table 1. Peptide-specific CD8\(^+\) T cell responses in PBMC

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>HLA*</th>
<th>Score†</th>
<th>Donor no. and HLA type</th>
<th>Response‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>M229–237</td>
<td>YLEKESIYY</td>
<td>A1</td>
<td>32</td>
<td>D1: A1, A3, B7, B37</td>
<td>0:02–0:21</td>
</tr>
<tr>
<td>M2151–159</td>
<td>RLPADVLKK</td>
<td>A3</td>
<td>29</td>
<td>D1: A1, A3, B7, B37</td>
<td>No response</td>
</tr>
<tr>
<td>NP306–314</td>
<td>NPKASLLSL</td>
<td>B7</td>
<td>23</td>
<td>D1: A1, A3, B7, B37</td>
<td>0:04–0:12</td>
</tr>
<tr>
<td>M264–72</td>
<td>AELDRTEEY</td>
<td>B44</td>
<td>27</td>
<td>D1: A1, A3, B7, B37</td>
<td>0:07–2:0</td>
</tr>
<tr>
<td>NS241–49</td>
<td>LAKAVIHTI</td>
<td>B51</td>
<td>28</td>
<td>D1: A1, A3, B7, B37</td>
<td>0:09–2:38</td>
</tr>
<tr>
<td>M195–203</td>
<td>IPYSGLLLV</td>
<td>B51</td>
<td>26</td>
<td>D1: A1, A3, B7, B37</td>
<td>0:12–0:46</td>
</tr>
</tbody>
</table>

*HLA allele used for peptide prediction.
†Prediction score in SYFPEITHI for binding to HLA allele (maximum score 32).
‡IFN-\(\gamma\) response of PBMC stimulated with peptide on day 0 and subsequently cultured for 10 days with IL2. The percentage of IFN-\(\gamma\)-positive CD8\(^+\) T cells at day 10 was compared for samples that received no second stimulation at day 10 (first number) and samples that were restimulated at this time point for 5 h with the peptide (second number).
§Peptide described by Goulder et al. (2000).
Lower epitope-specific T cell response in PBMC stimulated with RSV is not due to interference from other dominant epitopes

The observation that the T cell response against a particular epitope is higher in cultures stimulated with the peptide compared with cultures stimulated with whole virus can be explained in several ways. One explanation may be that, within the context of the total repertoire of peptides displayed on APC exposed to whole virus, the T cell response against some epitopes might be masking the response against others. However, when RSV-infected PBMC were restimulated with APC infected with RSV, the T cell response detected was still lower than the response measured following restimulation with the peptide (Fig. 3). Therefore, we concluded that, in RSV-infected PBMC, T cell activation is less efficient compared with T cell activation induced by stimulation with peptides. Hence, a lower epitope-specific T cell response after RSV infection did not appear to reflect subdominance of the epitopes that were identified in this study.

RSV-specific responses can be detected in 50–60 % of randomly chosen healthy adults

The donors that were used for the experiments in Fig. 1 and Table 1 had been used previously to identify epitopes recognized by RSV-specific CD4\(^+\) T cells. They were selected from a slightly larger panel because they had readily detectable RSV-specific CD4\(^+\) T cell responses. We found a response against one of the five epitopes that we identified in almost every donor in this pre-selected panel. To establish whether responses against the newly identified HLA-A1 epitope were a general event in a panel of randomly chosen healthy adults, we performed an experiment in which PBMC from 13 additional donors were stimulated...
with the A1 peptide and cultured for 10 days with rHuIL2. At day 10, expanded CD8⁺ T cells specific for the M229–237 peptide were visualized by tetramer staining. Using this procedure, we could detect responses in seven of 13 HLA-A1-positive donors (Table 2). Similarly, we found a response against NP306–314 in four out of six HLA-B7-positive donors.

**Fig. 3.** Dominant epitopes presented on cells infected with RSV. (a) PBMC of an HLA-A1-positive donor were stimulated with RSV (m.o.i. of 5) or peptide M229–237 (HLA-A1) or left unstimulated, and cultured in the presence of IL2. After culturing for 10 days, peptide-specific cells were identified by extracellular staining with the M229–237-containing tetramer. An HLA-A1-negative donor served as a negative control. (b) PBMC of donor 8 (HLA-A3, -A11, -B51, -B35) were infected with RSV at an m.o.i. of 5 at day 0 and cultured with IL2 for 10 days. On day 10, the cells were either not restimulated or restimulated with peptide NS241–49 (HLA-B51) or M195–203 (HLA-B51). Cells were intracellularly stained for IFN-γ production. (c) PBMC from two different donors were stimulated with peptide M2151–159, peptide M264–72 or RSV (m.o.i. of 5) at day 0 and expanded in the presence of IL2. On day 10, cells were not restimulated (1 and 3), restimulated with peptide (2 and 4) or restimulated with autologous APC infected with RSV (5). Cells were intracellularly stained for IFN-γ production. In all parts of the figure, CD3⁺ cells were gated in a lymphocyte/lymphoblast gate. The percentage of IFN-γ-producing CD8⁺ T cells is given in the upper right quadrant of the figures.

**Frequency and phenotype of RSV-specific memory CD8⁺ T cells**

The frequency of RSV-specific memory CD8⁺ T cells in PBMC was low. It was necessary to culture PBMC with antigen in vitro in order to detect RSV-specific CTL responses using intracellular staining assays. To determine
the frequency of CD8+ memory T cells, we performed direct tetramer staining experiments using PBMC from five healthy HLA-A1-positive donors. Using this procedure, we determined that the frequency for the HLA-A1 epitope M229–237 varied between 0.4 and 3 per 10^4 CD8+ T cells. All tetramer-positive cells were CD45RA dull, CD27+, CD28+ and about 80% were CCR7−, thus resembling an extra-lymphoid memory T cell phenotype. A representative example of the experiments performed is shown in Fig. 4.

**DISCUSSION**

In this paper, we have reported the identification of five novel RSV-derived epitopes that are presented on the surface of RSV-infected cells by HLA class I molecules A1, A3, B44 and B51 (two). These epitopes are recognized by CD8+ memory T cells present in peripheral blood of healthy adult blood donors. We performed this identification focussing on some of the most prevalent HLA alleles to ensure that the information obtained would be applicable in a large population in future studies aimed at unravelling the mechanism of the development of RSV-specific primary and memory immune responses. To our knowledge, five RSV-derived epitopes that are recognized by human CD8+ T cells have been described before. The first epitope that was identified was HLA-B7 restricted. This epitope was found by testing a series of overlapping peptides derived from the NP of RSV in enzyme-linked immunospot assays using PBMC from healthy adults (Goulder et al., 2000). Using a similar procedure, an HLA-B8-restricted epitope in the NP and an HLA-A1-restricted epitope in the F protein were identified (Venter et al., 2003; Rock & Crowe, 2003). Two additional epitopes derived from the F protein were found to be presented by HLA-B57 and HLA-C12, relatively uncommon HLA alleles (Brandenburg et al., 2000). These two epitopes were recognized by T cell clones that were cultured from PBMC of RSV-infected children. Our newly defined epitopes thus add to this repertoire two epitopes derived from the M protein, two derived from the M2 protein and one from the NS2 protein. Interestingly, an early study using vaccinia recombinants claimed that human T cell responses directed against the RSV NP, SH, F, M, M2 and NS1 molecules are most prevalent (Cherrie et al., 1992). The responses against the NS2 and P proteins were each found in one donor out of the panel of nine donors tested. However, the HLA type of donors was not revealed in that paper. Hence, we do not know whether the HLA molecules for which we determined

<table>
<thead>
<tr>
<th>Donor</th>
<th>Tetramer+/CD8+ (%)</th>
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<tbody>
<tr>
<td>HLA-A1</td>
<td></td>
</tr>
<tr>
<td>D13</td>
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<td>D14</td>
<td>0.40</td>
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<tr>
<td>D15</td>
<td>0.98</td>
</tr>
<tr>
<td>D16</td>
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<td>D17</td>
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<td>D19</td>
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<tr>
<td>D20</td>
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<td>&lt;0.01</td>
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<td>D24</td>
<td>&lt;0.01</td>
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<td>D25</td>
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<tr>
<td>HLA-B7</td>
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<td>D27</td>
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<tr>
<td>D28</td>
<td>0.08</td>
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<tr>
<td>D29</td>
<td>0.16</td>
</tr>
<tr>
<td>D30</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>D31</td>
<td>&lt;0.01</td>
</tr>
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</table>

Table 2. CD8+ T cell responses against peptides M229–237 and NP306–314 in PBMC of randomly chosen healthy adult donors

Values are percentages of proliferating tetramer-positive cells out of total CD8+ T cells after 10 days stimulation with peptide and IL2.

Fig. 4. Frequency and phenotype of RSV-specific CD8+ T cells. PBMC from healthy HLA-A1-positive adults were extracellularly stained for tetramer HLA-A1/M229–237, CD8 and a combination of differentiation markers. The position of the quadrant was determined on a live gate (right panels), while the differentiation of tetramer-specific cells was determined on a live tetramer-positive gate (left panels). This experiment was repeated in five HLA-A1-positive donors.
the epitopes in the present paper were expressed in this donor population and, if so, how frequently.

The epitope prediction program SYFPEITHI proved very useful in selecting candidate epitopes recognized by CD8\(^+\) T cells. For HLA-B8 only, we did not find a new peptide among the top eight predicted peptides. Indeed, the HLA-B8-restricted nonamer epitope N\(_{235–263}\) that was described in the literature by Venter et al. (2003) had a SYFPEITHI score of 19 (the maximum score for HLA-B8 is 33), ranking this peptide number 74. Of the peptides that we identified in this paper, three had the highest score in the SYFPEITHI program (M\(_{229–237}\)/HLA-A1; M\(_{264–72}\)/HLA-B44; N\(_{241–49}\)/HLA-B51). The other two peptides were among the seven highest (M\(_{151–159}\)/HLA-A3) and four highest (M\(_{195–203}\)/HLA-B51) scoring peptides. The SYFPEITHI score for the NP\(_{306–314}\) peptide described by Goulder et al. (2000) was 23, the maximum score for HLA-B7, which was shared by three other peptides. However, in our experiments only the NP\(_{306–314}\) peptide induced IFN-\(\gamma\) production in CD8\(^+\) memory T cells (data not shown). Rock & Crowe (2003) characterized a decamer epitope, F\(_{109–118}\), in the F protein of RSV. A shorter nonamer version of this peptide could also induce a CD8\(^+\) T cell response but was somewhat less effective. The SYFPEITHI scores for these peptides were, respectively, 4 and 22, ranking the nonamer peptide number 19. By our selection method we would have overlooked these epitopes. In conclusion, the SYFPEITHI programme is a useful tool to predict candidate CD8\(^+\) T cell epitopes. However, not all epitopes will be successfully predicted.

We found that memory CD8\(^+\) T cells specific for RSV epitopes are present in small numbers in healthy adult donor blood. To detect CD8\(^+\) T cell responses by measuring the production of IFN-\(\gamma\) with intracellular staining, an in vitro expansion of CD8\(^+\) T cells was necessary to measure responses above the detection limit of the assay. In Table 2, we show the results of this type of experiment performed in a small set of randomly chosen HLA-A1 and HLA-B7 donors. In about 60% of the donors, we found a response against the M\(_{229–237}\) and NP\(_{306–314}\) peptides, meaning that responses against these epitopes are probably frequently occurring events in the population. The PBMC used in these experiments were isolated from donor blood sampled both outside and during the RSV season. However, to date we have not found a direct relation between the level of the responses and the time the blood samples were taken. To answer the question of whether there is a difference in the magnitude of the T cell response during and outside the RSV season one would need to study RSV-specific T cell responses in one patient at different time points, rather than comparing the responses of different individuals. We are currently addressing this issue in more depth in a study underway in our laboratory, whereby the CD4\(^+\) and CD8\(^+\) T cell responses against RSV are monitored at different time points during and outside the RSV season in a panel of healthy adult donors.

We directly compared the magnitude of the CD8\(^+\) T cell response against the published HLA-B7 epitope with the magnitude of the response of the HLA-A1-restricted epitope from the M protein of RSV described in this paper. When tested in donors that expressed both HLA-A1 and HLA-B7, peptide-specific CD8\(^+\) responses against the two peptides were comparable. Moreover, the number of tetramer-specific T cells found was similar to that described by Goulder et al. (2000) who first used the B7/NP\(_{306–314}\) tetramer.

In donors that expressed more than one of the HLA alleles presenting known epitopes, T cell responses against two or more epitopes could often be detected. However, we also found donors that carried the right HLA restriction elements who did not respond to the newly identified epitopes. It may be that in these donors the RSV-specific T cell frequency was too low to detect a response, even after expansion of CD8\(^+\) T cells. Whether these donors were low responders for RSV, or whether other dominant epitopes were recognized, is not known. This issue was not further pursued, because of the inefficient expansion of CD8\(^+\) T cells in PBMC exposed to intact RSV. We showed that, in cultures that had been stimulated with the intact virus, CD8\(^+\) T cells could be expanded that recognized the newly identified epitopes. However, it appeared that CD8\(^+\) T cell expansion by culturing PBMC with virus is less efficient than the T cell expansion obtained with synthetic epitopes. Thus, from these experiments we could not make a reliable estimation of the contribution of the T cells that recognized the single epitopes to the total memory T cell response.

Several factors might explain the suboptimal expansion of T cells after culturing PBMC with intact virus. First, RSV only efficiently enters monocytes when PBMC are exposed to the virus, while synthetic peptides will be presented on all MHC class I-positive cells. Thus, the number of APC expressing peptide MHC complexes will be much larger when peptides are added to the PBMC cultures. Secondly, the number of MHC peptide complexes per cell might be larger on peptide-loaded cells than after intracellular processing of viral proteins. Thirdly, RSV is known to have a suppressive effect on T cell proliferation (Schlender et al., 2002; Salkind et al., 1991). Thus, the presence of live virus might be responsible for an inefficient expansion of CD8\(^+\) T cells in the cultures. This might result in an under-estimation of the total number of CD8\(^+\) T cells that are specific for the virus. A fourth factor might be the presence of multiple viral epitopes presented on APC after infection with virus. T cells specific for the different epitopes thus would have to compete for the interaction with the APC and/or different epitopes might have to compete for binding to a certain MHC allele. However, these latter phenomena are less likely to have contributed to the lower peptide-specific CD8\(^+\) T cell responses that we observed within RSV-stimulated PBMC. We found that the response was also low against virus-infected target cells, i.e. cells
expressing the same epitope repertoire to which the T cells reacted initially (Fig. 3c).

The use of MHC tetramers has provided the technical means to phenotype CD8+ T cells during different stages of the immune response. Thus, phenotypic information has been obtained during primary responses and in the memory stage of the immune response against different chronic virus infections, such as human cytomegalovirus, Epstein–Barr virus (EBV), hepatitis C virus (HCV) and human immunodeficiency virus (He et al., 2003; Callan et al., 1998; Hislop et al., 2001; Urbani et al., 2002; Appay et al., 2002). In the late chronic stage of infection, CD8+ T cells specific for these viruses differ significantly with respect to the surface expression of the CD27 and CD28 molecules that were used as markers to type the maturation stage of the memory T cells. In this study, we showed that RSV-specific CD8+ T cells are CD45RA dull, CD27+, CD28+ and CCR7+. According to the expression of these markers, the phenotype of RSV-specific cells resembles that of influenza A virus-, EBV- and HCV-specific CD8+ memory T cells (Appay et al., 2002; He et al., 2003; van Lier et al., 2003). All donors analysed for the frequency of RSV-specific CD8+ T cells directly ex vivo were healthy and showed no signs of respiratory infections. Blood was sampled outside the RSV season. These factors indicated that the RSV-specific CD8+ cells in the peripheral blood were ‘resting’ memory cells. Overall, these observations suggest that healthy adult donors indeed have an antiviral immunity to RSV, characterized by the presence of low-frequency IFN-γ-producing CD8+ CD45RA−, CD28+, CD27+ and CCR7− T cells.

In conclusion, we have identified five new human RSV-derived CD8+ T cell epitopes that were HLA-A1, -A3, -B44 or -B51 restricted, a significant addition to the formerly published epitopes since they are presented by HLA alleles that are highly prevalent in the Caucasian population. The knowledge of antigenic epitopes will allow us to address the role of RSV-specific CD8+ T cells in the delicate balance of controlling infection and the possible role these cells may have in immune-mediated pathology during primary RSV infection.

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