CD8⁺ T Cell Responses in Bronchoalveolar Lavage Fluid and Peripheral Blood Mononuclear Cells of Infants with Severe Primary Respiratory Syncytial Virus Infections


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CD8+ T Cell Responses in Bronchoalveolar Lavage Fluid and Peripheral Blood Mononuclear Cells of Infants with Severe Primary Respiratory Syncytial Virus Infections

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A protective role for CD8+ T cells during viral infections is generally accepted, but little is known about how CD8+ T cell responses develop during primary infections in infants, their efficacy, and how memory is established after viral clearance. We studied CD8+ T cell responses in bronchoalveolar lavage (BAL) samples and blood of infants with a severe primary respiratory syncytial virus (RSV) infection. RSV-specific CD8+ T cells with an activated effector cell phenotype: CD27+/CD28+; CD45RO+CCR7−CD38+CD44HIHLA-DR+Granzyme B+CD127+ could be identified in BAL and blood. A high proportion of these CD8+ T cells proliferated and functionally responded upon in vitro stimulation with RSV Ag. Thus, despite the very young age of the patients, a robust systemic virus-specific CD8+ T cell response was elicited against a localized respiratory infection. RSV-specific T cell numbers as well as the total number of activated effector type CD8+ T cells peaked in blood around day 9–12 after the onset of primary symptoms, i.e., at the time of recovery. The lack of a correlation between RSV-specific T cell numbers and parameters of disease severity make a prominent role in immune pathology unlikely, in contrast the T cells might be involved in the recovery process. The Journal of Immunology, 2007, 179: 8410–8417.

Respiratory syncytial virus (RSV) is the most common cause of severe lower respiratory tract infections in infants. Fifty to 70% of all children become infected during the first year of life, which usually results in symptoms of mild upper respiratory tract infection. However, a considerable proportion of infants develops lower respiratory tract disease and 1–3% of the entire birth cohort requires hospitalization because of respiratory distress and feeding problems (1, 2). Of these hospitalized children, ∼10% needs mechanical ventilation (3, 4). Morbidity is highest during the first 6 mo of life and in infants with chronic lung disease of prematurity, congenital heart disorders, immune system disorders, and in infants born preterm.

Since the implementation of non-bronchoscopic bronchoalveolar lavage (NB-BAL) in very young infants, several groups have studied the cellular immune response to primary RSV infection in ventilated patients (5–7). It has consistently been shown that neutrophils are the predominant cells in NB-BAL, accounting for up to 85% of the total cell count, and that the total number of lymphocytes is low (0.1–9%). Interestingly, postmortem studies of children who died from RSV infection showed a lymphocytic peribronchiolar infiltrate in the lungs (8, 9).

The role of CD8+ T cells, while appreciated as a crucial component of an anti-viral immune response, has not been extensively addressed in studies of human RSV infections. Studies in mouse models and in patients with deficiencies in T cell responses provided evidence for an important role for CD8+ T cells in clearance of the virus (10, 11). However, it has been shown in the mouse model that they can also cause immune pathology (12–14). Indications for immune mediated pathology in humans were also found during a vaccine trial in the 1960s, in which infants were vaccinated with formalin-inactivated whole virus. Exposure of vaccinated infants to natural RSV infection resulted in enhanced morbidity and mortality (15, 16). At present it is still an issue of debate whether T cell responses are the cause or cure during severe primary RSV infections (17, 18).

In previous studies several groups have described the presence of RSV-specific T cells in peripheral blood and/or nasal washes of RSV-infected infants (19–23). However, these studies used in vitro expansion protocols to be able to enumerate and functionally test virus-specific T cells. Moreover, to our knowledge no information besides differential cell counts is available on T cells present in the airways during RSV infections in infants. With the introduction of HLA class I tetrameric complexes a method of direct detection of virus-specific cells was introduced. The recent characterization by our group (24) and others (23, 25–27) of several dominant RSV T cell epitopes provided the necessary tools to study the role of CD8+ T cells during RSV infection.

In the present study, we describe our investigation into the dynamics and function of the primary CD8+ T cell response in the airways and peripheral blood during a localized severe primary respiratory infection. RSV-specific CD8+ T cells were positively
identified and characterized as activated effector cells. In blood, virus-specific T cell responses were heterogeneous and RSV-specific T cell numbers peaked at the moment of discharge from the intensive care unit.

Materials and Methods

Study population and sample collection

Thirty-two infants admitted to the pediatric intensive care unit of the Wilhelmina Children’s Hospital were included during the winter seasons of 2002–2006. They all required mechanical ventilation because of respiratory failure due to RSV lower respiratory tract infection. RSV status was confirmed by indirect immunofluorescence or PCR on nasopharyngeal aspirates. RSV-specific T cell responses in the context of HLA-A2, the allele most frequently expressed in the human population are very low in adults (23) and antigenic epitopes for HLA-A2 had not been identified at the time we started our study, we included patients expressing HLA-A1 and HLA-B7 for which immunodominant epitopes were known (24, 27). Excluded from the study were children above 52 wk of age and infants with known immune deficiencies. In 2006, a new T cell epitope was characterized by elution from HLA-A2 molecules purified from RSV-infected dendritic cells (DCs) (28). We used this peptide (NS132–34 KLHLTLNAL) in some functional studies with PBMC of HLA-A2-positive infants.

The control group consisted of 18 infants that underwent surgery for nonrespiratory pathology. The control group was born after the RSV season and was included before the next season had started. We also studied some functional studies with PBMC of HLA-A2-positive infants.

To be able to perform HLA tetramer staining of fresh BAL samples, immunization by elution from HLA-A2 molecules purified from RSV-infected dendritic cells (DCs) (28). We used this peptide (NS132–34 KLHLTLNAL) in some functional studies with PBMC of HLA-A2-positive infants.

The control group consisted of 18 infants that underwent surgery for nonrespiratory pathology. The control group was born after the RSV season and was included before the next season had started. We also studied some functional studies with PBMC of HLA-A2-positive infants.

Direct HLA typing of the patients

To be able to perform HLA tetramer staining of fresh BAL samples, immediate limited HLA typing was performed. Therefore, 50 µl of whole blood was stained with HLA-specific Abs (IHB-Hu-037 for HLA-A1/A36, and IHB-Hu-035 for HLA-B7 kindly supplied by Dr. A. Mulder (LUMC, Leiden, The Netherlands). After 30 min of incubation at room temperature, cells were stained with FITC-conjugated IgG1 for IHB-Hu-035 and IgMx for IHB-Hu-037, DAKO F0315 and F0317). After another 30 min of incubation at room temperature, cells were washed in FACS buffer (PBS containing 2% FCS, 0.1% sodium azide, and 2 mM EDTA). Erythrocytes were lysed using lysis buffer (BD Bioscience) for 15 min. Cells were washed twice in FACS buffer and analyzed by FACS.Calibur flow cytometer and CellQuest software (BD Biosciences). The HLA type of the patients was confirmed at a later stage by PCR using stage-specific EBV B cell lines of the patients. PCR-SSP was performed according to the instructions of the manufacturers (Biotest).

Phenotyping of CD8+ T cells and RSV-specific T cells

To maximize cell viability, BAL samples were processed directly after sampling. PBMCs were isolated by Ficoll-Paque gradient centrifugation (Pharmacia Biotech), stored in liquid nitrogen, and stained at the same time after all samples of one patient were collected. Cells were washed in FACS buffer and blocked in FACS buffer containing 10% pooled human AB serum (HPS) for 30 min on ice. Cells were stained with 10 µl of the appropriate HLA-A1, or B7 tetramer. The allophycocyanin-conjugated HLA-B7 tetramer containing the peptide NP299S134 (NP306–314) and the HLA-A1 tetramer containing peptide YLEKESIYY (M229–237) were purchased from Sanquin.

After 20 min incubation at room temperature, cells were washed and further stained with different extracellular mAbs, FITC-conjugated anti-CD3, -CD4, -CD8, -CD14, -CD16, -CD19, -CD27, -CD45RA, -CD45RO, PE-conjugated anti-CD3, -CD4, -CD8, -CD14, -CD27, -CD28, -CD38, -CD45RO, -CD56, -HLA-DR, -CCR7, and allophycocyanin-conjugated anti-CD3, -CD8, -CD28 were all purchased from BD Pharmingen except CD8-PE-FTC (Sanquin; specifically used in combination with tetramers) and CD127-PE (Immunotech). For intracellular staining, cells were permeabilized and fixed using FACS permeabilizing/fixation solution (Perm/Fix; BD Pharmingen). Cells were stained intracellularly with FITC-conjugated anti Ki-67, PE-conjugated anti-Granzyme B (GzmB) or their isotype control (BD Pharmingen). For surface stained samples, 7-amino-actinomycin D (7AAD; BD Pharmingen) was added just before FACS analysis to visualize cell viability. Cell staining was analyzed on a FACS.Calibur using CellQuest software (BD Bioscience).

IFN-γ expression upon in vitro stimulation

Peptide- or virus-exposed monocyte-derived DCs were used to study the virus-specific CD8+ T cell response in peripheral blood of RSV patients. Because we had only small frozen aliquots of patient PBMC, DCs were made from monocytes derived from adult volunteers that shared HLA alleles with the patients. The culture procedure and infection of DCs was performed as described before (29). As control cells, we used immature DCs cultured for 48 h with 50 ng/ml TNF-α (Strathmann) and 50 ng/ml IL-1β (Strathmann) to obtain mature DCs with a similar pattern of maturation markers as the RSV-infected DCs. Infection levels and maturation status were checked by measuring the expression of the viral F protein at the cell surface and the expression level of costimulatory molecules CD80/86 and HLA class II as well as the mature DC marker CD83. 1 × 10^5 DC- and 5 × 10^6 patient-derived PBMC were cocultured for 5 h. Total RSV-specific responses were compared with the responses against synthetic peptides. These peptides (NP306–314: M229–237, and NS133–142 at 1 µM) were added to cocultures of PBMC and uninfected mature DC. After 1 h, brefeldin A was added to the cultures to accumulate IFN-γ inside the responding T cells. Surface and intracellular staining was performed with allophycocyanin-conjugated anti-CD8 and PE-labeled IFN-γ-specific mAb (BD Pharmingen; clone 340449).

To check whether RSV-infected DCs efficiently presented viral epitopes to CD8+ T cells adult HLA-B7+, PBMC were cultured for 9 days in the presence of peptide NP306–314 and 20 U/ml recombinant human IL-2. The IFN-γ response of expanded peptide-specific T cells was compared when peptide exposed and virus-infected DCs were used in an in vitro 5 h stimulation assay.

Statistical analysis

Differences in absolute cell numbers and percentages of activation and differentiation were assessed with the nonparametric Mann-Whitney U rank sum test. Tests were two tailed and p ≤ 0.05 was regarded as statistically significant.

Results

Highly activated CD8+ effector T cells accumulate in the airways during acute RSV infections

During the RSV seasons of 2002–2006, NB-BAL samples were collected from 32 patients on mechanical ventilation for RSV bronchiolitis and from 18 control patients without respiratory infections who had to be intubated before surgery. The average age of admission was 8.7 (SEM 1.3) weeks for the RSV patients and 13.9 (SEM 2.3) weeks for the controls, which was a nonsignificant difference. The mean time from onset of clinical symptoms to intubation was 6.3 days (range 2–21) in RSV patients and the mean time of ventilation was 11.1 days (range 2–21). We studied the BAL leukocyte composition in detail by cytospin and FACS analysis. Neutrophils were the most abundant cell type found in the cysitpsins of BAL of RSV patients (85%, range 71–96), while in controls, monocyte/macrophages were the predominant cell type (79%, range 75–83). Lymphocytes were found at 1.5% (range 0–5) in RSV patients vs 8% (range 0–15) in controls. Eosinophils were rare in both RSV patients (1%, range 0–7) and controls (0.3%, range 0–1). By FACS analysis, CD16 was found on 76%...
young infants, most CD8+ T patients and compared them to controls. In contrast to PBMC of only migrated to the lower airways, in patients as well as controls, memory phenotype (CD45RA negative staining (mean of tested patients).

To measure RSV-specific T cell responses in the airways during severe RSV infection we used HLA-A1 and HLA-B7 tetrameric complexes containing RSV epitopes M229–237 and NP306–314. Both epitopes are derived from a well conserved region of RSV proteins, and no strain differences have so far been reported. In 22 of 23 infants that expressed either HLA-A1 or HLA-B7 (confirmed by PCR-SSP) we were able to detect an RSV-specific CD8+ T cell response, although at low percentages (average 0.58% of CD8+ T cells). T cells of the HLA-A1/B7 controls that had never been exposed to RSV did not stain positive with the tetramers. There was no correlation found between the percentage of RSV-specific CD8+ T cells and time after intubation, age, duration of mechanical ventilation or severity of disease. To determine the differentiation and activation status of RSV-specific cells, BAL samples were stained with HLA tetramers and different cell markers. The RSV-specific T cells had the phenotype of effector cells: CD45RO+, CD27+, CD28+, CCR7-, similar to the total CD8+ T cell population. Most CD8+ T cells (>93%) were CD127 negative, at all time points during ventilation. The percentage of activated CD38+GzmB+ cells was higher in the tetramer-positive fraction compared with the total CD8+ T cell population (Fig. 1, D and E). To study whether RSV-specific CD8+ T cells were proliferating, tetramer stained cells were co-stained for Ki-67. Forty-eight percent (range 30–61%) of tetramer-positive CD8+ T cells positively stained for Ki-67 indicating that they were actively proliferating cells.

The absolute number of virus-specific T cells that we detected with tetramer staining in BAL was very low. An explanation for the small numbers of tetramer-positive cells might be the time and location of sampling, i.e., in the airways. In the mouse it was observed that effector T cell numbers in BAL increased when the peak of the response in tissue had subsided (30). During the 2005–2006 RSV season two children with a tracheotomy came to our hospital who had recovered from a severe primary RSV infection respectively one and three months earlier. In these two cases we had the opportunity to sample tracheal aspirate at a later time point than the BAL samples that we obtained from ventilated patients who were usually extubated around day 11 after onset of symptoms. Indeed in these two patients we found higher percentages of tetramer-positive CD8+ T cells (3.7% and 6.7% after respectively one and three months) than in any of the HLA-A1-positive patients tested at earlier time points during infection, where the RSV-specific response never exceeded 1.8% (Fig. 2).

### Numbers of activated CD8+ T cells in PBMC of RSV-infected infants peak at the time of extubation

In addition to T cell response in BAL we studied CD8+ T cells in PBMC of RSV-infected infants who were usually extubated around day 11 after onset of symptoms. In these two patients we found higher percentages of tetramer-positive CD8+ T cells (3.7% and 6.7% after respectively one and three months) than in any of the HLA-A1-positive patients tested at earlier time points during infection, where the RSV-specific response never exceeded 1.8% (Fig. 2).

### Table I. FACS analysis of BAL samples of RSV-infected children

<table>
<thead>
<tr>
<th>Cell Marker</th>
<th>Percentage of Cells</th>
<th>Range</th>
<th>Range of Absolute Cell Number (10⁵/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16 (granulocytes)</td>
<td>76.4</td>
<td>65–97</td>
<td>1.5–10.9</td>
</tr>
<tr>
<td>CD14 (monocytes)</td>
<td>25.0</td>
<td>19–36</td>
<td>0.4–4.5</td>
</tr>
<tr>
<td>CD19 (B cells)</td>
<td>0.4</td>
<td>0.1–0.5</td>
<td>0.0–0.5</td>
</tr>
<tr>
<td>CD56 (NK cells)</td>
<td>0.4</td>
<td>0.3–0.4</td>
<td>0.1–0.6</td>
</tr>
<tr>
<td>CD3 (T cells)</td>
<td>1.9</td>
<td>0.4–4.4</td>
<td>0.3–5.9</td>
</tr>
<tr>
<td>CD4+ of CD3+</td>
<td>28.1</td>
<td>21–39</td>
<td>0.1–1.2</td>
</tr>
<tr>
<td>CD8+ of CD3+</td>
<td>62.2</td>
<td>50–80</td>
<td>0.2–3.0</td>
</tr>
</tbody>
</table>

* The percentage of cells from total live cells, based on FCS/SSC and 7AAD negative staining (mean of tested patients).
* CD16 is mainly expressed on granulocytes but also on minor populations like macrophages and NK cells.
of the patients as determined by different surface marker combinations: RSV-specific CD8+ T cells in peripheral blood of RSV patients

The total number of activated CD8+ T cells in the peripheral blood of the patients as determined by different surface marker combinations: GzmB+/Ki-67+ cells, HLA-DR+/CD27low or CD38high/CCR5+ cells was in all patients around 100-fold higher than the number of virus-specific CD8+ T cells that we could detect with single peptide containing HLA tetrameric complexes. This observation might suggest that only an extremely small fraction of the virus-specific response was visualized by the HLA tetramers that we could detect with single RSV epitopes. To address this issue we performed experiments that could give an impression of the magnitude of the RSV-specific component in the total activated CD8+ T cell population. We previously showed that monocyte derived DCs could be infected with RSV. Although the infection with the virus strongly inhibits the ability of such DC to support proliferative responses in T cells (29), the class I Ag presentation route was intact and virus-specific memory T cells responded functionally with the production of IFN-γ to stimulation with infected DC (unpublished results and Fig. 4A). Because we had only small amounts of cryopreserved PBMC of the patients available, we used monocyte derived DCs of adult blood donors as APCs that partially matched the HLA type of the patients. Infected and uninfected DC were cocultured with PBMC samples of the RSV patients obtained at different time points after hospitalization. Fig. 4B shows an example of the RSV-specific T cell response in the context of a set of three HLA alleles that were matched between the patient T cells and the adult APC. This response was substantially higher than the response against a single virus epitope, NS133–41, restricted to HLA-A2. Moreover, the T cell response visualized by stimulation with RSV-infected DC showed the same kinetics as the appearance of Ki-67+/GzmB+ cells and CCR5+ cells and represented roughly one third of the total activated T cell response in this patient. Fig. 5 shows the RSV-specific T cell response in PBMC of a second patient after exposure to RSV-infected DC sharing HLA-A2 alone or HLA-A2 in combination with HLA-B35 or A24 and B44. This experiment shows that the response against the only known epitope restricted to HLA-A2 represented only a minor fraction of the HLA-A2 restricted response in this patient when epitopes of the entire virus are presented. Moreover, the HLA-B35 and A24 plus B44 alleles significantly contributed to the response. As in the experiment shown in Fig. 4B the RSV-specific CD8+ T cell response in blood was larger by the time the patient had recovered. Table II summarizes the RSV-specific T cell responses in 9 patients. The fraction of IFN-γ producing RSV-specific T cells
stimulated by APC sharing two to three alleles with patient T cells varied from 3.4–47% of total activated T cells based on Ki-67/H11001/GzmB/H11001 staining. The responses against synthetic peptides M229–237 and NP306–314 that were earlier identified as dominant epitopes in adult memory responses were around the detection limit of the assay (data not shown). These data confirm that virus-specific CD8⁰ T cell responses were substantially higher than the responses visualized with HLA tetramers. We did not find a correlation between the total number of activated CD8⁰ T cells in peripheral blood of RSV patients and parameters of disease severity.

Discussion

In the present study we showed that substantial numbers of CD8⁰ T cells infiltrate the airways during severe primary RSV infections. Furthermore, we positively identified RSV-specific CD8⁰ T cells in this local site as activated effector type cells. As expected in a peripheral site (34, 35), the CD8⁰ T cells had an activated phenotype in both patient and control BAL samples. Most cells were CD45RO⁰, CD28⁰, CD27⁰, CCR7⁰, CD127⁰. The high proportion of CD8⁰ T cells in the BAL of patients expressing GzmB and HLA-DR and a somewhat lower expression of CD27 (36) further indicated that preferentially highly differentiated effector type CD8⁰ T cells migrated to the airways. These findings resemble those of a recent murine study showing that predominantly the effector CD8⁰ T cell population migrated from alveolar capillaries into the lung interstitium (37). It was shown in this study that this process was dependent on G protein-coupled receptor triggering in T cells. In noninflamed lungs constitutive expression of RANTES/CCL5 by airway epithelial cells might explain a continuous recruitment of effector cells from the circulation that express CCR5 the receptor for RANTES. RSV infection leads to increased levels of RANTES expression in the lungs which might explain the enhanced recruitment of CCR5⁰ effector T cells to the lungs during an ongoing RSV infection (38, 39).

Naive and memory T cells depend on IL-7 for maintenance. IL-7Ra (CD127) expression is down-regulated on effector T cells during acute infection and can therefore be used as a marker of effector cells (31). After clearing of Ag, memory T cells re-express CD127 (35). We found that in both the control group and RSV patients CD8⁰ T cells in BAL were CD127 negative. Recently, de Bree et al. (36) evaluated the presence of virus-specific T cells in lung tissue of elderly lung carcinoma patients, who had at the time of lobotomy no symptoms of upper respiratory tract infection. They found a higher frequency of T cells directed against cleared respiratory viruses in lung tissue compared with blood. It was shown that virus-specific T cells had an activated phenotype, CD45RO⁺ CD27low, but in contrast to the situation of acute RSV infection shown by us, the expression of CD127 was high which

FIGURE 2. RSV-specific CD8° T cells during convalescence. One (A) and 3 (B) mo after a severe primary RSV infection, the number of RSV-specific CD8° T cells was determined in tracheostoma aspirates of two patients. Cells were gated for live CD8° T cells.

FIGURE 3. Activated CD8° T cell numbers in peripheral blood peak at the time of recovery after a primary RSV infection. PBMC were drawn on admission, day 4, at the time of extubation and during the convalescence phase. Day 1 indicates the first day after intubation, which was 5 days after onset of clinical symptoms in this patient. Cells were gated on a CD8° gate and a live lymphocyte gate based on the forward side scatter. The experiment shown is a representative example of 15 individual patients. In all patients, a similar pattern of activation markers and chemokine receptor expression was observed. Moreover, in all patients, activated effector CD8° T cell numbers were highest at the time of extubation. Only the level of the response varied significantly.
recently shown in mice that T cells found in the airways after viral clearance are effector type cells, continuously replenished from blood. This process continues for months, as long as a reservoir of processed viral Ag is present in the draining lymph nodes (41).

Interestingly, in the two tracheostoma patients the percentage of RSV-specific CD8\(^+\) T cells seemed higher in the airways during the convalescence phase of the response than at earlier time points (Fig. 2), which might also reflect the continuous recruitment of effector cells. Some caution is required because we were unable to make this comparison longitudinally in the same patients. The opportunity to obtain material from the “lower” airways in the two tracheostomy patients that had recently recovered from a proven severe RSV infection were unique events. Therefore, we only had two samples that could be used to determine the level of the RSV-specific CD8\(^+\) T cell response at a later time point. However, the observation that later after infection T cell numbers increased in the airways is similar to observations we made in mice (30).

It is not clear what percentage of CD8\(^+\) T cells in BAL of patients reflected the RSV-specific response. The percentage of virus-specific cells detected with two different tetramers was low (<1.8%). However, there was a difference in the percentage of Ki-67-positive proliferating cells during acute infection in RSV patients vs controls (22.5% vs 4.1%) which points to recent activation of these cells in patients. In the tetramer-positive fraction the percentage of Ki-67-positive cells was even higher: ~47%. Because we found that in peripheral blood of patients the number of CD8\(^+\) T cells responding to RSV-infected APCs was much higher than the number of tetramer\(^+\) cells, it is likely that also in BAL the Ki-67 \(^+\) CD8\(^+\) T cells reflected for a large part virus-specific cells. Unfortunately, for technical reasons we were unable to perform stimulation assays with T cells derived from BAL samples using RSV-infected DC to directly measure the total RSV-specific response.

Because the total number of virus-specific T cells in peripheral blood was much higher than the numbers we detected with single tetramers or single peptide stimulation assays it seemed likely and was indeed shown in Figs. 4 and 5 that there were additional RSV epitopes restricted by different HLA alleles (24). The HLA tetramers used contained epitopes that were found to be immunodominant in memory responses of healthy adults. However, immunodominance during primary and secondary responses may not be the same as was previously shown for influenza epitopes that were dominant during primary infection in mice, but less dominant during secondary infections (42, 43). Also for RSV we have found in mice that the response during secondary infection is focused on a single epitope derived from the matrix protein while the primary response is more heterogeneous (30). During primary RSV infection there might thus be other epitopes with a significant contribution to the response that we missed because we based the choice for the epitopes contained in the tetramers on the specificity of adult memory T cell responses. Obviously, also T cells responding to HLA alleles for which we have not performed epitope searches might significantly contribute in the total antiviral response as is shown for HLA-B35 and HLA-A24 and B44 (Fig. 5). Because we used partially HLA matched DC as APCs and the infection level of the DC varied, the total virus-specific response might approach the total activated T cell response even closer than is shown in Figs. 4 and 5 and Table II.

During primary RSV infections in mice there is a large lymphocytic influx into the lungs. It was shown that although depletion of CD8\(^+\) T cells delayed viral clearance, it diminished illness severity (14). This observation led to the hypothesis that this delicate balance between viral clearance and immune pathology might account for the differences in severity of illness observed in infants during primary RSV infection. Because it is not easy for ethical
reasons to obtain BAL samples from infants with mild infections, we have so far been unable to perform extensive studies to link levels of CD8⁺ T cell responses in the airways to disease severity. Within the presented patient group there was no correlation between any of the clinical parameters and the level of the CD8⁺ T cell response in BAL or peripheral blood. This might suggest that CD8⁺ T cells were not involved in immune pathology, but in contrast contributed to the recovery process. Several studies have shown that at the time of hospitalization for severe RSV infections, which is usually 3–6 days after the onset of primary symptoms, the viral load is already declining in nasal aspirates and BAL samples (44–47). We found that CD8⁺ T cell responses in blood peaked at the time of extubation which was at least 4–7 days after hospital admission and presumably also 4–7 days after the peak of the viral load. Thus during primary RSV infections severe airway symptoms preceded the peak of the T cell response in blood. It is currently unclear how the kinetics of the CD8⁺ T cell response measured in blood relates to the infiltration of virus-specific effector T cells in lung tissue. Welliver et al. recently reported that low numbers of lymphocytes were present in post mortem lung tissue samples of children with severe untreated RSV infections (17). Their findings suggested that T cells might not contribute to the disease process.

In summary in the present report we present the first time evidence that RSV-specific CD8⁺ T cells are present in NB-BAL during primary severe RSV infection. Although we found a low frequency of RSV-specific T cells by using HLA tetramers, these were activated effector cells. Comparisons with T cell responses in patients with mild disease could in the future contribute to discriminate between a role for viral load vs immune pathology as parameter affecting disease course.

Acknowledgments
We thank patients and parents for participation in this study, nurses, anesthetists, and assistants for their help in obtaining BAL samples and Dr. Caroline Lindemans for providing blood samples. We thank Professor

Table II. Overview of patient characteristics and RSV-specific CD8⁺ T cell responses in peripheral blood

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Age (days)</th>
<th>Days Hospitalized</th>
<th>% GzmB⁺/Ki-67⁺</th>
<th>% CCR5⁺</th>
<th>% RSV-Specific CD8⁺ T Cells</th>
<th>Number of Matching Alleles</th>
<th>Infection Level DC (%)</th>
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<tr>
<td>505</td>
<td>29</td>
<td>8</td>
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<td>22.2</td>
<td>6.48</td>
<td>3</td>
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References


