Acute lung injury in children: from viral infection and mechanical ventilation to inflammation and apoptosis

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Activation of the Granzyme Pathway in Children with Severe RSV Infection

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ABSTRACT

Granzymes, serine proteases present in granules of effector lymphocytes, are involved in several host immune responses, including the activation of cell death and inflammatory pathways. The main goal of this study was to determine whether the local cell-mediated granzyme pathway is activated during severe respiratory syncytial virus (RSV) lower respiratory tract illness (LRTI) in children. Tracheal aspirates from 23 children with RSV-LRTI and 12 controls without pulmonary disease were analyzed for granzyme (Gr) A and B. Bronchoalveolar lavage fluid samples from 7 children with RSV-LRTI were analyzed for cellular expression of GrB. Levels of GrA and GrB in tracheal aspirate were significantly increased in RSV patients as compared to controls and both granzymes showed preserved activity. Granzyme levels correlated with the total leukocyte counts and IL-8 levels in the airways at several time points. However, no correlation between granzyme levels and release of caspase-cleaved cytokeratin-18 was found. There was evidence for marked expression of GrB by both CD8+ and CD4+ T-cells and natural killer cells in the respiratory tract. These findings suggest activation of the cell-mediated granzyme pathway during severe RSV-LRTI in children.
INTRODUCTION

Respiratory syncytial virus (RSV) is a major respiratory pathogen among infants and young children. Although in general RSV infection is limited to the upper respiratory tract the disease may progress to the lower airways leading to acute hypoxemic respiratory failure. The most severe cases of RSV-lower respiratory tract illness (RSV-LRTI) fulfilling the criteria of acute lung injury (ALI) or the acute respiratory distress syndrome (ARDS) are described. Despite decades of research the exact mechanisms that determine the development of severe RSV-LRTI in previously healthy children remain unclear.

One hypothesis proposes that activation of cell death pathways directed against RSV-infected cells and/or uninfected bystander cells contributes to disease severity. Although regulated cell death or apoptosis seems an important mechanism for RSV clearance, Welliver et al. showed marked expression of the apoptosis marker caspase-3 in airway epithelium of children with fatal RSV-LRTI, suggesting an imbalance in this process. Interestingly, increasing evidence implicates epithelial apoptosis in the pathogenesis of ALI/ARDS. Several studies demonstrated increased expression of classical apoptotic mediators such as FasL and granzymes in the lungs of adults with ALI/ARDS correlating with disease severity. These findings implicate enhanced activation of apoptotic pathways in the pathogenesis of acute inflammatory lung diseases and may also be relevant for severe RSV-LRTI in children.

In anti-viral host immune response an important cell death pathway is the granule exocytosis pathway involving the serine proteases granzyme (Gr) A and GrB, which is exploited by CD8+ T-lymphocytes (CTLs) and natural killer (NK) cells (reviewed elsewhere). Granzymes induce rapid cell death when directed into a target cell, and this mechanism is facilitated by the protein perforin, but may also occur independently from perforin. Detection of free extracellular GrA and GrB is considered to reflect cytotoxic activation of the cell-mediated immune response. However, proteolytic active extracellular granzymes may also contribute to the activation of pro-inflammatory cytokine release and degradation of the extracellular matrix, indicating that granzymes may be involved in several mechanisms of the host immune response.

Studies investigating the cell-mediated immune response in the lungs of children with RSV-LRTI have reported limited numbers of effector lymphocytes, although in rodents both CTLs and NK cells are recruited to the lungs upon primary RSV infection where they appear to contribute to disease pathogenesis. In the present study we hypothesized that severe RSV-LRTI in children is associated with local activation of the granzyme pathway by the cell-mediated host immune response. To test this, we investigated extracellular GrA and GrB and cellular expression of GrB in the respiratory tract of infants with RSV-LRTI.
METHODS

Patients and sample collection
All protocols were approved by the Academic Medical Center ethical committee and informed consent was obtained from parents. Tracheal aspirate (TA) samples were obtained from 23 children with RSV-LRTI and 12 age-matched controls without a pulmonary condition. All patients were admitted to the intensive care unit for mechanical ventilation (MV) between November 2003 and March 2006. Infection with RSV was proven by direct immunofluorescence assay (Imagen, DakoCytomation, UK) of nasopharyngeal aspirate. As part of an international randomized placebo-controlled trial on the use of glucocorticosteroids during RSV-LRTI (www.star-trial.com), RSV patients received dexamethasone 0.15 mg/kg/dose, intravenous or placebo (QID, 8 doses in total) starting within 24 h after start of MV. Oxygenation index calculated as (FiO2 x mean airway pressure (cmH2O) x 100)/ PaO2 (kPa), during the first 24 h was used to assess severity of oxygenation anomaly. If more than one arterial blood gas was obtained, the best index was chosen. Pediatric risk of mortality (PRISM) scores were used as a measure of disease severity.

TA was collected as described before. Aspirate was collected without previous installation of fluids on the day of start of MV and in the RSV-patients on day 2 and 4 as long as the patient was intubated. An arterial blood sample was obtained from 15 RSV-patients on the day of start of MV.

Sample processing
After determination of the volume of the aspirate sample, an equal volume of cold 10 mM dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO) in 25 mM Hepes (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) pH 8.0 was added at 4°C followed by 15 minutes mixing. If the aspirate remained mucoid this process was repeated once with a similar amount of DTT. Remaining cellular aggregates were cleared by adding DNsase (Sigma-Aldrich) at 4°C. Cells in processed aspirate were collected by 10 min centrifugation at 450 x g. Supernatant was aspirated, aliquoted and stored at -80°C. The cell pellet was resuspended in PBS and total white blood cell (WBC) were counted in a Bürker bright line counting chamber. Air dried cytospins were stained with Romanovsky (Diff-Quick) and differential WBC counts were obtained by counting 300 leukocytes using a standard light microscope. Reliable differential counts could not be obtained in 18% of the samples because of debris and some degeneration of cells.

Immunoassays
Extracellular GrA and GrB were measured by immunoassays (Sanquin, the Netherlands) according to the manufacturer’s description. Interleukin (IL)-8 was measured by immunoassay using monoclonal antibodies against IL-8 (R&D Systems) for capture and detection. Extracellular caspase-cleaved cytokeratin-18 (CK18), a marker of epithelial cell apoptosis, was
measured using the M30-Apoptosense sandwich immunoassay (Peviva AB, Sweden). The M30 antigen levels are expressed as Units/mL, where one Unit corresponds to 1.24 pmol of recombinant M30-containing peptide. Assay lower limit: 0.7 U/mL.

TA samples were diluted minimally 1:100 (GrA/B and IL-8) and 1:20 (cleaved CK18) to overcome interference of DTT with the assays and serial dilutions were tested. All reported antigen levels in TA are corrected for the sample processing dilution factor.

**Granzyme activity**

Activity of GrA was measured by a recently developed immunoassay as described elsewhere. For measurement of GrB activity, GrB in samples was captured on microtiter plates coated with anti-GrB mAb GB-11 (2 μg/ml in 0.1M sodium carbonate/bicarbonate buffer, pH 5.5). The plates were then washed and incubated with GrB-specific chromogenic substrate Ac-Ile-Glu-Thr-Asp-pNA (Alexis Biochemicals, Lausen, Switzerland; 0.4mM in 50mM Tris, 100mM NaCl and 0.1% Tween (v/v), pH 7.4). GrB activity was measured for 4 hours at 37°C at an absorbance (A) of 405nm on a Titer-Tek Multiscan (Labsystems, Helsinki, Finland) and is expressed as deltaA per hour.

**Fluorescent-activated cell sorting (FACS) analysis**

FACS analysis was performed in bronchoalveolar lavage fluid (BALF) samples from mechanically ventilated children with RSV-LRTI in the winters of 2006-2008. BALF was obtained by three subsequent instillations of 1 ml/kg of 0.9% saline through a wedged suction catheter passed through the endotracheal tube. After each instillation fluid was suctioned (mean ± SE recovery: 36% ± 4). The last 2 samples were pooled and 10 min centrifuged at 450 x g. The pooled BALF cells in FACS buffer (0.5% BSA, 0.02% potassium-EDTA in PBS) containing 10% normal human serum were labeled with APC-labeled anti-CD3, PerCP/Cy5-labeled CD8 or -CD4 (BD Pharmingen, San Jose, CA) and FITC-labeled anti CD16 (Sanquin, the Netherlands) and -56 (BD Pharmingen). For intracellular GrB staining cells were fixed and permeabilized with fixation/permeabilization solution (BD Pharmingen) and stained with PE-labeled anti-GrB mAb (Sanquin) or a PE-labeled isotype control (BD Pharmingen). Cells were analyzed using a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences).

**Statistical analysis**

Not normally distributed data are expressed as medians and were analyzed by using the Mann-Whitney U test for differences between groups. For normally distributed data, a student’s t-test was used to compare group means. Spearman’s correlation coefficient was calculated to assess the degree of association between granzymes and studied markers. Proportions in the patient groups were compared by Fisher’s exact test. A two-sided p value of < 0.05 was considered statistically significant.
RESULTS

Baseline patient characteristics
The baseline characteristics of the patients are shown in Table 1. Ten RSV patients (43%) had received dexamethasone as part of the aforementioned randomized placebo-controlled trial. Total WBC counts and differentials in TA samples of controls and RSV patients are shown in Table 2.

Table 1. Baseline patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>RSV</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>12</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Age, months, median (IQR)</td>
<td>3.5 (6.6)</td>
<td>1.5 (1.0)</td>
<td>0.10</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>6 (50)</td>
<td>15 (65)</td>
<td>0.48</td>
</tr>
<tr>
<td>Duration of symptoms, days, median (IQR)</td>
<td>n.a.</td>
<td>3 (2)</td>
<td>0.48</td>
</tr>
<tr>
<td>PRISM score, median (IQR)</td>
<td>6 (6)**</td>
<td>10 (8)</td>
<td>0.09</td>
</tr>
<tr>
<td>Oxygenation index, median (IQR)</td>
<td>1.4 (0.5)§</td>
<td>4.4 (4.3)¶</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Positive bacterial culture TA, n (%)¶</td>
<td>0 (0)</td>
<td>5 (22)</td>
<td>0.14</td>
</tr>
<tr>
<td>Duration of MV before 1st sample, hr, median (IQR)</td>
<td>9.5 (12.8)</td>
<td>19 (9)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* Reason for admission and mechanical ventilation of control patients were: metabolic disorder (n=1), intracardial rhabdomyoma (n=1), convulsions (n=1), pneumococcal meningitis (n=1), cardiomyopathy (n=1) and, postoperative (n=7: abdominal surgery (n=4), craniosynostosis (n=1), closure of persistent ductus arteriosis (n=1), congenital diaphragmatic hernia, (n=1)).
** (n=9) Of 3 controls no PRISM score was available at the time of TA sampling. § (n=8) No arterial blood gas analysis was obtained in 4 controls. ¶ On day of start of mechanical ventilation. MV: mechanical ventilation.

Table 2. Total white blood cell (WBC) counts and differentials in TA samples of mechanically ventilated patients without pulmonary disease (controls) and RSV patients on the day of start of mechanical ventilation.

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>RSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC count (x 10^6/ml), median (IQR)</td>
<td>6.8 (7.7)</td>
<td>28.6 (54.8)*</td>
</tr>
<tr>
<td>Differentials in %, median (range):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>81.4 (36.0-92.7)</td>
<td>88.5 (70.0-94.3)</td>
</tr>
<tr>
<td>Monocytes/macrophages</td>
<td>18.3 (7.3-64.0)</td>
<td>11.2 (5.0-30.0)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0 (0-2)</td>
<td>1.0 (0-2)</td>
</tr>
</tbody>
</table>

* p < 0.05, Mann-Whitney U test

Elevated GrA and GrB in TA during severe RSV-LRTI
The median (range) level of GrA in TA on the day of start of mechanical ventilation was 0.6 (0.3-14.3) ng/ml and 11.1 (0.3-98.4) ng/ml in controls and RSV-patients respectively (p<0.01, Figure 1A). Likewise, the median (range) level of GrB in TA was higher in the RSV patients (69.0, 3.1-728.0, ng/ml) as compared to the controls (1.7 ng/ml, 0.5-39.6, ng/ml) (p < 0.01, Figure
There was a significant correlation between the levels of GrA and GrB in TA (Spearman $r = 0.67$, $p<0.001$). In the RSV patients, the plasma levels of GrA (median 63.0, range 3.0-180.0, pg/ml) and GrB (median 35.0, range 13.0-92.0, pg/ml) on the first day of MV were significantly lower than the levels in TA ($p<0.001$ for both comparisons, data not shown).

GrA and GrB in TA have retained activity
To ascertain biological activity of GrA and GrB in the airways of RSV patients we measured active GrA and GrB with enzyme capture assays. Because the sensitivity of these assays was 50 pg/ml and 1 ng/ml respectively, only 10 samples of TA containing high levels of GrA and GrB in TA were detected throughout the course of RSV-LRTI, but no effect of dexamethasone as compared with placebo on the granzyme levels was found (Figure 1C and D). No difference in granzyme levels was found between RSV patients with or without a positive bacterial culture of TA.

1B). There was a significant correlation between the levels of GrA and GrB in TA (Spearman $r = 0.67$, $p<0.001$). In the RSV patients, the plasma levels of GrA (median 63.0, range 3.0-180.0, pg/ml) and GrB (median 35.0, range 13.0-92.0, pg/ml) on the first day of MV were significantly lower than the levels in TA ($p<0.001$ for both comparisons, data not shown).

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Granzymes in TA correlate with inflammatory markers, but not with cleaved-CK18

There was a positive correlation between GrA and GrB and total WBC counts and IL-8 in TA in the RSV patients and this correlation tended to be stronger after the first days of MV (Figure 3). However, no correlation between granzyme levels and disease severity at baseline, as determined by PRISM scores and oxygenation index, was found (data not shown).

To study lung epithelial apoptosis in relation to extracellular granzymes, we analyzed the correlation between GrB and cleaved-CK18 in TA. Cytokeratin-18 is an epithelium-specific intermediate filament protein which is cleaved by caspases early during apoptosis.

Table 3. Mean percentages ± SE (of total cells) of cells expressing CD3, CD3*CD8 and CD3-CD16/56 (NK cells) in BALF of mechanically ventilated children with severe RSV infection, as determined by FACS analysis.

<table>
<thead>
<tr>
<th>Day</th>
<th>Lymphocyte subpopulation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CD3</td>
</tr>
<tr>
<td></td>
<td>% of total cells</td>
</tr>
<tr>
<td>start of MV</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>2</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>4</td>
<td>2.1 ± 0.8</td>
</tr>
</tbody>
</table>

MV: mechanical ventilation. First day of MV: n = 7, day 2: n = 6, day 4: n = 4.
and may be subsequently released from cells into the extracellular space. Caspases are activated by GrB, but not by GrA. There was no significant correlation between GrB and cleaved-CK18 in TA at any time point. Also, no statistically significant difference between cleaved-CK18 levels (U/mL) of controls (median 14.5, IQR 39.2) as compared to the RSV patients (median 8.4, IQR 17.7) was found (p=0.20).

**GrB expression by lymphocytes in BALF**

In order to find a potential source of granzymes in the lungs of children with severe RSV-LRTI, we performed FACS analysis in BALF samples in an additional cohort of mechanically ventilated children (mean age 1.4 ± 0.2 months, n=7) with RSV-LRTI. These patients did not differ from the original cohort in terms of age, gender distribution and duration of illness (data not shown). T-cells and NK cells were detected in low numbers throughout the course of RSV-LRTI (Table 3). GrB-positive cells were found predominantly among cells within the side/forward scatter region of the lymphocyte population (Figure 4A-E). In addition to CD8+ T-cells and NK cells, CD8- T-cells were found to express GrB (Figure 4F-J). This latter observation suggests that besides CD4-CD8- T-cells (which may include gamma/delta T-cells) CD4+ T-cells may express GrB, which was confirmed in an additional FACS analysis of 4 RSV patients (Figure 4K). The percentages of GrB-positive T-cells and NK cells and corresponding mean fluorescence intensity are shown in Figure 5.

**Figure 3.** A, correlation between the levels of GrA (●) or GrB (○) and total white blood cell counts (A) and IL-8 levels (B) in TA of RSV patients on day 4 after start of MV. Spearman $r > 0.63$ (p<0.05) for all comparisons.
Figure 4. Representative examples of FACS scatter plots of RSV patients. A, Side/anti-GrB mAb scatter plot showing the GrB-positive cell population (arrow), which is not detected in the same sample stained with an isotype control for GrB (B). C, Side/forward scatter plot of the gated GrB-positive cell population. D, Side/anti-CD3 mAb scatter plot showing a CD3-positive population (arrow). E, Side/forward scatter plot of the gated CD3-positive cell population. Note that the GrB-positive cell population (see C) is detected in the same side/forward region as the CD3-positive cell population. F, anti-CD3/anti-CD8 mAb scatter plot of a gated lymphocyte region (see E) showing CD3+CD8+ cells (*) and CD3+CD8- cells (**). G, anti-CD3/anti-CD16/56 mAb scatter plot of a gated lymphocyte region (see E) showing CD3-CD16/56+ (NK) cells. H, anti-GrB/anti-CD8 mAb scatter plot of a gated CD3+CD8+ cell population (L, corresponding GrB isotype control plot). I, anti-GrB/anti-CD3 mAb plot of a gated CD3+CD8- cell population (M, corresponding GrB isotype control plot). J, anti-GrB/anti-CD16/56 mAb plot of a gated CD3CD16/56+ cell population (N, corresponding GrB isotype control plot). K, anti-GrB/anti-CD4 mAb plot of a gated CD3+CD4+ cell population (O, corresponding GrB isotype control plot).
DISCUSSION

The main goal of this study was to determine whether severe RSV infection in children is associated with local activation of the granzyme pathway by the cell-mediated host immune response. We report high levels of proteolytic active extracellular GrA en GrB and marked expression of GrB by T-lymphocytes and NK cells in the respiratory tract of children during the course of severe RSV-LRTI. The levels of extracellular GrA and GrB correlated with total WBC counts and IL-8 levels in the airways after the acute-onset of RSV disease, but no association with the epithelium apoptosis marker cleaved-CK18 could be demonstrated.

The present study extends our understanding of cell-mediated cytotoxic responses during severe RSV-LRTI in children. Numerous rodent studies have shown that both NK cells and CTLs are recruited to the lungs during primary RSV infection 15-17. Graham et al. reported that although CTLs are involved in the clearance of RSV, depletion of these lymphocytes diminishes clinical illness upon exposure to RSV 15. Treatment of RSV-infected CTL depleted mice with high dose RSV-specific CTLs results in viral clearance, but augments lung injury by causing severe haemorrhage and neutrophilic infiltration 21. These findings suggest that cell-mediated cytotoxic responses play an important role in RSV disease pathogenesis in mice, but the relevance in humans is unclear. Studies in vitro show that CTLs isolated from peripheral blood of RSV infected infants lyse infected autologous target cells 22,23. However, Welliver et al. recently reported the near absence of CTLs and NK cells in lung tissues from 9 infants who died of RSV-LRTI 5. Previous analysis of BALF samples of RSV-infected children showed that a small percentage of the total cells in the lungs is CD8-positive 14. Similarly, in the present study we found low...
numbers of T-lymphocytes and NK cells in the lungs. However, despite these low cell counts, we report high levels of extracellular granzymes and marked GrB expression in these cell populations in the respiratory tract.

The activation of the cell-mediated granzyme response in children with severe RSV-LRTI raises the possibility that this cell death pathway is involved in RSV disease pathogenesis. Direct killing of infected cells by granzymes is considered a major host defence mechanism against viruses in general. In mice, both GrA and GrB are essential in controlling ectromelia and cytomegalovirus infection. Interestingly, viruses have been reported to encode proteins that inhibit granzymes, suggesting pathogen immune evasive adaptations. On the other hand enhanced death of infected cells and/or bystander cells may contribute to the development of disease by causing tissue dysfunction as has been suggested for severe inflammatory lung diseases. Welliver et al. found marked expression of caspase-3 in airway epithelium of children with fatal RSV-LRTI, a finding consistent with studies performed in adult patients with ALI/ARDS. The study of Welliver et al. suggests an imbalance in apoptosis during severe RSV-LRTI, but the involved cell death pathways remain unclear. Our findings may point toward a role for the granzyme pathway in apoptotic cell death during severe RSV-LRTI, but contrary to our expectations, we found no correlation between the levels of extracellular GrB and caspase-cleaved CK18 in the airways. The release of caspase cleaved CK18 is a surrogate marker of epithelial apoptosis but has not been studied extensively in vivo. The detection of cleaved CK18 in serum was found to correlate well with tissue damage in patients with hantavirus infection, but it is unclear how this protein behaves and reflects epithelial injury in other body fluids, including BALF. Histological analysis of apoptosis to confirm our data is highly preferred but could not be performed in our study for obvious ethical reasons.

In addition to CTLs and NK cells, CD4+ T-cells and basophils may express granzymes. Recently, neutrophils also were shown to express granzymes, although this has been debated by others. In the present study, GrB-positive cells were only found among lymphocytes. CTLs seemed to contribute to GrB expression the most, but actual degranulation and subsequent granzyme release may have differed between the studied cell populations. The highest GrB expression by NK cells occurred on the first day of admission, before the peak of GrB expression by T-cells. Similarly, in RSV infected mice the cytotoxic activity of NK cells is highest before CTLs are recruited to the lungs. Remarkably, granzyme levels in the airways during severe RSV-LRTI were not affected by treatment with dexamethasone, although several studies in vitro have shown suppression of CTL and NK cytotoxic activity by corticosteroids.

Our finding of GrB expression by CD4+ T-lymphocytes is interesting because it suggests that the granzyme pathway may be utilized to modulate immune responses to RSV. Devadas et al. have shown that T\(_h\)2 cells express GrB which induces their own cell death. Furthermore, they reported that GrB deficient mice have augmented T\(_h\)2 cytokine
responses and lung cellular infiltrations in a model of allergic inflammation. Grossman et al. have shown that the granzyme pathway is exploited by natural T-regulatory cells (CD4+CD25+) against autologous activated CD8+ and CD4+ T-cells. These findings suggest that granzymes function to control immune responses, in addition to their role in direct virus-infected cell killing. In contrast, another line of research proposes that granzymes actively participate in the activation of pro-inflammatory responses. For example, studies in vitro have shown that proteolytic active GrA induces the release of IL-6 and IL-8 from epithelial cells and lung fibroblasts. In the present study, granzyme levels significantly correlated with IL-8 levels in the airways during severe RSV-LRTI and with total WBC counts after the acute phase of respiratory failure. Moreover, we found evidence that active forms of GrA as well as GrB were present, confirming the potential to exert a biological function.

This study has three potential limitations. First, all patients had severe acute hypoxemic respiratory failure necessitating MV and thus represent the end of the spectrum of RSV disease. This withholds us from drawing firm conclusions when relating the extent of activation of the granzyme pathway with markers of disease severity such as PRISM scores and oxygenation index. Second, because all patients (including controls) were subjected to MV and supplemental oxygen it is possible that this might have had (synergistic) effects on granzyme levels, inflammation or apoptosis. Third, soluble granzymes were measured in TA that may reflect more localized (and thus variable) proximal epithelial lining fluid.

In conclusion, severe RSV-LRTI in children is associated with high levels of proteolytic active extracellular GrA and GrB and expression of GrB by lymphocytes in the respiratory tract. These findings suggest that the granzyme pathway is activated by the local cell-mediated host immune response to RSV. Further studies should elucidate the exact role of granzymes in the lungs during RSV disease.
REFERENCES


