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

Bern, R.A.

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Potential Role of Soluble TRAIL in Epithelial Injury in Children with Severe RSV Infection

Reinout A. Bem1
Albert P. Bos1
Roelie M. Wösten-van Asperen 1
Martijn Bruijn1
Rene Lutter2
Martin R. Sprick3
Job B.M. van Woensel1

1 Pediatric Intensive Care Unit, Emma Children’s Hospital; 2 Departments of Pulmonology and Experimental Immunology, and 3 Laboratory of Experimental Oncology and Radiobiology Academic Medical Center, Amsterdam, The Netherlands

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ABSTRACT

Lower respiratory tract infection by respiratory syncytial virus (RSV) is a frequent cause of acute lung injury in young children and infants. Studies in adults and animals suggest that tumor necrosis factor receptor (TNFR) ligands may mediate lung injury by causing apoptosis of epithelial cells. The main goal of the present study was to determine whether the TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) pathway may be implicated in epithelial injury during severe RSV infection in children. We report elevated levels of soluble (s)TRAIL released by leukocytes in bronchoalveolar lavage fluid (BALF) of patients with RSV-associated respiratory failure (n=22) as compared to mechanically ventilated patients without pulmonary illness (n=7). Primary bronchial epithelial cells of children without pulmonary disease obtained by non-bronchoscopic cytobrushing expressed both death receptors TRAIL-R1 and -R2, and were found to be susceptible for cell death by human recombinant sTRAIL in vitro. Furthermore, BALF from a RSV patient induced cell death in these cells, which was partly attenuated by inhibiting TRAIL signaling. These data suggest that the TRAIL pro-apoptotic pathway may contribute to lung epithelial injury in severe RSV infection in children.
INTRODUCTION

Lower respiratory tract infection (LRTI) by respiratory syncytial virus (RSV) remains a frequent cause of acute respiratory failure in young children and infants worldwide. RSV-LRTI may present as bronchiolitis with obstructive airway symptoms, and/or pneumonia, involving the alveolar compartment. Furthermore, severe RSV-LRTI accounts for up to 20% of the admissions for acute lung injury (ALI) or the acute respiratory distress syndrome (ARDS) in the pediatric intensive care unit. The precise mechanisms by which RSV infection progresses to acute respiratory failure in previously healthy children are largely unknown. Currently, treatment of severe RSV-LRTI is limited to supportive measures.

Histopathology studies in children have shown that fatal RSV disease is associated with marked expression of pro-apoptotic markers, such as active caspase-3, in airway and alveolar epithelial cells. However, it is difficult to interpret these findings: on the one hand it may indicate RSV disease can progress despite an ongoing host defense mechanism of apoptosis of infected cells, on the other hand it may suggest extensive and imbalanced cell death during RSV infection leads to enhanced lung injury. In fact, increased caspase-3 activity in lung epithelial cells is also found in adult patients who died of ALI/ARDS, and numerous animal models of ALI/ARDS, including a murine model for severe RSV infection, have shown that lung injury is associated with enhanced lung epithelial cell apoptosis. More importantly, several of these animal studies also demonstrated that strategies inhibiting pro-apoptotic signalling may attenuate lung inflammation, alveolar-capillary permeability and histopathological alterations. These observations suggest that under certain circumstances activation of pro-apoptotic pathways in the lungs can contribute to lung injury, and this may also play a role in the pathogenesis of severe RSV-LRTI.

An important pro-apoptotic pathway is triggered by the activation of specific membrane “death” receptors belonging to the tumor necrosis factor receptor (TNFR) superfamily. One of the ligands for this TNFR pathway is TNF-related apoptosis-inducing ligand (Apo2L/TRAIL), which initiates caspase activation upon ligation of either one of two TNFR members: TRAIL-R1 and -R2. TRAIL is a type 2 transmembrane protein, but a soluble form (sTRAIL) can be generated after cleavage of the extracellular domain by cysteine proteases or through secretion in microvesicles. In the lungs, TRAIL is potentially expressed by a number of different activated leukocytes, including monocytes/macrophages, neutrophils and lymphocytes. Interestingly, RSV infection of human carcinomic alveolar basal epithelial cells (A549 cell line) in vitro upregulates the surface expression of TRAIL-R1 and -R2, which suggests the TRAIL pathway may contribute to host defense by limiting RSV replication and spread. However, in a recent study by Herold et al. mice with influenza virus pneumonia and TRAIL deficient macrophages or treated with anti-TRAIL mAb showed decreased lung...
epithelial cell apoptosis, together with decreased lung permeability and mortality, despite delayed viral clearance. These results suggest that TRAIL-induced lung epithelial cell death may be an important pathogenic mechanism of lung injury in severe viral lung infection.

In the present study we investigated the hypothesis that the activation of the TRAIL pathway is a potential mechanism of lung epithelial injury in severe RSV-LRTI in children. We measured levels of sTRAIL in bronchoalveolar lavage fluid (BALF) of children receiving mechanical ventilation for RSV-LRTI, and explored the susceptibility of normal primary bronchial epithelial cells of children to TRAIL-induced cell death in vitro.

**METHODS**

All patient sampling protocols were approved by the Academic Medical Center ethical committee and informed consent was obtained from parents.

**Bronchoalveolar lavage fluid samples**

*Patients.* Between November 2007 and January 2009 BALF samples were obtained from 22 children receiving mechanical ventilation for RSV-LRTI and 7 age-matched patients receiving mechanical ventilation for non-pulmonary conditions. Infection with RSV was proven by direct immunofluorescence assay (Imagen, DakoCytomation, UK) of nasopharyngeal aspirate. RSV patients with bilateral infiltrates on chest radiography and a PaO₂/FiO₂ ratio of < 200 mmHg in the absence of cardiac failure were designated to fulfil the American-European consensus criteria of ARDS.

*Sampling and processing.* BALF was obtained on the day of start of mechanical ventilation, and in the RSV patients also on day 2 and 4 thereafter. 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 % recovery ± SE: 36.9 ± 1.8 for RSV patients and 28.7 ± 3.5 for controls). The last 2 samples were pooled and 10 min centrifuged at 450 x g. Supernatant was aspirated, aliquoted and stored at -80°C. The mucoid cell pellet was resuspended in 10 mM dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO) in 25 mM Hepes (N'-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid) pH 8.0 at 4°C followed by 10 minutes mixing. After again 10 min centrifugation at 450 x g the cell pellet was resuspended in PBS and total white blood cells (WBC) were counted in a Bürker bright line counting chamber. Air dried cytopsins were stained with Romanovsky (Diff-Quick) and differential WBC counts were obtained by counting 200 leukocytes using a standard light microscope.

In several patients the remainder of the BAL cells was incubated with RPMI containing L-glutamine (Invitrogen Ltd, Paisley, UK), supplemented with 0.1% penicillin/streptomycin
and 10% fetal bovine serum (Invitrogen Ltd), for 18 hr at 37°C, 5% CO₂, after which the supernatant was collected.

ELISA. sTRAIL in BALF and cell supernatants was measured by ELISA (R&D systems, Minneapolis, MN), according to the manufacturer’s description.

FACS analysis. For detection of TRAIL expression on BAL cells in 10 RSV patients, BAL 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 or anti-CD14 (BD Biosciences, Franklin Lakes, NJ), FITC-labeled anti-CD16 (Sanquin, the Netherlands) and -56 (BD Pharmingen) and PE-labeled anti-TRAIL mAb (BD Biosciences) or a PE-labeled isotype control (BD Biosciences). The gating strategy used has been described previously. The FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc, Ashland, OR) were used for analysis.

Primary lung epithelial cell cultures
Patients. Primary bronchial epithelial cells were obtained by non-bronchoscopic cytobrush sampling in 6 children (age 0-4 yr) who were endotracheally intubated for elective minor surgery for non-pulmonary disorders.

Sampling and culturing. Briefly, a sheathed cytology brush (2.4 mm, Olympus, Hamburg) was introduced through the endotracheal tube directly after intubation. In wedge position the sheath and brush were withdrawn ~2 cm after which gentle brush sampling was performed over a range of ~2 cm. The obtained cells were shaken of the brush and cultured at 37°C, 5% CO₂ in collagen- (Vitrogen-100, Cohesion Technologies, Palo Alto, CA) coated cell culture plates (Corning Costar, Schiphol-Rijk, The Netherlands) in BEGM-Bulletkit bronchial epithelial medium containing the following supplements/growth factors: bovine pituitary extract, hydrocortisone, hEGF, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, and gentamicin/amphotericin-B (Cambrex Cooperation, NJ) and cyproxin (Bayer BV, Mijdrecht, The Netherlands). After ~2 wks of culturing and before passage 2-3, the cells were used for experiments at ~70-80% confluency.

Virus exposure. RSV-A aliquots were a kind gift from L. van der Hoek, Department of Experimental Virology AMC, Amsterdam, The Netherlands. Cells at ~70-80% confluency in 24-well plates (Corning Costar) were exposed to a total of 3x10⁷ copies RSV-A, as determined by qPCR. This dose was based on previous experiments which showed intracellular RSV replication by direct immunofluorescence assay (Imagen, DakoCytomation, UK) at day 6-7 post-infection.

Immunocytochemistry. Cytospin preparations were fixed in 4% paraformaldehyde, treated with peroxidase block (Envision system, DAKO, Carpinteria, CA) and blocked with 3% bovine...
serum albumin in PBS/TritonX-100 0.1%. Thereafter, the cells were exposed to one of the following primary antibodies: mouse anti-human cytokeratin 1-8, 10, 13-16 and 19 mAb (AE1/AE3 clone, DAKO), anti-human TRAIL-R1/DR4 or anti-human TRAIL-R2/DR5 (Axxora, San Diego, CA) for 1 hour at RT, followed by incubation with labelled polymer-HRP anti-mouse and AEC+ substrate chromogen (Envision system, DAKO) according to the manufacture’s description. The cells were counterstained with haematoxylin.

**FACS analysis.** For detection of TRAIL-R1 and -R2 expression cells in FACS buffer (0.5% BSA, 0.02% potassium-EDTA in PBS) were labeled with anti-human TRAIL-R1 or anti-human TRAIL-R2 (Axxora, San Diego, CA), and detected with RPE-labelled rabbit anti-mouse Ab (DAKO). The FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc, Ashland, OR) were used for analysis.

**Cytotoxicity assays.** To investigate the susceptibility to TRAIL induced apoptosis in vitro, cells were exposed to 2 different forms of human recombinant sTRAIL: superkillerTRAIL (Axxora, San Diego, CA) diluted in 20mM HEPES, pH 7.4, containing 300mM NaCl, 0.01% Tween 20, 1% sucrose and 1mM DTT or FLAG-TRAIL (Axxora) either with or without M2-anti-FLAG antibody at a ratio of 2 μg antibody to 1μg TRAIL to establish crosslinking. To determine the cytotoxicity of sTRAIL in BALF, BALF was concentrated 40-fold by ultrafiltration (Centricon Ultracel YM-10, Millipore, Billerica, MA), sterile-filtered, and then added to the cells at a 1:50 dilution. For specific blocking of TRAIL-mediated effects, the BALF-concentrate was incubated with 10 μg DR5-Fc fusion protein (R&D systems, Minneapolis, MN) for 15 min at room temperature before addition to the cells. Cell death was determined with a flow-cytometer upon incubation of the cells with FITC-labeled Annexin V (Nexins, Kattendijke, The Netherlands) or propidium iodide.

**Statistical analysis**

Statistical analysis was performed with SPSS 16.0 software. 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 or paired t-test was used to

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**Table 1. Patient and BALF characteristics**

<table>
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<tr>
<th></th>
<th>no pulmonary disease</th>
<th>RSV</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Number of patients</td>
<td>7</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Age, months, mean (SD)</td>
<td>1.1 (1.0)</td>
<td>2.8 (2.7)</td>
<td>0.10</td>
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<tr>
<td>Male, n (%)</td>
<td>5 (71)</td>
<td>16 (73)</td>
<td>1.0</td>
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<tr>
<td>BALF total cell count (x 106), median (IQR)</td>
<td>1.4 (0.8)</td>
<td>22.5 (28.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Differentials in %, median (range):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>26 (0-72)</td>
<td>72 (12-89)</td>
<td></td>
</tr>
<tr>
<td>Monocytes/macrophages</td>
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<td>28 (11-87)</td>
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<tr>
<td>Lymphocytes</td>
<td>0 (0-1)</td>
<td>0 (0-6)</td>
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</table>
compare means. 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

Patient characteristics
The baseline characteristics of the patients are shown in Table 1. Reasons for admission and mechanical ventilation of patients without pulmonary/RSV disease were postoperative abdominal surgery (n=5), meconium ileus associated with respiratory distress (n=1) and laryngeal granuloma (n=1). The (first) BALF sample was obtained at a mean (±SD) of 18.3 (±15.0) hr after start of mechanical ventilation in the patients without pulmonary/RSV disease,

**Figure 1.** A, levels of sTRAIL (pg/ml) in BALF from children without pulmonary/RSV disease (n=7) as compared to children with RSV infection (n=22) on the day of start of mechanical ventilation (MV) and individual peak levels during the course of RSV infection. * p <0.05 (Mann-Whitney U test) as compared to children without pulmonary/RSV disease. B, levels of sTRAIL (pg/ml) in BALF on the day of start of mechanical ventilation from children with or without ARDS during the course of RSV infection (p=0.07 by t-test). C, levels of sTRAIL (pg/ml) in BALF from children during the course of RSV infection. Data are shown as box plots depicting the median, interquartile range and range.
and at 15.2 (±5.9) hr in the RSV patients (p = 0.4). The mean (±SE) tidal volume at time of BALF sample was 8.0 (±0.5) for patients without pulmonary/RSV disease, as compared to 6.6 (±0.3) for RSV patients. In total, 5 of the RSV patients had a history of preterm birth, and 3 of the RSV patients had a pulmonary or cardiac abnormality. Seven (31%) of the RSV patients fulfilled the criteria of ARDS during admission.

Elevated levels of sTRAIL in the lungs of children with severe RSV infection
On the day of start of mechanical ventilation, the median (IQR) level of sTRAIL in BALF of the RSV patients was 298 (287) pg/ml, as compared to 147 (110) pg/ml in the patients without pulmonary disease (p< 0.05) (Figure 1A). RSV patients who fulfilled the criteria of ARDS during the course of RSV disease had a median (IQR) sTRAIL level of 332 (503) pg/ml in BALF, as compared to 253 (229) pg/ml in RSV patients without ARDS, but this difference did not reach statistical significance (Figure 1B). Because it was unknown at what time point actual infection by RSV had occurred in the patients, we also examined levels of sTRAIL in BALF at day 2 and 4 after start of mechanical ventilation (Figure 1C). Although levels of sTRAIL within patients at the different time points were variable, the median levels of sTRAIL remained high during the course of RSV infection. The median (IQR) of the individual peak levels of sTRAIL in BALF of the RSV patients was 393 (299) pg/ml (Figure 1A). There was no correlation between levels of sTRAIL in the BALF and tidal volume or duration of mechanical ventilation (data not shown).

sTRAIL is released from lung leukocytes
In viral infected lungs, TRAIL may be expressed by several activated leukocytes, including monocytes/macrophages, neutrophils and lymphocytes 16-20. Specifically, activated monocytes and neutrophils have been shown to release sTRAIL 16. To determine whether sTRAIL is released by lung leukocytes in RSV infection, BAL cells were isolated by centrifugation from the BALF from RSV patients and incubated in RPMI medium containing 10% fetal bovine serum. The supernatants of the BAL cells collected after 18 hr incubation all contained sTRAIL (mean ± SEM level per 5x10^6 cells: 128 ± 28 pg/ml for RSV patients, and 32 ± 24 pg/ml for patients without pulmonary disease) (Figure 2A). RPMI medium and fetal bovine serum did not contain human sTRAIL.

In the lungs of mice infected with influenza virus marked expression of TRAIL is found predominantly in macrophages 23. In line with this observation, we found evidence for expression of membrane-bound TRAIL in CD14+ BAL cells (monocytes/macrophages) during RSV infection by FACS analysis (Figure 2B). In contrast, no clear expression of TRAIL was found in CD3+ BAL cells (T-lymphocyte population) and CD3-/CD16+/56+ BAL cells (NK cell population) (Figure 2B).
Primary normal bronchial epithelial cells of children express TRAIL-R1 and -R2, which is increased by RSV infection

Activation of TNF death receptor-ligand systems, including the TRAIL pathway, has been implicated in the development of lung epithelial injury \textit{in vivo} \cite{13,23,28}. This led us to hypothesize that TRAIL in the lungs of children with severe RSV infection may activate the death receptors TRAIL-R1 and/or -R2 on epithelial cells. Because epithelial cells in the developing lungs of young children may differ from primary adult or cancer-derived cell lines we isolated and cultured bronchial epithelial cells of children, age 0-4 year, by nonbronchoscopic cytobrushing \cite{25}. More than 98\% of the cells obtained after culturing for a maximum of \~{}2 weeks stained positive for epithelial specific cytokeratins (data not shown). Moreover, primary bronchial epithelial cells expressed both TRAIL-R1 and -R2 death receptors as determined by immuncytochemistry and FACS analysis (Figure 3A and 3B).

In vitro exposure to RSV and influenza virus has been reported to increase the expression of TRAIL receptors in A549 and mouse alveolar epithelial cells respectively \cite{22,23}. Similarly, exposure of primary bronchial epithelial cells of children to RSV-A \textit{in vitro} resulted in increased expression of both TRAIL-R1 and -R2 at 6 days post-infection (Figure 3C). Intracellular RSV replication in primary bronchial epithelial cells at this time point was confirmed by direct immunofluorescence assay. The pattern of expression of TRAIL-R1

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\textbf{Figure 2.} A, levels of sTRAIL (pg/ml) in supernatants from BAL leukocytes (5x10^6) from children without pulmonary/RSV disease (n= 2) and children with RSV infection (n=6). Data are shown as bars depicting the mean and standard error. B, FACS histogram plots showing expression of membrane bound TRAIL in CD14+ BAL leukocytes in a RSV patient (upper left panel), and child without pulmonary/RSV disease (upper right panel), and in CD3+ and CD3-/CD16+56+ BAL leukocytes (lower panels, representative for both RSV patients and patients without pulmonary/RSV disease). Grey surface plots represent isotype control for anti-TRAIL.
and TRAIL-R2 8 days post-infection differed between samples, as we observed either a moderate further increase or down regulation (data not shown).

**Figure 3.** A, immunocytochemistry for TRAIL-R1 and -R2 of cytopsins of primary bronchial epithelial cells of children (magnification 500x). B, representative examples of FACS histogram plots of primary bronchial epithelial cells of children stained for membrane bound TRAIL-R1 and -R2. Negative control (grey surface plot) is without primary antibody. C, representative examples of FACS histogram plots for membrane bound TRAIL-R1 and -R2 staining on primary bronchial epithelial cells of children, 3 and 6 days post-infection (dpi) with RSV-A in vitro. Uninfected cells are shown in grey surface plots.

and TRAIL-R2 8 days post-infection differed between samples, as we observed either a moderate further increase or down regulation (data not shown).

**Primary bronchial epithelial cells of children are susceptible to TRAIL-induced cell death in vitro**

The susceptibility to TRAIL-induced cell death of primary bronchial epithelial cells of children *in vitro* was analyzed in three different ways. First, we exposed cells to human recombinant superkillerTRAIL (Axxora), which is a form of sTRAIL that has been cross-linked to enhance its cytotoxic potential. As compared to exposure to dilution buffer only, primary bronchial epithelial cells exposed to superkillerTRAIL at a concentration of 50 ng/ml for 6 hr showed
Figure 4. A, fold increase in the percentages of annexin V positive cells upon 6 hr exposure to superkillerTRAIL, as compared to cells treated with dilution buffer only. Experiments were performed in duplicates with primary bronchial epithelial cells obtained from three different children. B, example of FACS histogram plots from primary bronchial epithelial cells stained for annexin V upon 6 hr exposure to superkillerTRAIL, as compared to cells treated with dilution buffer only. C, percentages of PI positive primary bronchial epithelial cells from one child upon 48 hr exposure to non cross-linked FLAG-TRAIL with or without M2-anti-FLAG antibody to establish crosslinking. D, percentages of PI positive cells upon 48 hr exposure to non-cross linked FLAG-TRAIL (33 ng/ml) or concentrated BALF from a RSV patient with or without DR5-Fc to neutralize TRAIL signalling. Experiments were performed in duplicates with primary bronchial epithelial cells obtained from two different children. * p <0.05 (paired t-test). Data are shown as bars depicting the mean and standard error. E, representative light microscopy photograph showing the apoptotic morphological appearance of primary bronchial epithelial cells of children in TRAIL cytotoxicity assays (zoomed in 250x magnification). Inset shows normal unexposed control cells (250x magnification).
a 2-fold increase in the percentage of annexin V-positive cells (Figure 4A and 4B). Next, we exposed primary bronchial epithelial cells to non cross-linked human recombinant FLAG-sTRAIL without or with M2-anti-FLAG antibody to establish crosslinking. Non cross-linked sTRAIL at a concentration of 33 ng/ml and higher also clearly induced cell death in primary bronchial epithelial cells, although the susceptibility to this form of sTRAIL appeared much lower than to the cross-linked sTRAIL (Figure 4C). Finally, we exposed primary bronchial epithelial cells to a BALF sample obtained from a RSV patient (RSV-BALF). This BALF sample contained sTRAIL at a concentration of approximately 600 pg/ml as determined by ELISA. The RSV-BALF sample was concentrated 40-fold and used at a dilution of 1:50 to get a biologically relevant concentration of sTRAIL, while culturing in a normal volume of medium, and thus strongly reducing background cell death. Exposure to RSV-BALF for 48 hr induced a 5-fold increase in cell death in primary bronchial epithelial cells which was partly (~30%) attenuated by the addition of DR5-Fc which binds and neutralizes TRAIL (Figure 4D). Cell death in the above mentioned experiments was accompanied by the appearance of apoptotic morphological features such as cell shrinkage, rounding, cell membrane blebbing and detachment (Figure 4E).

Neutralization of TRAIL induced cell death by DR5-Fc was found to be complete in the highly TRAIL-sensitive BJAB cell line (Supplemental figure 1A), and neither DR5-Fc or human IgG exerted direct cytotoxicity in BJAB cells or the primary bronchial epithelial cells (Supplemental figure 1A-B).

**DISCUSSION**

The goal of this study was to determine whether severe RSV infection in children is associated with local activation of the pro-apoptotic TRAIL pathway, and whether this may be a mechanism of lung epithelial injury. We found elevated levels of sTRAIL in BALF of children receiving mechanical ventilation for RSV-LRTI. Normal primary bronchial epithelial cells of children expressed both death receptors TRAIL-R1 and -R2, and human recombinant sTRAIL as well as sTRAIL in RSV-BALF induced cell death in these cells in vitro. These findings suggest that sTRAIL may contribute to lung epithelial injury in children with severe RSV infection.

The present study extends our insight into cell death mechanisms in lung injury. Pro-apoptotic signaling is an essential process in growth, development and homeostasis in the lungs. However, accumulating evidence links enhanced activation of specific TNFR death receptors on lung epithelial cells to the pathophysiology of ALI/ARDS. For example, Albertine et al. found that alveolar wall cells of patients with fatal ALI/ARDS show prominent immunohistochemical staining for apoptotic markers such as terminal dUTP nick-end labeling, caspase-3, Bax, and p53, as well as the TNFR Fas. Furthermore, increased levels of soluble Fas ligand (sFasL) are present in BALF of patients with fatal
ALI/ARDS, and this has been implicated in apoptosis of primary lung epithelial cells of adults in vitro. Numerous studies with animal models for ALI/ARDS using (in)direct lung hits such as LPS, live bacteria, cecal ligation and puncture, hemorrhagic shock, mechanical ventilation and hyperoxia have shown extensive apoptosis of lung epithelial cells is associated with dysfunction of the alveolar-capillary barrier and histopathological injury. Interestingly, the lung injury in several of these animal studies can be attenuated by pharmacological apoptosis inhibitors or gene targeting of the Fas/FasL system, highlighting the relevance in the search for new therapeutic strategies.

Activation of TNFR-mediated apoptosis causing epithelial injury appears to be a general lung injury response, although the responsible mechanisms remain incompletely understood and may actually differ between injurious events such as mechanical ventilation and bacterial- or viral infection. We hypothesized that activation of the TNFR system may also play a role in the development of severe RSV infection in children, as characteristic lung pathological changes of fatal RSV-LRTI include small airway entrapment of apoptotic cellular debris and increased expression of active caspase-3 and Fas in airway and alveolar epithelial cells. However, previously, we have failed to detect sFasL in BALF of children with severe RSV infection by 2 different commercially available ELISAs (unpublished data), although there are concerns that this may in part be related to low specific antibody affinity as compared to anti-FasL antibodies used in other previous studies. In the present study we investigated the alternative, highly homologous TNFR-ligand system involving TRAIL and its death receptors -R1 and -R2. We found increased levels of sTRAIL, in the range of 0.1-1.5 ng/ml, in the lungs of children with severe RSV-LRTI. sTRAIL was released by lung leukocytes, and caused apoptosis of primary epithelial cells of children at a biologically relevant concentration in vitro. Interestingly, a recent study by Herold et al. showed a critical role for TRAIL-induced apoptosis of lung epithelial cells in disease pathogenesis of influenza virus pneumonia in mice. They showed that treatment with specific anti-TRAIL mAb antibodies which inhibited TRAIL signaling causes decreased lung epithelial cell death, lung permeability and mortality. Taken together, these findings suggest that the activation of the TRAIL pathway may be a mechanism of epithelial injury in severe viral infection, including influenza virus and RSV.

Several activated leukocytes, including macrophages, neutrophils and lymphocytes, are a potential source of TRAIL expression in the lungs. Monocytes/macrophages and neutrophils activated by type I interferon or LPS release sTRAIL with intact pro-apoptotic activity by signaling through both TRAIL-R1 and -R2. sTRAIL is released through secretion by microvesicles, but can also be generated by cleavage of the extracellular domain of membrane bound TRAIL by cysteine proteases. In the aforementioned study by Herold et al. TRAIL expression in the lungs during influenza virus infection in mice was confined to macrophages. In line with this observation we found evidence for expression of membrane bound TRAIL on lung monocytes/macrophages, and no clear expression in CD3+ and NK lymphocytes. However, because we investigated TRAIL
expression in a limited number of RSV patients and did not include a specific neutrophil marker we cannot draw firm conclusions from this.

To model epithelial cells in the lungs of children we cultured primary bronchial epithelial cells from children without pulmonary disease by non-bronchoscopic cytobrushing. An advantage of this *in vitro* model is that the apoptotic responses of these cells may reflect the developing pediatric lungs to a greater extent than primary adult or cancer-derived lung epithelial cells. Although in severe RSV infection both airway and alveolar epithelial cells appear to undergo extensive apoptosis, our *in vitro* model may not necessarily be representative for the apoptotic responses of alveolar epithelial cells. Even more pronounced apoptotic responses of distal as compared to proximal lung epithelial cells upon activation of Fas have been reported, but at this moment we can only speculate that this may be true for TRAIL as well.

In this study, primary lung epithelial cells of children were exposed to three different forms of sTRAIL: non-cross linked and cross-linked human recombinant sTRAIL, and finally sTRAIL in RSV-BALF at a biologically relevant concentration. In a study by Wajant *et al.* cross-linked sTRAIL was found to behave similar to membrane bound TRAIL acting through both TRAIL-R1 and -R2, while non cross-linked sTRAIL like natural sTRAIL activated only TRAIL-R1. However, in a more recent study Tecchio *et al.* showed that sTRAIL released by neutrophils and monocytes can activate both TRAIL-R1 and -R2. Here we show that normal primary lung epithelial cells of children expressed both TRAIL-R1 and -R2, and were susceptible to cell death induced by both non cross-linked and cross-linked recombinant sTRAIL. More importantly, RSV-BALF caused considerable cell death in primary epithelial cells of children, and this was partly attenuated by the addition of DRS-Fc which blocks TRAIL signalling. However, this observation necessitates several considerations. First, apart from sTRAIL a number of different cytokines and enzymes in RSV-BALF (e.g. TNFα, sFasL), and MMPs and other (serine) proteases) may have (in)directly activated cell death programs, and moreover, they may have enhanced the sensitivity of primary epithelial cells of children to TRAIL *in vitro*. In addition, it is possible that by concentrating the BALF though filtration low molecular weight bioactive material, such as degraded cytokine fragments, with potential effects on cell survival has been lost. Second, actual effective local levels of sTRAIL in the lungs *in vivo* may be higher, because epithelial lining fluid is diluted in BALF and sTRAIL bound at the surface of target cells may not be washed out. Furthermore, we have to take into account that there are a number of additional factors, including the treatment with mechanical ventilation, that may affect the lung microenvironment including TRAIL/apoptotic susceptibility.

It is important to consider that viral infection itself may sensitize cells to TRAIL-induced cell death by increasing TRAIL receptor expression, as has previously been shown for RSV infected A549 cells by Kotelkin *et al.* and for influenza virus infected mouse lung epithelial cells by Herold and co-workers. In line with these results we found evidence that *in vitro* RSV infection increased TRAIL-R1 and -R2 surface expression on normal primary
bronchial epithelial cells of children. This may suggest a complex interplay between host defence against virus infected cells on the one hand, and immunopathogenic mechanisms by sTRAIL against bystander (uninfected) epithelial cells on the other hand. However, in the case of widespread viral infection this theoretical concept of bystander versus viral infected cell, in terms of death of bystander cells is ‘bad’, whereas death of infected cells is ‘good’, may just be too simple. The seemingly beneficial effect of activation of pro-apoptotic pathways specifically directed against viral infected cells may be completely lost when a high number of cells is infected, leading to widespread cell death and therefore serious injury to the lungs and airways. This is supported by the study by Herold et al. which showed that inhibition of the TRAIL pathway attenuates lung injury despite decreased influenza viral clearance in mice. Our results show that sTRAIL may cause death of human airway epithelial cells, either infected by RSV or not, but whether this mechanism contributes to severe RSV disease in vivo remains to be elucidated.

In the present study we did not observe a statistically significant relation between disease severity (ARDS and duration of mechanical ventilation) and the levels of sTRAIL in the lungs. Remarkably, in general the development of ARDS during RSV infection leads to a minor increase in mortality in young children. This suggests that patients admitted for RSV-acute respiratory failure may represent the end of spectrum of RSV disease, regardless of fulfilling the clinical criteria of ARDS, and this withholds us from drawing firm conclusions about our findings in this cohort. The fact that several animal studies have convincingly shown that TNFR ligands, including TRAIL, can under certain circumstances contribute to the development of lung injury, to our believe warrants further research on this topic.

In summary, severe RSV-LRTI in children is associated with elevated levels of sTRAIL in the lungs. Primary lung epithelial cells of children are susceptible for sTRAIL-induced apoptosis in vitro. We speculate that activation of the TRAIL pro-apoptotic pathway is a mechanism of lung epithelial injury in severe RSV infection in children.
REFERENCES


Supplemental figure 1. A, percentages of PI positive BJAB cells upon 48 hr exposure to DR5-Fc (10μg/ml), human IgG (10μg/ml) or human cross-linked recombinant TRAIL (10-1000 μg/ml) with and without DR5-Fc or human IgG. Note the complete neutralization of TRAIL signaling by DR5-Fc. B, percentages of PI positive primary bronchial epithelial cells of children upon 48 hr exposure to DR5-Fc (10μg/ml) or human IgG (10μg/ml). Note the absence of direct Fc- or antibody cytotoxicity.