Acute lung injury in children: from viral infection and mechanical ventilation to inflammation and apoptosis
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Citation for published version (APA): Bern, R. A. (2010). Acute lung injury in children: from viral infection and mechanical ventilation to inflammation and apoptosis

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Mechanical Ventilation Enhances Lung Inflammation and Caspase Activity in a Model of Mouse Pneumovirus Infection

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ABSTRACT

Severe infection with respiratory syncytial virus (RSV) in children can progress to respiratory distress and acute lung injury (ALI). Accumulating evidence suggests that mechanical ventilation (MV) is an important cofactor in the development of ALI by modulating the host immune responses to bacteria. This study investigates whether MV enhances the host response to pneumonia virus of mice (PVM), a mouse pneumovirus that has been used as a model for RSV infection in humans. BALB/c mice were inoculated intranasally with diluted clarified lung homogenates from mice infected with PVM strain J3666 or uninfected controls. Four days after inoculation the mice were subjected to 4 hr of MV (Vt 10 ml/kg), or allowed to breathe spontaneously. As compared with mice inoculated with PVM-only, the administration of MV to PVM-infected mice resulted in increased bronchoalveolar lavage fluid (BALF) concentrations of the cytokines MIP-2, MIP-1α (CCL3) and IL-6; increased alveolar-capillary permeability to high molecular weight proteins; and increased caspase-3 activity in lung homogenates. We conclude that MV enhances the activation of inflammatory and caspase cell death pathways in response to pneumovirus infection. We speculate that MV potentially contributes to the development of lung injury in patients with RSV infection.
INTRODUCTION

Respiratory syncytial virus (RSV) is an important cause of severe lower respiratory tract infections in infants and young children 1. Treatment remains largely limited to supportive measures such as mechanical ventilation 2. Severe RSV infection may progress to acute lung injury (ALI) or its more severe form, the acute respiratory distress syndrome (ARDS) 3,4. ALI/ARDS is a syndrome associated with prominent neutrophil recruitment to lung tissues accompanied by increased permeability of the alveolar epithelial barrier. The development of ALI/ARDS in children with RSV infection frequently necessitates prolonged mechanical ventilation, which may lead to long-term morbidity and related functional consequences 4-6. At the present time, the factors that determine whether RSV infection progresses to ALI/ARDS remain unclear.

Recent studies suggest that mechanical ventilation may contribute to the development of lung injury by enhancing the host response to bacteria and bacterial products 7-11. Mechanical ventilation with tidal volumes of 10 ml/kg amplifies the pulmonary inflammatory responses of mice to transpharyngeal LPS by a mechanism involving activation of genes involved in inflammatory and stress responses, including the genes coding for the cytokines CCL3 (MIP-1α), CXCL2 (MIP-2) and IL-6; components of the mitogen-activated protein kinases (MAPK) pathway (e.g. GADD45-α, β and □), and transcription factors (e.g. IRF-7) 8. O’Mahony et al. extended these findings by demonstrating that mechanical ventilation enhances the lung inflammatory response to systemic LPS, and Dhanireddy et al. confirmed that mechanical ventilation enhances lung injury in response to live bacteria 10,11. The exact mechanisms whereby mechanical ventilation alters the host inflammatory response remain unclear, but studies in vitro have shown that the application of cyclic pressure-stretch to primary human alveolar macrophages and alveolar epithelial-like A549 cells induces IL-8 release, which is enhanced when the cells are exposed to LPS 12-14. These findings suggest that mechanical ventilation results in an inflammatory response that may be augmented by co-stimulation with bacteria. One important question is whether the interaction between mechanical ventilation and host defences is exclusive to bacteria or bacterial products, or instead is a more general phenomenon extensive to viral pathogens such as RSV.

A limitation to the study of pathogenic mechanisms of RSV infection in vivo is the relative resistance of mice to RSV 15,16. RSV shows limited replication in mouse lungs, and causes little to no overt clinical disease in mice even when inoculated with high titers 16. As an alternative, a mouse model using pneumonia virus of mice (PVM) is being increasingly recognized as having unique features that mimic human RSV lower respiratory tract infections 15,17. PVM is a natural rodent pathogen belonging to the same virus family, subfamily and genus as RSV, and shows robust replication in mice 15,17. PVM and RSV share the same gene order and are structurally similar to one another 18,19. Most importantly, murine PVM and human RSV infections induce similar clinical and
pathological responses, including predominance of peribronchiolar cellular inflammation in the lungs and a cytokine profile dominated by CC and CXC chemokines. It is important to emphasize that RSV and PVM are different viruses, and therefore PVM remains an imperfect model of human RSV. However, because of the lack of better murine models of RSV infection, and because the responses elicited by PVM in mice are similar to those elicited by RSV in humans, we used PVM in this study as a model to investigate acute lung injury and inflammation by mechanical ventilation.

In this study, we test the hypothesis that mechanical ventilation enhances the lung response to lower respiratory tract infection by PVM in mice by investigating the lung inflammatory, permeability and apoptotic responses of mice subjected to mechanical ventilation 4 days after intranasal inoculation with the virulent PVM strain J3666.

**METHODS**

**Viral stock preparation**

Pneumonia virus of mice (PVM) strain J3666, was a gift from Dr. A.J. Easton and was kept virulent by continuous passage in mice. For this study, lungs from five BALB/c mice infected with PVM were homogenized at 4°C in 1 mL Iscove's modified Dulbecco's medium (IMDM) (Life Technologies, Gaithersburg, MD), then spun at 13,000 x g for 5 min at room temperature. The supernatants were pooled and stored as individual aliquots in liquid nitrogen. The virus titer in the aliquots was 1.1 x 10^{10} copies of PVM / ml. For control inoculations, clarified lung homogenates were generated from a healthy BALB/c mouse by identical methods. On the day of each experiment, one aliquot was thawed and diluted 30-fold in RPMI medium (Cellgro, Mediatech Inc, Herndon, VA).

**Animal protocols**

The animal protocols were approved by the Animal Care Committee of the VA Puget Sound Healthcare System, Seattle, WA. Female BALB/c mice weighing 18 to 22 g (Jackson Laboratory, Bar Harbor, ME) received intranasal instillations of diluted virus stock in a total volume of 80 μL. Four days later the mice were anesthetized with inhaled isoflurane. Some mice were allowed to recover from anesthesia, and some were intubated endotracheally and subjected to 4 hr of mechanical ventilation using tidal volume=10 ml/kg; respiratory rate=150 breaths/minute; fraction of inspired oxygen=0.21; and PEEP= 2 cm H2O. Airway pressures and electrocardiogram were monitored continuously, and the rectal temperature was kept at 36-37°C with external heating. At the end of the experiment the mice were euthanized with intraperitoneal pentobarbital (120 mg/kg) and exsanguinated by closed intracardiac puncture. The left lung was removed and flash-frozen in liquid nitrogen. Bronchoalveolar lavage (BAL) was performed in the right lung by instilling four separate 0.5 ml aliquots of 0.9 % NaCl containing 0.6 mM EDTA. An aliquot of BAL fluid (BALF) was processed immediately...
for cell counts and differentials. The remainder of the BALF was spun at 200 x g for 10 min at 4°C and the supernatants were stored in individual aliquots at -80°C. Immediately after the BAL procedure, the right lung was fixed with 4% paraformaldehyde at 15 cm H₂O for histological analysis.

**Experimental design**

First, to determine the optimal day for mechanical ventilation, defined as the onset of inflammation, mice received intranasal instillations of clarified lung homogenates containing PVM, diluted 1:100, 1:30 and 1:10 in RPMI medium; control mice were instilled with RPMI medium containing no virus (n=5/group). The mice were evaluated for clinical evidence of disease once per day using a score previously tested in other PVM studies (Table 1) (modified from Cook et al. 25). Next, to determine the optimal dose of virus, mice were euthanized at the onset of clinical signs of disease and evaluated for cellular, inflammatory, permeability and apoptotic responses.

Once the time and dose of virus were identified, we studied 4 groups of mice: mice inoculated with control homogenates followed by spontaneous breathing on day 4 (“SB”, n = 6); mice inoculated with control homogenates followed or mechanical ventilation on day 4 (“MV”, n = 5); mice inoculated with PVM followed by spontaneous breathing on day 4 (“PVM + SB”, n = 6) and mice inoculated with PVM followed by mechanical ventilation on day 4 (“PVM + MV”, n = 6).

**Measurements**

**Lung Injury.** Total cell counts were performed on an aliquot of the BALF, using a hemacytometer. Differential cell counts were performed on cytospin preparations using the Diff-quick method (Andwin Scientific, Addison IL). BALF total protein was measured with the bicinchoninic acid method (BCA assay, Pierce Co., Rockford, IL). BALF IgM, α-macroglobulin, and MIP-1α (CCL3) were measured with immunoassays (Bethyl Laboratories Inc., Montgomery, TX for Ig-M; Life Diagnostics, Inc. West Chester, PA for α-macroglobulin and R&D systems, Minneapolis, MN for MIP-1α). BALF TNF-α, IL-1β, MIP-2, KC, IL-6, IL-10, MCP-1 (CCL2), IFN-γ, GM-CSF, and VEGF were measured using a multiplex fluorescent bead assay (R&D systems, Minneapolis, MN).

<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>Score</th>
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<tbody>
<tr>
<td>Healthy, no signs of illness</td>
<td>1</td>
</tr>
<tr>
<td>Subtle ruffled fur</td>
<td>2</td>
</tr>
<tr>
<td>Evident ruffled fur with hunched posture</td>
<td>3</td>
</tr>
<tr>
<td>Evident lethargy with abnormal breathing pattern</td>
<td>4</td>
</tr>
<tr>
<td>Moribund</td>
<td>5</td>
</tr>
<tr>
<td>Dead</td>
<td>6</td>
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**Table 1.** Scores associated with clinical signs caused by PVM infection in mice.
Apoptosis. Caspase-3 activity was measured in lung homogenates with the caspase-3/CPP32 Fluorometric Assay kit according to manufacturer instructions (Biovision Inc.). The concentrations of FasL and granzyme B in lung homogenates were measured using immunoassays (R&D systems, Minneapolis, MN and eBioscience, San Diego, CA respectively). Caspase-3 immunohistochemistry was performed using rabbit anti-cleaved caspase-3 mAb (BD Pharmingen, San Jose, CA) and biotinylated goat anti-rabbit IgG (Vector Laboratories) as previously described previously. DNA nick-end labeling assays (TUNEL) were performed using the TACS XL Blue Labeling kit from Trevigen Inc. (Gaithersburg, MD). TUNEL results were quantified by counting the number of positive cells in 12 randomly generated high power fields. The counts were done in a blinded fashion.

Viral loads. The viral sh gene (GenBank: AY573815) was used as marker of PVM. RNA was isolated from frozen lungs with the Absolutely RNA® Miniprep kit (Stratagene, La Jolla, CA) and reverse transcribed to cDNA (High-capacity cDNA kit, Applied Biosystems, Foster City, CA). Copies of the sh gene of PVM were detected in RT-PCR reactions containing 1 μl cDNA, Taqman PCR Master Mix (Applied Biosystems, Foster City, CA), 77 nM TAMRA probe (5’-6FAM-CGCTGATAATGGCCTGCAGCA TAMRA-3’), 200 nM primers (5’-GCCTGATGTCAGTGCAGTT-3’ and 5’-GCCTGATGTCAGTGCAGTT-3’) (Ellis et al. 2007). The gapdh housekeeping gene was detected in cDNA samples using rodent gapdh primers (100nM) and VIC-probe (200nM) of (Applied Biosystems). Standard curves with known concentrations of the full-length sh gene and gapdh decatemplate (Ambion, Foster City, CA) were used for quantification. Results are expressed as copies of PVM-sh per 10⁹ copies of gapdh.

Statistical analysis
Comparisons between multiple groups were performed using one-way ANOVA. Significance between groups was determined with the Fisher’s Least Significant Difference (LSD) post-hoc test. A p value of < 0.05 was considered statistically significant. Data are reported in the text as means ± SEM, and shown in the figures as individual data points and boxplots depicting the median, interquartile range and range.

RESULTS

Determination of time for mechanical ventilation and dose of PVM
The mice instilled with 1 x10⁷ copies of PVM, and mice instilled with medium only developed no clinical evidence of disease (Figure 1A). The mice instilled with 3 x10⁷ copies of PVM developed clinical evidence of disease on day 4 (mean clinical score = 3), and the mice treated with 9 x10⁷ copies of PVM developed a clinical score of 2 on day 3 and a clinical
score of 5 on day 4. Based on these data, we chose day 4 as the day for the ventilation studies. Next, to confirm the dose of virus, we evaluated the key variables of lung injury on day 4. Mice treated with 3 × 10^7 copies of PVM developed mild increases in total BALF PMN and cytokines (Figure 1B and C), and minimal increases in permeability and apoptosis markers (Figure 1D and E). Based on these data, we chose the dose of 3 × 10^7 copies of PVM as the optimal dose to identify an enhancing effect of mechanical ventilation.

**Figure 1.** Mean clinical score in mice treated with medium only (no virus) or 3 different doses of PVM (low: 1 × 10^7; mid: 3 × 10^7; high: 9 × 10^7 copies of PVM), evaluated on days 1 – 4 after inoculation (A). Total BALF macrophages (AM), neutrophils (PMN) and lymphocytes (Ly) (B); BALF cytokine concentrations (C); BALF permeability markers (IgM and α-macroglobulin) (D) and lung homogenate caspase-3 activity (E) in mice treated with medium only (no virus) or 3 different doses of PVM (low: 1 × 10^7; mid: 3 × 10^7; high: 9 × 10^7 copies of PVM) on day 4 after inoculation. n= 5 per group. * p < 0.05 as compared to the uninfected mice.
Lung leukocyte response

The number of alveolar macrophages in the BALF of uninfected, spontaneously breathing mice was $41.5 \pm 5.0 \times 10^4$ cells (Figure 3A). In comparison, the number of macrophages in the BALF of each of the other groups was significantly decreased: $26.1 \pm 2.9 \times 10^4$ cells in mice exposed to mechanical ventilation alone; $21.3 \pm 4.7 \times 10^4$ cells in PVM-treated mice breathing spontaneously, and $18.0 \pm 2.4 \times 10^4$ cells in mice exposed to both PVM and mechanical ventilation. In contrast, significant neutrophil recruitment was observed in response to PVM infection ($1.6 \pm 0.9 \times 10^4$ cells), but mechanical ventilation did not result in a large additional response ($2.3 \pm 0.9 \times 10^4$ cells) (Figure 3B). PVM infection resulted in lymphocyte recruitment, and the addition of mechanical ventilation augmented lymphocyte recruitment approximately 4-fold (Figure 3C).

Lung cytokine response

PVM infection is accompanied by local production of numerous cytokines, including CC and CXC chemokines\(^20,28\). In particular, MIP-2 and MIP-1\(\alpha\) (CCL3) correlate with the severity of murine PVM infection and of human RSV disease, and MIP-1\(\alpha\) (CCL3) is a key mediator for granulocyte recruitment to lung tissue following PVM infection\(^17,24,28\). In the present study, the concentrations of the chemokines MIP-2 and MIP-1\(\alpha\) (CCL3) showed a small increase in spontaneously breathing mice infected with PVM, and a greater increase in PVM infected mice subjected to mechanical ventilation (Figure 4A-B). The concentrations of KC and IL-6 in were also significantly increased in the PVM + MV mice (Figure 4C-D). The concentrations of VEGF showed a trend towards increase in response to mechanical ventilation plus PVM.
**Figure 3.** Total BALF macrophages (AM, A), neutrophils (PMN, B) and lymphocytes (C) detected in uninfected mice allowed to breathe spontaneously (SB, n = 6) or subjected to mechanical ventilation (MV, n = 5); and in PVM infected mice allowed to breathe spontaneously (PVM + SB, n = 6) or subjected to mechanical ventilation (PVM + MV, n = 6). * p < 0.05. Data are shown as individual data points and boxplots depicting the median, interquartile range and range.

infection (Figure 4E). The cytokines TNF-α, IL-1β, IL-10 and GM-CSF were all below the limit of the assay. Likewise, low levels of MCP-1 (CCL2) and IFN-γ were detected in the BALF of mice of PVM infected mice only (data not shown).

### Lung permeability

Lung permeability was assessed by measuring the BALF concentrations of total protein and of the high-molecular weight serum proteins IgM and α-macroglobulin. In uninfected mice breathing spontaneously the mean IgM concentration in the BALF was $33.7 \pm 8.9$ ng/ml (Figure 5A). Mechanical ventilation alone or PVM alone resulted in a small increase of IgM in BALF ($69.4 \pm 10.9$ ng/ml for the MV group and $43.0 \pm 10.2$ ng/ml for the PVM + SB group); these increases did not reach statistical significance. In contrast, the mean IgM concentration in the BALF of mice exposed to both PVM and mechanical ventilation was $101.7 \pm 20.3$ ng/ml; this represented a significant increase compared to spontaneously breathing mice with or without PVM infection. The α-macroglobulin concentrations in BALF followed a similar pattern to IgM (Figure 5B), and the total protein in BALF showed a trend towards increased concentrations in the mice exposed to both PVM and mechanical ventilation (Figure 5C).
Lung apoptotic response

To assess the apoptotic response we measured lung homogenate caspase-3 activity, tissue expression of cleaved caspase-3 by immunohistochemistry, TUNEL staining, and lung homogenate concentrations of sFasL and Granzyme-B. Mechanical ventilation did not alter caspase-3 activity in the uninfected mice, but led to a significant increase in caspase-3 activity in the mice infected with PVM (Figure 6A). Caspase-3 activity was localized to cells of the

Figure 4. Cytokine concentrations in the BALF of uninfected mice allowed to breathe spontaneously (SB, n = 6) or subjected to mechanical ventilation (MV, n = 5); and in PVM infected mice allowed to breathe spontaneously (PVM + SB, n = 6) or subjected to mechanical ventilation (PVM + MV, n = 6). * p < 0.05. Data are shown as individual data points and boxplots depicting the median, interquartile range and range.
alveolar walls and macrophages, and this was independent of the treatment group (Figure 6E-H). Overall, there was less than 1 cell staining for caspase-3 in each high power field. TUNEL staining was increased in all of the mice infected with PVM, regardless of mechanical ventilation (p=NS) (Figure 6B). There was a trend towards increased concentrations of sFasL and granzyme B in the lungs of the PVM infected mice subjected to mechanical ventilation, but this did not reach statistical significance (Figure 6C, D).

Microscopic lung histology
There were no prominent differences observed in an examination of lung tissue from uninfected mice subjected to mechanical ventilation compared to those breathing spontaneously (Figure 7A and 7B). In the mice from the PVM + SB group, the lungs showed variable degrees of mixed mononuclear and neutrophilic peribronchial infiltrates (Figure 7C), consistent with previous findings \( ^{15} \). The lungs of the PVM infected mice subjected to mechanical ventilation showed tissue findings similar to those of the PVM + SB mice (Figure 7D).
Figure 6. Caspase-3 activity in lung homogenates (A); number of TUNEL-positive cells per 12 high-power fields in lung tissue sections (B); BALF soluble FasL concentrations (C) and BALF granzyme B (GrB) concentrations (D) in uninfected mice allowed to breathe spontaneously (SB, n = 6) or subjected to mechanical ventilation (MV, n = 5); and in PVM infected mice allowed to breathe spontaneously (PVM + SB, n = 6) or subjected to mechanical ventilation (PVM + MV, n = 6), * p < 0.05. The cellular distribution of caspase-3 activation is shown in merged differential interference contrast (DIC) and fluorescence images (cleaved caspase-3 in pink, nuclei in blue) from a mouse in the SB group (E); MV group (F), PVM group (G), and PVM + MV group (H). The figures show the presence of signal in cells of the alveolar walls (open arrows) or macrophages (closed arrow).
Functional lung responses in ventilated mice

To assess whether the patchy distribution of peribronchial inflammation led to increases in airway flow resistance, we measured peak airway pressures continuously for the duration of mechanical ventilation. The peak inspiratory pressures in the MV and PVM + MV mice were similar during the period of mechanical ventilation (Figure 7E). In both mechanically ventilated groups, the peak inspiratory pressures increased with time. The transcutaneous CO2 pressure in the mice in the MV group was 46 ± 2.2 mmHg, and 54 ± 2.8 mmHg in the PVM + MV group (p = 0.09).

Figure 7. H&E stained lung tissue sections of lung tissue from uninfected mice allowed to breathe spontaneously (SB, A) or subjected to mechanical ventilation (MV, B); and in PVM infected mice allowed to breathe spontaneously (PVM + SB, C) or subjected to mechanical ventilation (PVM + MV, D); 400X magnification. Note the peribronchiolar cellular infiltration in both the PVM + SB and PVM + MV group (arrows). E, mean peak inspiratory pressures in uninfected mice subjected to mechanical ventilation (n = 5) and PVM infected mice subjected to mechanical ventilation (n = 6). Means ± standard error.
DISCUSSION

The goal of this study was to determine whether mechanical ventilation enhances the response of mice to pneumoviral infection. The mice were infected with PVM and subjected to a mechanical ventilation strategy that results in minimal injury when applied alone. The main findings are that exposure to 4 hours of mechanical ventilation in the setting of PVM infection enhances the local release of the cytokines MIP-2, MIP-1α (CCL3) and IL-6; increases alveolar-capillary permeability to high molecular weight proteins; and induces an increase in lung caspase-3 activity. These data suggest that mechanical ventilation may serve to exacerbate local inflammatory responses and lung injury in pneumovirus infection.

Human and animal studies have demonstrated that mechanical ventilation may worsen lung injury and outcome in children and adults with ALI/ARDS. In adults, the ARDS Network trial showed that ALI/ARDS mortality can be decreased by reducing tidal volumes. Interestingly, in the same cohort lower tidal volumes were also associated with lower circulating concentrations of pro-inflammatory cytokines such as IL-8 and IL-6, suggesting the existence of a direct link between mechanical ventilation and inflammation. In the pediatric population a recent retrospective study evaluating children with ALI/ARDS showed that the change in ventilatory practice towards the use of a lower tidal volume in 2000-2004 was associated with decreased mortality and increased ventilator-free days. Thus, tidal volumes are associated with mortality and with the severity of the inflammatory response in adults and children with ALI/ARDS. In contrast to patients with ALI/ARDS, patients with spinal cord injury or other neuromuscular diseases that need mechanical ventilation usually receive large tidal volumes, and yet these patients do not develop lung injury. Thus, the association between tidal volume, mortality and inflammation seen in patients with ALI/ARDS is not dependent exclusively on mechanical factors associated with stretch, but requires the presence of additional factors associated with the ALI/ARDS lung microenvironment, that are magnified by the addition of mechanical ventilation.

The possibility that mechanical ventilation enhances the inflammatory response of the lungs to pathogens has been underscored by studies demonstrating that mechanical ventilation at tidal volumes that do not induce injury in healthy lungs, may induce injury in lungs exposed directly or indirectly to bacterial products or to live bacteria. The synergism between mechanical ventilation and bacteria/bacterial products is characterized by increased alveolar neutrophils, increased cytokine concentrations, and disruption of the epithelial barrier. The present study demonstrates that in addition to enhancing the lung response to LPS and bacteria, mechanical ventilation also enhances the lung response to viruses such as PVM. The amplifying effect of mechanical ventilation on PVM infection has several features in common with previous models based on LPS or bacteria, including increased release of MIP-2, MIP-1α (CCL3) and IL-6. This suggests that the enhancing effect of mechanical ventilation on lung injury is not
limited to bacteria or LPS, but instead represents a more general response to a variety of viral and bacterial pathogens that signal through different receptor pathways. This has broad implications for the treatment of critically ill patients.

The cellular mechanisms linking mechanical ventilation with enhancement of the innate immune response to pathogens remain unclear. Human macrophages and alveolar epithelial cells exposed to cyclic stretch in vitro show alterations in membrane lipid trafficking and changes in cytoskeleton-protein interactions that result in activation of mitogen-activated protein kinases (MAPK) including JNK and p38, and activation of transcription factors such as NF-κB, Erg-1 and the AP-1 component c-fos. Activation of pro-inflammatory pathways following these intracellular processes, most notably the activation and translocation of NF-κB, is thought to be a key event in stretch induced lung injury in vivo. The magnitude of the cytokine response of lung cells to in vitro stretch varies with the cell type, with macrophages showing greater release of TNF-α, IL-8 and IL-6 as compared to epithelial cells. In addition to the induction of pro-inflammatory events, stretch induced by mechanical ventilation may result in the activation of cell death pathways. Mice exposed to 4 hr of mechanical ventilation with tidal volumes of 20 ml/kg show evidence of caspase-dependent apoptosis in lung endothelial and alveolar epithelial cells by a mechanism involving activation of the MAPK member p38. Together these data suggest that stretch activates intracellular pathways leading to inflammatory and cell death responses in epithelial and immune cells of the lungs.

The inflammatory and cell death responses of lung cells to stretch are modulated by bacterial products. The release of pro-inflammatory cytokines by human macrophages and human and rat lung epithelial cells exposed to stretch is enhanced by LPS in vitro. Tremblay et al. showed increased concentrations of TNF-α, MIP-2 and IL-6 in BALF of ex vivo rat lungs exposed to LPS, as compared to saline, after high volume ventilation. These observations have been extended by studies showing that unique sets of genes are upregulated in mice exposed to the combination of LPS and mechanical ventilation. These genes include the chemokines CCL3 and CXCL2; IL-6, IL-1β and components of the MAPK cascade such as GADD45-γ. A transcriptional analysis performed on the same dataset revealed enrichment of several transcription factors including NF-κB, ETF, USF2, IRF7 and Myc. These findings indicate that the interaction between mechanical ventilation and pathogens results in the activation of a specific transcriptional response that may result in enhancement of downstream pro-inflammatory pathways.

A number of studies suggest that apoptosis of lung epithelial cells and migrating leukocytes, such as neutrophils and macrophages, is a key event in the development of ALI/ARDS in adults. As mentioned above, the same may be true in children with severe RSV, who have marked caspase-3 activation in the airway epithelium; and studies in vitro showed that alveolar epithelial cells isolated from septic rats are more susceptible to cell death in response to cyclic stretch than alveolar epithelial cells from non-septic
rats. This suggests that stretch due to mechanical ventilation may enhance cell death pathways in the lungs. In our study, mice infected with PVM and subjected to mechanical ventilation had increased caspase-3 activity, which was primarily localised to cells in the alveolar walls and to macrophages; however, the number of TUNEL-positive cells was not significantly increased by mechanical ventilation. One possible explanation for the discrepancy between the observed caspase-3 activity and TUNEL staining is that caspase-3 activation is generally considered an early event in apoptosis, whereas DNA fragmentation occurs at later stages. Alternatively, the activation of caspases may trigger pathways separate from apoptosis, in particular pathways leading to inflammation. Therefore, the role of apoptosis in the synergism between mechanical ventilation and viral pathogens remains to be fully clarified.

The presence of caspase activation in cells of the alveolar walls and also in alveolar macrophages raises the question of whether the mechanism of caspase activation was the same for these two cell types. Some bacterial agents, such as Legionella pneumophila and Streptococcus pneumoniae, are capable of inducing apoptosis of both alveolar epithelial cells and macrophages; in contrast other agents, such as Chlamydia muridarum induce apoptosis in macrophages only. RSV has been associated with both enhancement and inhibition of apoptosis of immortalized lung epithelial cell lines in vitro. This differential effect of RSV on apoptosis may be explained in part by predominant effects of different RSV proteins such as the F protein and nonstructural proteins NS1 and NS2. SH induces p53-dependent apoptosis in A549 cells by a mechanism involving activation of the caspase-pathway, eventually resulting in cell shedding, in contrast NS1 and NS2 delay apoptosis by a pathway that may involve NF-kB. RSV infected A549 epithelial cells seem more susceptible for death receptor ligands such as FasL and TRAIL. Finally, studies of lung tissue of children with fatal RSV infection showed marked caspase-3 staining of the bronchiolar epithelium, suggesting a pro-apoptotic effect of RSV infection in vivo. Thus, the effects of infectious agents on apoptosis of lung cells appear to depend on the species of the infectious agent and on what specific pathogen pathways are triggered, and may involve enhancement or inhibition of apoptosis in epithelial cells, macrophages or both. The present study suggests that both macrophage and epithelial cell apoptosis are present in mice infected with PVM, but additional studies will be necessary to determine whether the mechanism of caspase activation is similar in these two cell types. The present study has several limitations. First, PVM infection resulted in patchy areas of peribronchiolar inflammation, which may have caused heterogeneous distribution of the tidal volume with some lung areas becoming exposed to higher stretch than others. The peak airway pressures were similar in the uninfected and PVM infected ventilated mice, suggesting the absence of large differences in airway obstruction between the two groups. However, peak airway pressures may not entirely reflect alveolar pressures, and we cannot fully exclude enhancement effects related to regional overdistention. As observed in studies using LPS, in the present study the mice...
treated with both mechanical ventilation and PVM showed greater variability in several measurements of lung injury \textsuperscript{7,10}. This observation may suggest differential responses of lung cells to mechanical forces, potentially resulting from regional volume/pressure differences in diseased lungs. Second, all of the mice subjected to mechanical ventilation in this study were kept anaesthetised with inhaled isoflurane. Volatile anesthetics appear to have immuno-modulatory effects \textit{in vitro}, but these are associated with immune suppression rather than activation \textsuperscript{57}. However, we cannot fully exclude that some of our findings are due to immunomodulation by the anesthetic used in this study. Finally, the PVM mouse model remains an imperfect model of human RSV infection, because PVM and RSV are not the same virus. However, because RSV is not a natural pathogen of mice, and because the molecular and pathological responses of mice to PVM share more features with human RSV infections than the murine responses to RSV, the PVM model has been used extensively as an experimental model of RSV infection \textsuperscript{15,17}.

In summary, mechanical ventilation enhanced the lung responses to viral infection caused by the murine pneumovirus PVM in mice. We conclude that mechanical ventilation may contribute to the pathogenesis of lung injury in response to pneumovirus infection by enhancing activation of inflammatory and cytopathological pathways in the lungs.
REFERENCES


(56) O’donnell DR, Milligan L, Stark JM. Induction of CD95 (Fas) and apoptosis in respiratory epithelial cell cultures following respiratory syncytial virus infection. Virology 1999;257:198-207.