Role of the Fas/FasL system in a model of RSV infection in mechanically ventilated mice

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van den Berg E, van Woensel JB, Bos AP, Bem RA, Altemeier WA, Gill SE, Martin TR, Matute-Bello G. Role of the Fas/FasL system in a model of RSV infection in mechanically ventilated mice. Am J Physiol Lung Cell Mol Physiol 301: L451–L460, 2011. First published July 8, 2011; doi:10.1152/ajplung.00368.2010.—Infection with respiratory syncytial virus (RSV) in children can progress to respiratory distress and acute lung injury necessitating mechanical ventilation (MV). MV enhances apoptosis and inflammation in mice infected with pneumonia virus of mice (PVM), a mouse pneumovirus that has been used as a model for severe RSV infection in mice. We hypothesized that the Fas/Fas ligand (FasL) system, a dual proapoptotic/proinflammatory system involved in other forms of lung injury, is required for enhanced lung injury in mechanically ventilated mice infected with PVM. C57BL/6 mice and Fas-deficient (“lpr”) mice were inoculated intratracheally with PVM. Seven or eight days after PVM inoculation, the mice were subjected to 4 h of MV (tidal volume 10 ml/kg, fraction of inspired O2 = 0.21, and positive end-expiratory pressure = 3 cm H2O). Seven days after PVM inoculation, exposure to MV resulted in less severe injury in lpr mice than in C57BL/6 mice, as evidenced by decreased numbers of polymorphonuclear neutrophils in the bronchoalveolar lavage (BAL), and lower concentrations of the proinflammatory chemokines KC, macrophage inflammatory protein-1α (CCL3), MIP-2 (CXCL2), and monocyte chemotactic protein-1 (CCL2), increased lung permeability, increased apoptosis, and robust viral replication. As a result, PVM is increasingly used as a model of severe RSV infection in mice (3, 34).

Using the PVM model of RSV infection, we recently investigated whether MV with noninjurious tidal volumes enhances the inflammatory and apoptotic responses of the lung to PVM (5). We selected a PVM inoculum low enough to cause only minimal lung injury and demonstrated that, in the presence of a minimally injurious ventilatory strategy, the lung’s inflammatory and apoptotic responses were enhanced, with increases in the lung concentrations of the proinflammatory mediators MIP-1α, MIP-2, and IL-6, and also in the lung activity of the proapoptotic protease caspase-3 (5). These findings were particularly remarkable in that the changes in the severity of injury occurred only after 4 h of MV, suggesting that mechanical stretch can have an important deleterious effect in lungs infected with pneumovirus.

The mechanism whereby MV alters the host inflammatory response is unclear, but one possibility is activation of the Fas/FasL system. Binding of FasL (CD178) to Fas (CD95) on target cells triggers apoptosis by aggregation of Fas-associated death domain-containing protein, leading to the activation of the caspase cascade (reviewed in Ref. 37). In addition to its proapoptotic function, the Fas/FasL system can also activate proinflammatory pathways that result in neutrophilic inflammation; these proinflammatory pathways appear to be independent...
of caspase-8 (20, 24, 26, 32, 33). The Fas/FasL system appears to be relevant for human lung injury because the concentrations of bioactive soluble FasL (sFasL) are increased in the bronchoalveolar lavage (BAL) and plasma of patients with ARDS, and the plasma sFasL concentrations are highest in patients who are ventilated with higher tidal volumes (1, 23, 27). Thus, MV upregulates the Fas/FasL system in the lungs, and, in turn, activation of the Fas/FasL system leads to apoptosis and inflammation, two key processes in the pathogenesis of ventilation-induced lung injury. This study tests the hypothesis that MV worsens lung injury in mice infected with PVM by a mechanism involving activation of the Fas/FasL system.

METHODS

Viral Stock Preparation

PVM strain J3666 originally was a gift from Dr. A. J. Easton and was kept virulent by continuous passage in mice (13). Clarified lung supernatants containing PVM were prepared by pooling the lungs of eight C57BL/6 mice infected with PVM. The pooled lungs were homogenized in 5 ml of PBS at 4°C and spun at 13,000 g for 5 min at 4°C. The supernatant was stored in individual aliquots in liquid nitrogen. The virus titer in the aliquots was 3.03 × 10^4 copies of PVM per microliter. On the day of each experiment, one aliquot was thawed and diluted 50-fold in PBS for subsequent inoculation in the mice as described below.

Animal Protocol

The animal protocols were approved by the Animal Care Committee of the University of Washington. Two strains of mice were used: C57BL/6 mice, which express Fas; and B6.MRL-Fas<sup>sh/lpr</sup> (lpr) mice, which are natural mutants deficient in Fas on a C57BL/6 background (Jackson Laboratory, Bar Harbor, ME). Briefly, male mice aged 7 to 13 wk old were anesthetized with inhaled isoflurane and intubated endotracheally with a 20-gauge Vialon angiocath (BD, Franklin Lakes, NJ). Placement of the catheter in the trachea was verified by visualizing the movement of a 100-μl bubble of water in an open syringe in response to respiratory efforts. The mice then received one 50-μl instillation of viral stock diluted 1:50 in sterile PBS, prepared as described above. The viral stock dilution was determined in pilot dose-response experiments. Following the instillations, the mice were returned to their cages with free access to water and food and monitored daily for clinical distress using a specific score system as previously described (Table 1) (10); a score of four or higher was a criterion for euthanasia. Seven or eight days after virus instillation, some of the mice were anesthetized with isoflurane and intubated endotracheally with a 20-gauge Vialon angiocath (BD, Franklin Lakes, NJ). Placement of the catheter in the trachea was verified by visualizing the movement of a 100-μl bubble of water in an open syringe in response to respiratory efforts. The mice then received one 50-μl instillation of viral stock diluted 1:50 in sterile PBS, prepared as described above. The viral stock dilution was determined in pilot dose-response experiments. Following the instillations, the mice were returned to their cages with free access to water and food and monitored daily for clinical distress using a specific score system as previously described (Table 1) (10); a score of four or higher was a criterion for euthanasia. Seven or eight days after virus instillation, some of the mice were anesthetized with isoflurane (induction 5%, maintenance 1.5%), intubated orally as described above, and subjected to MV with a rodent ventilator Type 845 (Mini-Vent; Hugo Sachs Elektronik Harvard Apparatus, March-Hugstetten, Germany) with the following settings: tidal volume 10 ml/kg; respiratory rate (RR) 150 breaths/min; fraction of inspired oxygen 0.21; and positive end-expiratory pressure of 3 cm H2O (31). The heart rate, airway pressure, rectal temperature, and end-tidal CO2 (EtCO2) were monitored continuously using a computerized monitoring system (Chart 4: AD Instruments, Colorado Springs, CO). The RR was adjusted to maintain the EtCO2 between 30 and 40 mmHg. The body temperature was maintained between 37 and 38°C with external heating. The mice were hydrated with a continuous intraperitoneal infusion of lactated Ringer solution at 500 μl/h. Muscle relaxation was attained with pancuronium bromide, 1 μg/g ip followed by 0.5 μg/g ip every hour. After 1 or 4 h of MV, the mice were killed with 0.30 ml/kg ip of Beuthanasia-D (Schering-Plough Animal Health, Union, NJ). The thorax was opened rapidly, and the mouse was exsanguinated by direct cardiac puncture. The heart was removed and fixed in 4% neutral buffered paraformaldehyde (PFA) for 30 min. The left lung was removed and flash-frozen in liquid nitrogen. The right lung was lavaged as described below, fixed in 4% PFA at an inflation pressure of 15 cm H2O, and embedded in paraffin for subsequent histological studies. Part of the liver, spleen, and kidney were flash-frozen in liquid nitrogen until ready for RNA isolation.

Experimental Design

We studied C57BL/6 or lpr mice inoculated with PVM and then allowed to breath spontaneously or subjected to MV for 4 h. These mice were studied at either 7 (n ≥ 6 mice/group) or 8 (n ≥ 7 mice/group) days after instillation of PVM. Additional C57BL/6 and lpr mice (n = 5 mice/group) were studied following MV for 1 h on day 8 after the instillation of PVM.

Sample Processing

The right lung was lavaged with PBS containing 0.6 mM EDTA at 37°C, one 0.6-ml aliquot followed by two 0.5-ml aliquots. The bronchoalveolar lavage fluid (BALF) was recovered by gentle suction, pooled, and placed on ice. A small portion of the BALF was processed immediately for total and differential cell counts. The remainder of the BALF was spun at 150 g for 5 min at 4°C, and the supernatants were stored at −80°C. Part of the left lung was homogenized for cytokine and myeloperoxidase (MPO) determinations as previously described (31).

Measurements

Viral loads. The viral sh gene (GenBank no. AY573815) was used as a marker for PVM. RNA was isolated from frozen lungs, hearts, livers, kidneys, and spleens with the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed to cDNA (high-capacity cDNA kit; Applied Biosystems, Foster City, CA). The sh gene and the gapdh gene (used as housekeeping gene) were detected by multiplex real-time PCR reactions containing 1 μl cDNA, SensiMix NoRef (Quantace, Taunton, MA), 100 nM sh probe (5′-GCCCTGACATCAACACAGTTGTG-3′ and 5′-GCCCTGATGGTCGAGTTTCTT-3′), 100 nM gapdh probe (5′-HEX-ATGACTGTGGTACGTAACCCCT BHQ1A-5HEX-3′), and 300 nM gapdh primers (5′-GACAATCTGGAGTTGGTGAGTT-3′ and 5′-AGTGAAGCTAGGAGTA-3′). Standard concentrations of the full-length sh gene and gapdh decatemplate (Ambion, Foster City, CA) were used for quantitation. Results are expressed as copies of PVM-sh per 10<sup>7</sup> copies of gapdh (19). All PCR assays were run in triplicates.

Lung inflammatory markers. Total BALF cell counts were determined by a dye exclusion-based method (Easycount Viasure Kit; Immunicon, Huntingdon Valley, PA). Cytospin preparations were stained with the Diff–quick method (Fisher Scientific, Kalamazoo, MI), and differential cell counts were obtained by counting 200 leukocytes using a standard light microscope. Tissue polymorphonu-
clear cells (PMNs) were assessed by MPO activity measured in lung homogenates using the Amplex red peroxidase assay kit (Invitrogen, Carlsbad, CA). The concentrations of MIP-1α (CCL3) and KC (CXCL1) were measured with immunoassays (R&D Systems, Minneapolis, MN). BALF IL-2, IL-4, IL-6, IL-10, IL-12, tumor necrosis factor (TNF)-α, IL-1β, MIP-2, interferon (IFN)-γ, granulocyte macrophage colony-stimulating factor (GM-CSF), and vascular endothelial growth factor were measured by multiplex fluorescent bead assay (Luminex; R&D Systems).

**Lung permeability.** BALF total protein was measured with the bicinchoninic acid method (BCA assay; Pierce, Rockford, IL). The high-molecular-weight protein α-macroglobulin was measured in BALF by enzyme-linked immunosorbent assay (Life Diagnostics, West Chester, PA).

**Lung apoptotic response.** Caspase-3 activity was measured in lung homogenates with the caspase-3/7/10/2 Fluorometric Assay Kit according to manufacturer instructions (Biovision, Mountain View, CA). Cleaved poly(ADP-ribose) polymerase (PARP) was measured in xylene, hydrated in graded alcohol, and penetrated for antigen retrieval in 10 mM citrate buffer for 20 min at 100°C. Staining was performed using rabbit anti-cleaved caspase-3 monoclonal antibody (mAb) (Asp-175; Cell Signaling, Danvers, MA) and a Leica Bond Polymer Refine Detection System (Leica Microsystems, Bannockburn, IL).

**Cardiac leukocyte infiltration.** Infiltration of leukocytes in the myocardium was detected by immunohistochemical staining with the pan-leukocyte marker CD45. The heart was fixed in 4% neutral buffered PFA for 30 min, embedded in paraffin, and cut into 5 μm sections. The heart sections were deparaffinized in xylene, hydrated in graded alcohol, and penetrated for antigen retrieval with 10 mM citrate buffer (Antigen Unmasking Solution; Vector Laboratories, Burlingame, CA) for 20 min at 100°C. The samples were labeled overnight in a moist chamber at 4°C using rat anti-CD45 antibody (BD Pharmingen, San Diego, CA) at a 1:100 dilution. Upon completion of primary antibody staining, the samples were labeled with the Polink-2 Plus HRP Rat-NM Dab detection kit (Golden Bridge International, Mukilteo, WA). The slides were scanned using the Olympus NanoZoomer digital pathology virtual microscope and analyzed using Visiomorph Image Analysis software (Visiopharm, Hoersholm, Denmark). The software was programmed to count the number of CD45-positive pixels (brown) and the total number of heart tissue pixels on random samples covering 10% of the total tissue area of each slide; data are expressed as the “CD45-to-tissue pixel ratio.” The assay was done in heart sections from three mice per group.

**Statistical Analysis**

The data were analyzed using GraphPad Prism 4.0 software (GraphPad, San Diego, CA). Comparisons between multiple groups were performed using a two-way factorial ANOVA, unless otherwise stated. Significance between groups was determined with the Bonferroni post hoc test. A P value of <0.05 was considered statistically significant. Data are reported as means ± SE.

**RESULTS**

Our first step was to compare the clinical response of C57BL/6 and lpr mice to PVM infection using a six-point clinical disease score developed specifically for use with PVM-infected mice (Table 1) (10). The onset and severity of clinical symptoms was similar in the C57BL/6 and lpr mice, with the earliest manifestations of disease occurring on day 4 after instillation and with mice from both strains reaching a clinical score of three (piloerection, deep breathing, and decreased alertness) by day 8 (Fig. 1). As an additional measurement of clinical illness, we monitored weight loss; the onset of weight loss occurred on day 7, rapidly increased by day 8, and was similar in C57BL/6 and lpr mice. We chose days 7 and 8 as the times for performing our studies.

**MV Enhances the Lung Inflammatory Response to PVM, and This Enhancement Requires a Functioning Fas/FasL System**

On day 7 after the instillation of PVM, all of the mice survived 4 h of MV. The lung viral loads were similar in the C57BL/6 and lpr mice regardless of whether the mice were allowed to breathe spontaneously (8.89 ± 2.84 vs. 4.77 ± 0.37 × 10^8 copies of PVM) or were subjected to MV (7.14 ± 1.52 vs. 9.07 ± 1.87 × 10^8 copies of PVM). No copies of the PVM sh gene were detected in homogenates of heart, liver, kidney, and spleen of PVM-infected mice.

In C57BL/6 mice, exposure to MV resulted in a significant increase in the total number of BAL neutrophils and in the concentration of proinflammatory chemokines such as KC, MIP-2, and MIP-1α compared with spontaneously breathing mice (Fig. 2. A–D). In contrast, in the lpr mice, the addition of MV had minimal or no effect on the lung inflammatory response. The number of PMN in the BALF of ventilated C57BL/6 mice was significantly higher than that of the lpr mice (1.97 ± 0.61 vs. 0.62 ± 0.11 × 10^5 cells, P < 0.05), as were the concentrations of the CXC chemokine KC (347.08 ± 24.89 pg/ml, P < 0.05) and the CC chemokine MIP-1α (92.92 ± 42.63 vs. 23.47 ± 3.08 pg/ml, P < 0.05). The number of lymphocytes and macrophages in the BALF of C57BL/6 and lpr mice was similar in each treatment group (data not shown), and the total lung neutrophil content, as determined by lung MPO activity, was similar in
C57BL/6 and lpr mice treated with PVM and MV (837.19 ± 40.88 vs. 755.90 ± 58.84 mU/ml). Thus, select cytokine production and PMN migration in the alveolar compartment in response to PVM was enhanced by MV in a Fas-dependent manner.

IFN-γ and IL-10 were significantly lower in the lpr mice compared with the C57BL/6 mice; however, in contrast to KC and MIP-2, IFN-γ and IL-10 did not show a ventilation-related effect (Fig. 2, E and F). Finally, IL-4, a Th2 cytokine, and TNF-α, IL-1β, and GM-CSF were all below the limit of detection (data not shown).

The lpr Mice Show Attenuated Permeability and Apoptotic Responses to PVM

Lung permeability was assessed by measuring the concentrations of total protein and α2-macroglobulin in the BALF. The latter is a high-molecular-weight protein (720 kDa) normally present in the serum only, and thus its appearance in the BALF is a marker of increased permeability to proteins across the alveolar-capillary barrier. We found significantly lower concentrations of both total protein and α2-macroglobulin in the BALF of spontaneously breathing lpr mice compared with C57BL/6 mice (Fig. 3, A and B). However, these differences were ventilation independent, suggesting a Fas-related difference irrespective of MV. To determine if the decrease in permeability seen in the lpr mice was associated with decreased injury of the alveolar epithelium, we measured the BALF concentrations of the receptor for advanced glycation end-products (RAGE), a marker of type I pneumocyte injury (42). The concentrations of RAGE were also decreased in the lpr mice and followed a similar pattern to that of the other permeability markers (Fig. 3). Thus, permeability disruption and type I injury were attenuated by Fas deficiency, and this was independent of the presence of MV.

It has been hypothesized that an important mechanism of disruption of the alveolar capillary barrier is apoptosis of alveolar epithelial cells mediated by the Fas/FasL system. To assess the apoptotic response, we measured lung homogenate caspase-3 activity and cleaved PARP. Caspase-3 activity (Fig. 4A) and cleaved PARP (Fig. 4B) showed a trend toward lower values in the lpr mice infected with PVM regardless of ventilation; these differences did not reach statistical significance. Immunohistochemistry studies suggested that cells expressing active caspase-3 were located in the alveolar spaces (Fig. 4C).

Histologically, the pattern of lung injury in C57BL/6 mice and lpr mice infected with PVM was characterized by variable...
degrees of intra-alveolar neutrophils and macrophages (Fig. 5). MV led to an increase in inflammatory cells in the alveolar spaces, thickening of the alveolar septa, and peribronchial wall thickening in B6 mice. These changes were less prominent in the lpr mice.

More lpr Mice Died When Ventilated After 8 Days of PVM Infection

Eight days after infection with PVM, the severity of disease had progressed in both C57BL/6 and lpr mice, as evidenced by
Fig. 5. The lpr mice infected with PVM had less ventilator-induced lung injury compared with C57BL/6 mice. Representative hematoxylin and eosin-stained lung tissue sections from C57BL/6 and lpr mice that were infected with PVM and allowed to breathe spontaneously (PVM + SB, row on top) or subjected to 4 h of MV (PVM + MV, row on bottom) 7 days after PVM infection. MV led to an increase in inflammatory cells in the alveolar spaces, thickening of the alveolar septa, and peribronchial wall thickening in C57BL/6 mice. These changes in response to MV were less prominent in the lpr mice. Magnification ×400.

Table 2. Lung response of C57BL/6 and lpr mice 8 days after instillation of PVM

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C57BL/6</th>
<th>lpr</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellular response</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>BAL PMN (total cells)</td>
<td>1.07 ± 0.26 × 10⁴</td>
<td>0.82 ± 0.20 × 10⁴</td>
<td>NS</td>
</tr>
<tr>
<td>BAL AM (total cells)</td>
<td>1.79 ± 0.47 × 10⁴</td>
<td>2.02 ± 0.30 × 10⁴</td>
<td>NS</td>
</tr>
<tr>
<td>BAL Lym (total cells)</td>
<td>0.39 ± 0.09 × 10⁴</td>
<td>0.30 ± 0.07 × 10⁴</td>
<td>NS</td>
</tr>
<tr>
<td>Lung MPO activity</td>
<td>459.07 ± 138.82</td>
<td>355.72 ± 42.38</td>
<td>NS</td>
</tr>
<tr>
<td>BAL cytokine response, pg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KC</td>
<td>96.24 ± 8.45</td>
<td>80.0 ± 17.76</td>
<td>NS</td>
</tr>
<tr>
<td>MIP-2</td>
<td>51.63 ± 6.47</td>
<td>45.61 ± 8.05</td>
<td>NS</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>44.02 ± 6.94</td>
<td>40.11 ± 13.83</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>823.02 ± 221.57</td>
<td>672.35 ± 238.53</td>
<td>NS</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.61 ± 0.00</td>
<td>0.74 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td>IL-4</td>
<td>36.2 ± 1.76</td>
<td>38.13 ± 2.17</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6</td>
<td>963.03 ± 279.68</td>
<td>623.61 ± 235.06</td>
<td>NS</td>
</tr>
<tr>
<td>IL-10</td>
<td>22.41 ± 4.16</td>
<td>8.36 ± 3.44</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>TNF-α</td>
<td>14.00 ± 0.00</td>
<td>14.25 ± 0.25</td>
<td>NS</td>
</tr>
<tr>
<td>VEGF</td>
<td>672.05 ± 69.81</td>
<td>396.31 ± 52.25</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td><strong>Permeability response</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>BAL total protein, μg/ml</td>
<td>976.90 ± 78.98</td>
<td>603.86 ± 68.54</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>BAL α-macroglobulin, ng/ml</td>
<td>6,379.15 ± 511.58</td>
<td>4,289.63 ± 657.09</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>BAL IgM, ng/ml</td>
<td>379.88 ± 66.52</td>
<td>473.55 ± 84.94</td>
<td>NS</td>
</tr>
<tr>
<td>BAL RAGE, ng/ml</td>
<td>31.30 ± 5.91</td>
<td>24.84 ± 2.01</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Lung apoptotic response</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Lung caspase-3 activity*</td>
<td>9,265.67 ± 2,536.47</td>
<td>9,065.02 ± 3,321.78</td>
<td>NS</td>
</tr>
<tr>
<td>Lung cleaved PARP*</td>
<td>0.02 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>NS</td>
</tr>
<tr>
<td>Viral load</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Lung</td>
<td>8.14 ± 1.55 × 10⁸</td>
<td>6.01 ± 2.12 × 10⁸</td>
<td>NS</td>
</tr>
<tr>
<td>Heart, liver, spleen, and kidney</td>
<td>Undetectable</td>
<td>Undetectable</td>
<td></td>
</tr>
</tbody>
</table>

BAL, bronchoalveolar lavage; PMN, polymorphonuclear neutrophils; AM, alveolar macrophages; Lym, lymphocytes; MIP, macrophage inflammatory protein; IFN, interferon; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; RAGE, receptor for advanced glycation end-products; PARP, poly(ADP-ribose) polymerase; NS, not significant. *Results are expressed as mean arbitrary fluorescence/absorbance units/mg lung tissue.
min) in the lpr mice. A comparison in physiological parameters between lpr and C57BL/6 mice was possible because the mice were monitored continuously; the EtCO₂ and peak airway pressures were similar, but there was a trend toward decreased heart rate in the lpr mice (Fig. 6, B-D). The decrease in heart rate raised the possibility of myocardial involvement; however, microscopic examination of the heart did not disclose myocardial infarction or right ventricular enlargement. Immunohistochemical measurements of leukocyte infiltration in the myocardium in mice that were not ventilated failed to reveal significant differences in myocardial leukocytes (Fig. 6E).

Because a direct comparison in lung injury parameters was precluded by the earlier time of death of the lpr mice, we studied additional mice 1 h after the onset of MV. No significant differences were found between C57BL/6 and lpr mice in the total numbers of BAL neutrophils (0.75 ± 0.25 vs. 1.38 ± 0.32 × 10⁵ cells), the concentrations of KC (137.29 ± 24.07 vs. 176.71 ± 42.34 pg/ml), MIP-2 (75.22 ± 14.14 vs. 84.03 ± 17.54 pg/ml), MIP-1α (78.80 ± 15.58 vs. 47.52 ± 9.26 pg/ml), IFN-γ (147.03 ± 31.38 vs. 151.97 ± 49.51 pg/ml), and IL-10 (16.08 ± 4.83 vs. 4.24 ± 2.49 pg/ml), total protein content (672.41 ± 107.32 vs. 739.33 ± 79.08 μg/ml), the concentrations of α-macroglobulin (4,579.40 ± 543.90 vs. 4,791.26 ± 566.72 ng/ml), and the concentrations of RAGE (35.29 ± 6.31 vs. 42.91 ± 3.64 ng/ml). Thus, the lpr mice had increased mortality compared with C57BL/6 mice when they were exposed to PVM and ventilated 8 days later, which was not preceded by a difference in lung inflammatory responses and injury parameters.

**DISCUSSION**

The main goal of this study was to determine whether MV enhances the host response to PVM infection by a mechanism involving the Fas/FasL system. C57BL/6 mice or Fas-deficient (“lpr”) mice received intratracheal instillations of PVM and 7 or 8 days later were subjected to MV. Seven days after PVM instillation, the ventilated lpr mice showed significantly less inflammation than the C57BL/6 mice. However, when the mice were ventilated 8 days after PVM instillation, there were more deaths among the lpr mice compared with the C57BL/6 mice.

Recent studies suggest an association between activation of the Fas/FasL system and RSV infection. The expression of Fas is markedly upregulated in respiratory epithelial cells infected with RSV in vitro (30) and also in the lung epithelium of
children who die with severe RSV disease (45). This increased expression of Fas may be associated with the severity of disease because mice lacking FasL (gld mice) show decreased weight loss after primary infection with RSV (36). Thus, the Fas/FasL system appears to be associated with ALI and with RSV infection, and this is true in humans and in experimental models.

The Fas/FasL system was initially described as a proapoptotic system, but a number of studies have demonstrated that Fas/FasL also plays an important role in the innate immune response. Previous studies have also linked activation of the Fas/FasL system with MV. Plasma sFasL was increased in patients with ARDS who underwent MV with a conventional strategy compared with a lung protective strategy (16), and Imai et al. (23) found that MV activates Fas/FasL in rabbits subjected to acid aspiration.

The first finding in our study was that MV for the relatively short period of 4 h was sufficient to enhance the inflammatory response to PVM infection in the lungs, as evidenced by increases in the total number of BAL neutrophils and the concentrations of the proinflammatory cytokines KC, MIP-2, and MIP-1α. Additionally, we and others have previously reported that a similar enhancement occurs when stretch is added to the administration of lipopolysaccharide (LPS), intratracheal bacteria, intraperitoneal bacteria, and PVM (2, 5, 6, 12, 31, 41). In the case of LPS, the enhancing effect of ventilation was associated with the activation of specific patterns of genes that were not activated with ventilation alone or with LPS alone. In the present study, the enhancement of inflammatory markers induced by MV was abrogated in the Fas-deficient mice, indicating that the Fas/FasL system has an important role in amplifying inflammatory responses in lungs subjected to MV. Thus, MV enhanced the inflammatory response to PVM in a Fas-dependent manner.

It is well known that Fas ligation can activate a number of proinflammatory pathways that lead to cytokine release (25, 44). The proinflammatory function of Fas is sufficient to cause significant neutrophilic alveolitis in vivo, and the primary target of Fas in the lungs appears to be nonmyeloid cells, probably alveolar epithelial cells, because mice lacking Fas in myeloid cells, and mice depleted of macrophages, are still able to develop a robust inflammatory response following Fas activation with Jo2 mAb (4, 26). In the specific case of alveolar epithelial cells, Fas ligation activates a pathway that involves the mitogen-activated protein kinases (MAPKs) ERK1/2 and JNK and activation of the transcription factor activator protein-1, with eventual release of KC (20). ERK1/2 and JNK are also involved in mechanotransduction pathways that are activated in lung epithelial cells in response to cyclic stretch (8, 28, 40). These pathways eventually lead to release of reactive oxygen species, activation of proinflammatory transcriptional programs, and release of proinflammatory mediators (7, 15, 22). Thus, it is possible that the Fas/FasL system amplifies proinflammatory mechanotransduction pathways by enhancing MAPK signaling.

Contrary to our expectations, the lack of functional Fas resulted in increased deaths in mice infected with PVM and ventilated 8 days later. However, the cause for the increased deaths remains unclear. Before the onset of ventilation, the C57BL/6 and lpr mice were similar in terms of clinical evidence of disease, markers of lung injury, and viral load in the lungs. Furthermore, there was no evidence of viral involvement of the heart or other distal organs. Thus, the increased number of deaths appeared to result directly from an added effect of MV. However, during ventilation, there was no difference in peak airway pressure in the C57BL/6 and lpr mice, suggesting that lung compliance values were similar. It is possible that the earlier death resulted from worsened lung injury in the lpr mice, but a direct comparison of markers of injury following the onset of ventilation was precluded by the fact that the lpr mice died earlier than the C57BL/6 mice. Another possibility was that the lpr mice died because of cardiovascular failure, although microscopic examinations of the heart did not disclose myocardial infarction or right ventricular enlargement. The findings of this study suggest that the Fas/FasL system enhances lung injury by MV; however, this enhanced lung injury is not necessarily associated with worsened outcome.

This study highlights the enhancement of lung injury by MV. Some of the changes induced by cyclic stretch in the lungs are of a physical nature. Overdistention of diseased alveoli by positive pressure promotes inflammation and further disruption of the alveolar-capillary barrier. This occurs by cellular stress failure, plasma membrane microrupture, and lung epithelial cell death (38, 43). In addition, stretch can elicit biochemical signaling events through activation of mechanical sensors present in lung cells, including stretch-activated ion channels, the cytoskeleton, and integrins (reviewed in Ref. 39). The MAPK enzyme family transduces many of these signals and mediates the cellular response to stretch. Farnand et al. (20) recently found that the relevant pathway linking Fas with inflammation involves myeloid differentiation factor 88 (MyD88) and MAPK activation (20) and subsequent KC release. These findings are relevant in the context of data by Chun et al. (9) who found that MV with moderate tidal volumes generates an endogenous ligand recognized by MyD88-dependent receptors other than Toll-like receptor 4 and that this mechanism can contribute to the development of ventilator-associated lung inflammation and injury. Together these data suggest that stretch leads to Fas activation and subsequent release of proinflammatory cytokines via a MyD88-dependent mechanism. Interestingly, in this study, MV did not enhance the permeability and apoptotic responses to PVM. This stands in contrast to our recent study using the PVM model in which the addition of MV resulted in an increase in BAL α-macroglobulin, BAL IgM, and lung capase-3 (5). One possible explanation is that different mouse strains were used in these two studies: the present study tested C57BL/6 mice, whereas the previous study tested BALB/c mice. BALB/c mice tend to be more susceptible to PVM infection than C57BL/6 mice.

This study has some limitations. Although PVM and RSV are both pneumoviruses, there is no animal model that displays all features of human RSV disease (34). The PVM infection resulted in patchy areas of inflammation, which may have caused heterogeneous distribution of the tidal volume. This is, however, comparable to the human situation with patchy rather than diffuse signs of infection and tissue damage during RSV infection. Another limitation of this study is that the duration of MV was only 4 h. This is in contrast to the clinical scenario in which patients are ventilated for long periods of time. Thus, this study applies only to the initial events that occur following the initiation of MV. In addition, it is
important to consider that this study was not a mortality study, since mortality is not a permissible outcome of animal experimentation at our institution.

In summary, activation of the inflammatory response of the lungs to PVM and MV was mediated by the Fas/FasL system. While inactivation of Fas attenuated the inflammatory response, this was not followed by an improvement in outcome in Fas-deficient mice with more advanced PVM disease.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


