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Fas-Mediated Acute Lung Injury Requires Fas Expression on Nonmyeloid Cells of the Lung

Gustavo Matute-Bello, Janet S. Lee, W. Conrad Liles, Charles W. Frevert, Steven Mongovin, Venus Wong, Kimberly Ballman, Steven Sutlief, and Thomas R. Martin

Fas (CD95) is a membrane surface receptor, which, in the lungs, is expressed in macrophages, neutrophils, and epithelial cells. In mice, Fas activation leads to a form of lung injury characterized by increased alveolar permeability. We investigated whether Fas-mediated lung injury occurs primarily as a result of Fas activation in myeloid cells (such as macrophages) or in nonmyeloid cells (such as epithelial cells). Chimeric mice lacking Fas in either myeloid or nonmyeloid cells were generated by transplanting marrow cells from lpr mice (which lack Fas) into lethally irradiated C57BL/6 mice (MyFas− group) or vice versa (MyFas+ group). Additional mice transplanted with marrow cells from their same strain served as controls (Fas+ ctr and Fas− ctr groups). Sixty days after transplantation, the mice received intratracheal instillations of the Fas-activating mAb Jo2 (n = 10/group), or an isotype control Ab (n = 10/group), and were euthanized 24-h later. Only animals expressing Fas in nonmyeloid cells (Fas+ ctr and MyFas−) showed significant increases in lung neutrophil content and in alveolar permeability. These same mice showed tissue evidence of lung injury and caspase-3 activation in cells of the alveolar walls. Despite differences in the neutrophilic response and lung injury, there was no statistical difference in the lung cytokine concentrations (KC and MIP-2) among groups. We conclude that Fas-mediated lung injury requires expression of Fas on nonmyeloid cells of the lungs. These findings suggest that the alveolar epithelium is the primary target of Fas-mediated acute lung injury, and demonstrate that apoptotic processes may be associated with neutrophilic inflammation. The Journal of Immunology, 2005, 175: 4069–4075.

Acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS), are important causes of morbidity and mortality in the United States, with an incidence as high as 64 cases per 105 person-years and a mortality rate of 39% (1). ALI in humans is characterized histopathologically by neutrophilic alveolitis and by disruption of the alveolar epithelium, leading to protein-rich alveolar edema (2–4). The damage to alveolar epithelial cells is one of the earliest events that occurs in the development of ALI/ARDS in humans, and the severity of the epithelial damage is associated with morbidity and mortality (4–6). The actual mechanisms leading to alveolar epithelial damage remain unclear, but are likely to involve a combination of necrosis and apoptosis (7, 8).

Emerging evidence suggests that activation of the Fas (CD95)/Fas ligand (FasL; CD178) system early in ALI may play a pivotal role in apoptosis of alveolar epithelial cells and disruption of the alveolar-epithelial barrier (8). For example, the concentration of soluble Fas ligand (sFasL) is increased in the bronchoalveolar lavage fluid (BALF) of patients with ARDS at concentrations that induce apoptosis of distal lung epithelial cells. In animal models, activation of Fas leads to a form of lung injury associated with increased alveolar permeability and apoptosis of cells of the alveolar walls (8–12). These data demonstrate that one target of sFasL in the lungs is the alveolar epithelium, which responds to Fas ligation by activation of apoptotic pathways, and suggest that Fas-mediated apoptosis of alveolar epithelial cells could play an important role in the pathogenesis of ALI/ARDS.

The response of lung cells to Fas ligation, however, depends on the cell type and does not necessarily lead to apoptosis. For example, bronchial epithelial cells express Fas, but do not become apoptotic following Fas ligation (13). Myeloid-derived pulmonary cells, such as human alveolar macrophages, are also resistant to Fas-mediated apoptosis and respond to Fas ligation by developing a proinflammatory phenotype with release of IL-8 and other cytokines (14). The activation of proinflammatory pathways following Fas ligation is associated with translocation of NF-κB and is independent of the pro-apoptotic pathway (15). The relevance of the proinflammatory function of Fas was highlighted in experiments involving transplantation of pancreatic islet cell lines overexpressing Fas into normal mice, which resulted in massive neutrophilic infiltration and eventual destruction of the transplanted islets (16, 17). Thus, the Fas/FasL system has a dual function, pro-apoptotic and proinflammatory, and although each of these functions is independent of the other, activation of either function can potentially result in tissue injury.

The main goal of this study was to determine whether activation of the Fas/FasL system causes lung injury primarily via activation of the pro-apoptotic pathway in alveolar epithelial cells, or instead by inducing activation of proinflammatory pathways in alveolar...
macrophages. To achieve this aim, we generated chimeric mice lacking Fas in either their myeloid or nonmyeloid cells and then exposed the mice to the Fas-activating mAb Jo2 using a protocol previously shown to induce injury in mouse lungs (10).

Materials and Methods

Animal protocol

The animal protocols were approved by the Animal Care Committee of the Veterans Administration Puget Sound Health Care System. Briefly, female mice weighing 25–30 g were irradiated with 900 cGy as described elsewhere (18). Following total body irradiation, the mice were returned to their cages with free access to food and water. Twenty-four hours later, the mice were injected intraperitoneally (i.p.) with 5 × 10^6 bone marrow cells, isolated from male mouse donors as described previously (18). The cells were administered in a volume of 200 μl by tail vein injection. Following marrow transplantation, the mice were returned to their cages with free access to food and water. During the first 2 wk after bone marrow transplantation, drinking water was supplemented with 22.7 mg/ml enrofloxacin (Bayer) and the food supply was supplemented with NutriCal (Evsco Pharmaceuticals).

On day 60 after bone marrow transplantation, mice were anesthetized with i.p. ketamine and xylazine and then received intratracheal instillations of either mAb Jo2 or isotype control mAb, at 2.5 μg in three separate aliquots. We have previously found that after marrow transplantation, 60 days is the optimal length of time required for full repopulation of the lungs with donor macrophages (18). Twenty-three hours after Ab instillation, each mouse received an i.p. injection of 200 μl of 5% human serum albumin (HSA) in PBS (Baxter). One hour later, the mice were euthanized with 120 mg/kg pentobarbital i.p. After euthanasia, the mice were exsanguinated by intracardiac puncture using syringes with trace amounts of heparin. The thorax was opened, the trachea was exposed and secured with suture, and the trachea was cannulated with a 20-gauge catheter. The left hilum was clamped and the left lung was removed and placed in 1 ml of sterile water for homogenization. The right lung was lavaged with four separate 0.5-ml aliquots of 0.9% NaCl containing 0.6 mM EDTA. Finally, the right lung was fixed with 4% paraformaldehyde at 15 cm of water for histological processing.

Throughout the duration of the experiment, the mice were monitored daily and euthanized if they developed weight loss >15% of the original body weight, had evidence of respiratory distress, or at least three of the following: 1) dehydration (evaluated by skin tenting), 2) lethargy and decreased movement, 3) pale eyes, 4) loose stools, 5) nasal and/or ocular discharge, or 6) neuromuscular signs (uncoordination or seizures).

Experimental protocol

Two strains of mice were used: C57BL/6, which express Fas, and lpr mice, which are natural mutants deficient in Fas on a C57BL/6 background. From these two strains, four groups of chimeric mice were generated. Group 1 was the Fas+ control (ctr) group composed of C57BL/6 mice receiving bone marrow from C57BL/6 donors; the resulting chimeras express Fas on all their cells (Fas+ ctr). Group 2 was the Fas–ctr group, composed of lpr mice receiving bone marrow from lpr donors, with the resulting chimeras lacking Fas expression in all cells (Fas–ctr). Group 3 was the myeloid (My) Fas group, composed of C57BL/6 mice receiving bone marrow from lpr donors, with the resulting chimeras expressing Fas only on their myeloid cells (MyFas–). Mice from each group received either the Fas-activating mAb Jo2 or an isotype control as described above.

Sample processing

The BALF aliquots were pooled. An aliquot of the BALF was immediately discharged, or 6) neuromuscular signs (uncoordination or seizures).

Measurements

Myeloperoxidase was measured in lung homogenates using the AmpliChip Red fluorometric assay according to instructions from the manufacturer (Molecular Probes).

Cytokine assays. MIP-2 and KC were measured in lung homogenates using mouse-specific immunoassays (R&D Systems). MIP-2 and KC were measured in BALF using a cytometric bead-based immunoassay system according to the manufacturer’s protocols (Luminex). The Luminex system was used because it offers a better sensitivity than the conventional ELISAs, allowing cytokines to be detected in BALF. Pilot studies using selected samples showed that the conventional ELISA and the Luminex correlated with each other for the measurement of MIP-2 and KC.

Lung alveolar permeability was measured by determining the concentration of human serum albumin (HSA) by immunoassay in BALF using rabbit anti-human albumin IgG (DakoCytomation) as capture Ab, HRP-labeled rabbit anti-human albumin IgG P0356 (DakoCytomation) as detecting Ab, and HSA (Albumin-25; Cen-Centec LLC) as the standard (19).

Capase-3 immunohistochemistry. Immunohistochemistry was performed using the Vector Elite ABC-HP kit (Vector Laboratories). Briefly, the slides were deparaffinized by heating at 57°C for 60 min and rehydrated by washing twice in Clear Rite (Richard Allan Scientific), twice in 100% ethanol for 3 min, twice in 95% ethanol for 3 min, and once in dH2O for 5 min. The slides were rinsed twice with PBS for 5 min and the samples were digested with citrate buffer (Vector Laboratories) in a microwave for 15 min at medium setting. Following digestion, the slides were cooled to room temperature for 10 min, rinsed twice with PBS for 5 min, and blocked with 5% normal goat serum in nonfat milk for 60 min at room temperature. The samples were labeled with rabbit anti-active caspase-3 (BD Pharmingen) overnight in a moist chamber at 4°C. Next, the slides were rinsed twice with PBS and labeled with goat anti-rabbit biotinylated Ab for 2 h at room temperature. The slides were rinsed twice with PBS, incubated with 0.3% H2O2 in MeOH for 60 min to block endogenous peroxidases, and rinsed again with PBS. The samples were then rinsed twice with PBS for 5 min, treated with 1% normal goat serum for 5 min, and incubated in a moist chamber for 90 min at room temperature, rinsed twice with PBS, and developed in a moist chamber with diaminobenzidine substrate (Sigma-Aldrich) for 12 min in the dark at room temperature. The
slides were rinsed with running deionized H2O for 5 min and counterstained with 1% methyl green for 6 min. The slides were dehydrated with ethanol, incubated in xylene for 5 min, and mounted with Permount.

The tissue sections (H&E stains) and the immunohistochemistry slides were read in a blinded fashion by an observer unaware of the treatments or animal groups.

**Statistical analysis**

Analysis of data from multiple groups was performed by one-way ANOVA. The Tukey post hoc procedure was performed to determine significance between individual groups. Data are shown as means ± SEM.

**Results**

Four animals died before reaching day 60 after bone marrow transplantation. All of these animals died in the first 15 days after marrow transplantation. The remaining animals survived to day 60 and received instillations of either Jo2 mAb or isotype control. In the Fas+ control group, six mice received Jo2 mAb and five mice received isotype control mAb. In the Fas+ control group, six mice received Jo2 mAb and six mice received the control mAb. In the MyFas− group, 10 animals received Jo2 mAb and 4 animals received control mAb. In the MyFas+ group, 10 mice received Jo2 mAb and 5 animals received control mAb. No animals died following the instillation of the Abs.

**Evidence of chimerism**

Chimerism was verified in two different ways. First, because all recipients were female and all of the donors were male mice, we used real-time PCR to quantify the Y chromosome gene sry in DNA isolated from whole blood extracted at the time of necropsy. Expression of sry isolated from BALF (Fig. 1). Fas expression was detected only in cells isolated from the Fas+ ctr mice and from the MyFas+ mice.

**Cellular response**

Neutrophil migration into the airspaces 24-h after the administration of Jo2 mAb or an isotype control mAb was evaluated by determining total polymorphonuclear leukocyte (PMN) counts in the BALF. As compared with animals treated with the control mAb, the total BALF PMN counts were significantly greater in the Fas+ ctr group (11.1 ± 5.8 × 103 vs 0.9 ± 0.4 × 103, p < 0.05); in the MyFas− group (27.6 ± 8.8 × 103 vs 2.8 ± 1.2 × 103, p < 0.05), and in the MyFas+ group (5.8 ± 3.0 × 103 vs 2.0 ± 0.8 × 103, p < 0.05) (Fig. 2A).

The total neutrophil content in the lungs was assessed by determining MPO activity in whole lung homogenates. Only animals expressing Fas in their nonmyeloid cells (Fas+ ctr and MyFas−) showed significantly greater MPO activity in response to Jo2 mAb, as compared with animals receiving isotype control mAb (Fas+ ctr, 0.85 ± 0.09 vs 0.27 ± 0.03, p < 0.05; MyFas− animals, 0.52 ± 0.03 vs 0.24 ± 0.03, p < 0.05) (Fig. 2B). In contrast, animals lacking Fas in their nonmyeloid cells (Fas+ ctr and MyFas+ cells) showed no significant difference in MPO activity between the Jo2-treated groups and the isotype control mAb-treated groups (Fas+ ctr, 0.29 ± 0.07 vs 0.16 ± 0.01; MyFas+, 0.14 ± 0.01 vs 0.17 ± 0.01). Other cells, such as lymphocytes, were present in very small numbers in the BALFs and their numbers were similar in all groups.

**Cytokine response**

The cytokine response was assessed by determining the concentration of MIP-2 and KC in BALF, which measures the soluble cytokine component, and in lung homogenates, which measures total cytokine content of the lung. As shown in Table I, none of the chimeric groups (MyFas+ and MyFas−) showed a significant difference in the BALF or lung homogenate concentrations of KC or MIP-2 between animals treated with Jo2 mAb and animals treated with the isotype control Ab.

**Permeability response**

Changes in the permeability of the alveolar-capillary barrier were determined by measuring the concentration of HSA in the BALF 1 h after an i.p. injection of HSA. Paralleling the neutrophilic response, only mice expressing Fas in their nonmyeloid cells (Fas+ ctr and MyFas−) showed a significant increase in the BALF concentration of HSA following Jo2 mAb administration, as compared with control mAb (Fas+ ctr, 10.3 ± 2.3 vs 1.6 ± 0.6, p < 0.05; MyFas−, 3.5 ± 1.6 vs 1.2 ± 0.2, p < 0.05). In animals lacking Fas in their nonmyeloid cells (Fas+ ctr and MyFas+), Jo2 administration did not result in significant increases in BALF HSA (Fas+ ctr, 1.0 ± 0.2 vs 0.7 ± 0.2; MyFas+, 0.6 ± 0.2 vs 0.7 ± 0.2) (Fig. 3A). The HSA concentration in BALF of the Fas− ctr mice was significantly higher than that of all of the other groups of mice, including the MyFas+ mice (p < 0.05). The total protein concentration in BALF was also determined as an additional measurement of permeability changes. As compared with animals receiving control Ab, the administration of Jo2 resulted in increased total BALF protein in the Fas+ ctr group (219.4 ± 23.9 vs 103.5 ± 26.7, p < 0.05) and in the MyFas− mice (291.0 ± 14.1 vs 189.2 ± 15.8, p < 0.05) (Fig. 3B).
Fas-mediated lung injury requires Fas in nonmyeloid cells

The main goal of this study was to determine whether Fas-mediated lung injury results from Fas activation of myeloid cells (such as neutrophils and macrophages), leading to cytokine release and neutrophilic inflammation or, alternatively, whether Fas-mediated lung injury results from Fas activation of nonmyeloid cells (such as epithelial cells), leading to apoptosis of alveolar wall cells, disruption of the alveolar epithelium, and secondary neutrophilic inflammation. The main finding was that Fas-mediated injury requires Fas expression in nonmyeloid cells and is associated with apoptosis of cells of the alveolar walls, increased alveolar permeability, and neutrophilic inflammation, without a clear cytokine response.

A growing body of evidence suggests that the Fas/FasL system plays a role in human lung disease. For example, FasL is present in the BALF from patients with ARDS, idiopathic pulmonary fibrosis (IPF), hypersensitivity pneumonitis, and bronchiolitis obliterans organizing pneumonia (8, 20–22). FasL is also present in the serum of patients with ARDS, IPF, pulmonary fibrosis associated with collagen vascular diseases, and pneumococcal pneumonia (21, 23–25). These studies show an association between FasL concentrations in either BALF or serum, and increased progression to multiple organ failure and mortality in ARDS, with disease activity in IPF, and with acute illness in pneumococcal pneumonia, suggesting that the Fas/FasL system may play a key role in the pathogenesis of lung disease (8, 20, 24, 25).

Animal studies have provided evidence supporting a key role for the Fas/FasL system in lung disease. We have shown that activation of the Fas/FasL system by either a Fas-activating mAb or human sFasL causes ALI in mice and rabbits (9, 10). Others have shown that Fas-mediated apoptosis contributes to the alveolar wall permeability changes and inflammation in a murine model of LPS-induced lung injury, and Fas-deficient mice show significantly reduced mortality from hypoxic-induced lung injury during *Legionella* pneumonia (12, 26). In addition, activation of the Fas/FasL system has also been associated with development of distant organ failures in models of injurious mechanical ventilation (23). Thus, a growing body of evidence from studies of humans and animal models links activation of the Fas/FasL system with lung injury. However, how the activation of Fas/FasL contributes to the development of injury in the lungs is currently unknown.

In the lungs, Fas is expressed in myeloid cells, including neutrophils, monocytes and alveolar macrophages, and also in nonmyeloid cells, including airway and alveolar epithelial cells and fibroblasts (9, 13, 20, 27, 28). Expression in pulmonary endothelial cells has been reported to be minimal or absent (28, 29). Activation of Fas appears to have markedly different results in each of the Fas-bearing cells of the lungs. Nonciliated lung epithelial cells develop apoptosis and, under certain circumstances, release cytokines following Fas activation (8, 30), whereas other nonmyeloid cells, such as fibroblasts and large airway epithelial cells, fail to develop apoptosis (13, 31). In contrast, alveolar macrophages and monocytes respond to Fas ligation with release of proinflammatory cytokines, including potent neutrophil chemoattractants such as IL-8 (14). Thus, depending on the target cell, Fas ligation in the lungs can lead to apoptosis of structural cells or, alternatively, to release of proinflammatory cytokines.

The ability of the Fas/FasL system to activate proinflammatory and pro-apoptotic pathways raises an important question: what is the main event involved in Fas-mediated lung injury, direct damage of the alveolar epithelium, or development of a neutrophilic response with secondary epithelial damage? At the heart of this question is the issue of how important is the neutrophilic response in the very early phases of lung injury. Previous studies have suggested that the development of a neutrophilic alveolitis is not necessarily associated with damage to the alveolar epithelial-capillary barrier. Early studies from our laboratory showed that instillation of the neutrophil chemoattractant leukotriene B4 into the lungs of healthy human volunteers is followed by a strong neutrophilic response but no permeability changes, and of course those volunteers did not develop ARDS (32). The dissociation between neutrophilic responses and lung injury in humans suggests that damage to the alveolar epithelial-capillary barrier is an essential step in the development of ALI/ARDS and constitute the basis of our interest in
identifying the mechanisms leading to that damage. Because bioactive FasL is present in the lungs of patients with ALI/ARDS, activation of the Fas/FasL system could be one mechanism of disruption of the alveolar epithelial-capillary barrier by inducing apoptosis or dysfunction of alveolar epithelial cells (8). However, activation of Fas has also been found to also induce cytokine release by macrophages and a mild neutrophilic response (9, 10, 14). Therefore, the specific goal of this study was to identify whether Fas-mediated injury was due primarily to its effects on the alveolar epithelium or to its effects on macrophages.

In this study, we created chimeric mice lacking Fas in either their myeloid or their nonmyeloid cells and studied the neutrophilic response, permeability changes, and the apoptotic response. We showed that Fas activation in nonmyeloid cells resulted in a moderate increase in total lung neutrophils and airspace neutrophils, associated with an increase in lung permeability. The minor neutrophilic response and the absence of measurable concentrations of the cytokines MIP-2 and KC, combined with the evidence of increased permeability, suggest that the major mechanism leading to Fas-mediated lung injury is via injury to the epithelium, rather than by inducing an inflammatory response. Furthermore, the neutrophilic response in the mice expressing Fas in myeloid cells only was very small and was not associated with permeability changes. This suggests that the overall contribution of macrophages to lung injury in response to Jo2 is small and that Fas-mediated lung injury is primarily driven by Fas activation in nonmyeloid cells.

The neutrophilic response seen in the mice lacking Fas in myeloid cells raises the question: how does Fas activation in nonmyeloid cells result in a neutrophilic response? One potential alternative is cytokine release from epithelial cells. Hagimoto et al. (30) have reported that Fas induces cytokine release in primary human small airway cells in vitro, including potent neutrophil chemotactants such as IL-8. However, we were unable to detect significant differences in the chemokine concentrations in BALF and whole lung homogenates among the different groups. A second possibility is that Fas activation results in epithelial disruption and/or endothelial dysfunction associated with apoptosis. The subsequent exposure of the basement membrane resulting from the denuded alveolar wall may lead to neutrophil activation, adhesion to endothelial surfaces, and eventual migration into the airspaces. This possibility is supported by the finding that alveolar permeability was increased in the mice expressing Fas in their nonmyeloid cells, and that these same animals showed significant increases in apoptotic cells in the alveolar walls and thickening of the alveolar walls. The increase in apoptosis, although significant, was limited to a small number of cells. These findings raise the possibility that epithelial cells may lose their barrier function before becoming apoptotic via a mechanism yet to be determined.

This study does not exclude a role for the proinflammatory function of the Fas/FasL system in the macrophage-driven, cytokine-mediated neutrophilic responses that occur in response to either live bacteria or in other experimental models of ALI. This study investigates the role of Fas activation in the absence of any other stimuli, and it remains possible that Fas activation on macrophages potentiates cytokine release induced by bacterial products or other

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<tr>
<th>Cytokine</th>
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**FIGURE 3.** Total HSA concentrations in BALF (A) and total protein concentration (B) in BALF 24 h after the intratracheal instillation of either Jo2 mAb or an isotype control mAb. HSA was injected i.p. 1 h before euthanasia. Fas⁺ ctr = animals expressing Fas in all cells (C57BL/6→C57BL/6); Fas⁻ ctr = animals deficient in Fas in all cells (lpr→lpr); MyFas⁺ = animals expressing Fas in myeloid cells only (C57BL/6→lpr); and MyFas⁻ = animals lacking Fas in myeloid cells (lpr→C57BL/6). Data shown as means ± SEM. *, p < 0.05 as compared with isotype control mAb.
cells is required for the development of increased protein permeability, alveolar neutrophilia, apoptosis, and lung tissue changes. We conclude that expression of Fas in nonmyeloid cells is necessary for the development of lung injury following Fas ligation and that the alveolar epithelium plays an essential role in mediating Fas-dependent ALI.

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Disclosures
The authors have no financial conflict of interest.

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