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Differential Role of the Fas/Fas Ligand Apoptotic Pathway in Inflammation and Lung Fibrosis Associated with Reovirus 1/L-Induced Bronchiolitis Obliterans Organizing Pneumonia and Acute Respiratory Distress Syndrome

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Bronchiolitis obliterans organizing pneumonia (BOOP) and acute respiratory distress syndrome (ARDS) are two clinically and histologically distinct syndromes sharing the presence of an inflammatory and fibrotic component. Apoptosis via the Fas/Fas ligand (FasL) pathway plays an important role in the development of acute lung injury and fibrosis characteristic of these and other pulmonary inflammatory and fibrotic syndromes. We evaluated the role of apoptosis via the Fas/FasL pathway in the development of pulmonary inflammation and fibrosis in reovirus 1/L-induced BOOP and ARDS. CBA/J mice were intranasally inoculated with saline, 1 × 10^6 (BOOP), or 1 × 10^7 (ARDS) PFU reovirus 1/L, and evaluated at various days postinoculation for in situ apoptosis by TUNEL analysis and Fas/FasL expression. Our results demonstrate the presence of apoptotic cells and up-regulation of Fas/FasL expression in alveolar epithelium and in infiltrating cells during the inflammatory and fibrotic stages of both reovirus 1/L-induced ARDS and BOOP. Treatment of mice with the caspase 8 inhibitor, zIETD-fmk, inhibited apoptosis, inflammation, and fibrotic lesion development in reovirus 1/L-induced BOOP and ARDS. However, CBA/KJms-Faslpr-cg/J mice, which carry a point mutation in the Fas cytoplasmic region that abolishes the ability of Fas to transduce an apoptotic signal, do not develop pulmonary inflammation and fibrotic lesions associated with reovirus 1/L-induced BOOP, but still develop inflammation and fibrotic lesions associated with reovirus 1/L-induced ARDS. These results suggest a differential role for the Fas/FasL apoptotic pathway in the development of inflammation and fibrotic lesions associated with BOOP and ARDS. The Journal of Immunology, 2009, 183: 8244–8257.

C hronic inflammation and tissue fibrosis are leading causes of morbidity and mortality in granulomatous and interstitial lung disorders as well as in the chronic stage of acute respiratory distress syndrome (ARDS)^4 (1–6). A number of distinct clinical entities are characterized by a fibrotic component, which may be distinguished by both the location of the fibrotic lesion within the lung and the presence of an interstitial pneumonia (1–6). Acute, inflammatory responses that occur in the distal air spaces of the lung (bronchioles, alveolar ducts, and alveoli) may develop into one of a limited number of nonspecific pulmonary disorders with a fibrotic component including bronchiolitis obliterans organizing pneumonia (BOOP) (3, 5, 7–12). BOOP lesions have a patchy distribution in the lung, are frequently associated with a peribronchiolar organizing pneumonia, and are associated with the presence of lipid-laden foam cells in the alveolar spaces (5, 7–12). While the structural integrity of the alveolar ducts and walls within regions of BOOP lesion development are normally preserved, the alveolar septa may be thickened with an infiltrate of inflammatory mononuclear cells (5, 7–12). ARDS is a clinical syndrome that is characterized by diffuse alveolar damage usually secondary to an intense host inflammatory response of the lung to an infectious, noninfectious, or extrapulmonary insult (6, 13–16). ARDS is a biphasic disease that progresses from an acute exudative phase, characterized by epithelial and endothelial cellular injury, neutrophilic aggregation, the formation of hyaline membranes, alveolar edema, and hemorrhage, to an organizing (chronic) phase, characterized by regeneration and healing via resolution or repair with persistent intraalveolar and interstitial fibrosis (6, 13–18). Thus, the histopathological changes observed in ARDS can be divided into the overlapping phases of exudation, regeneration, and healing, which may be distinguished by either resolution or repair leading to fibrosis (6, 13–18).

We have previously described small animal models of BOOP and ARDS elicited by respiratory infection with reovirus serotype 1, strain Lang (reovirus 1/L) (19–23). CBA/J mice inoculated by the intranasal (i.n.) route with 1 × 10^6 PFU reovirus...
I/L develop a clinically and histopathologically severe infection with the elicitation of a nonspecific fibrotic response of the lung that is characteristic of histopathology of human BOOP lesions (19, 22, 23). These BOOP lesions, like those observed in humans, are characterized by the patchy distribution of intraluminal plugs of granulation tissue that are chiefly composed of fibroblast-like cells and limited amounts of collagen (19). The development of BOOP lesions in this animal model is preceded by a similarly patchy distribution of peribronchiolar mononuclear cell inflammatory lesions that progress to characteristic well-developed BOOP lesions (19). In contrast, CBA/J mice inoculated with 1 × 10⁷ PFU reovirus I/L develop ARDS, providing a model that recapitulates both its acute exudative phase, including the formation of hyaline membranes, as well as its regenerative phase with healing by repair, leading to intraalveolar and interstitial fibrosis (20–23). As with human ARDS, histologically, our model exhibits diffuse alveolar damage, a protein-rich edema leading to the formation of hyaline membranes, hemorrhage due to vascular leakage, and disruption of the alveolar epithelium (20, 21). Additionally, diffuse infiltrates composed primarily of neutrophils (PMNs) and macrophages are predominant, which is similar to human ARDS (20–23). The chronic phase of our ARDS model demonstrates fibrosing alveolitis, which can be observed in human ARDS patients that exhibit healing by repair, leading to fibrosis (20–23). Thus, these small animal models of BOOP and ARDS provide very relevant models for deciphering common underlying cellular, biochemical, and molecular mechanisms that may alter the pulmonary environment, leading to inflammation and fibrosis.

Apoptosis, the process of programmed cell death, plays a major regulatory role in homeostasis by maintaining a balance between cell proliferation and cell death (24). In human respiratory diseases, including ARDS and other pulmonary fibrotic disorders, apoptosis may play a role in the pathogenesis of the disease process by two distinct mechanisms: (1) via delayed leukocyte apoptosis and/or (2) through enhanced endothelial and epithelial cell apoptosis (25–28). Recent studies have suggested that apoptosis of the alveolar epithelium via the Fas/Fas ligand (FasL) pathway may be an important determinant in the pathogenesis of pulmonary fibrosis in idiopathic pulmonary fibrosis (IPF), BOOP, oblitative bronchiolitis, and in acute lung injury such as ARDS (29–37). These observations support the hypothesis that apoptosis of alveolar epithelial cells potentially through the Fas/FasL pathway is involved in the pathophysiology of at least some forms of pulmonary fibrosis. We hypothesize that apoptosis through the Fas/FasL pathway plays a crucial role in inflammation and fibrosis associated with reovirus I/L-induced BOOP and/or ARDS, two in vivo models that closely resemble the pathophysiology of their human counterparts. We demonstrate an up-regulation of both Fas and FasL as well as a significant induction of apoptosis in situ in both reovirus I/L-induced BOOP and ARDS. Additionally, treatment of mice with the caspase-8 and caspase-6 inhibitor, N-benzoyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl-ketone (zIETD-fmk) (Kamiya Biomedicals) or the caspase-8 (FLICE) inhibitor N-benzoyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl-ketone (zIETD-fmk) (Kamiya Biomedicals) at 5 mg/kg in 100 μl of volume. zVAD-fmk and zIETD-fmk were prepared fresh daily as a stock solution at 1 mg/ml in 10% DMSO (in PBS). Based on a series of preliminary experiments using caspase inhibitor concentrations of 1, 5, and 7.5 mg/kg, 5 mg/kg was chosen as a final dosage after evaluating inflammatory infiltration and fibrosis via H&E staining. This dosage is consistent with other published protocols evaluating caspase inhibitors in vivo (39–45). Saline-inoculated control mice treated with either zVAD-fmk or zIETD-fmk did not demonstrate any pulmonary pathology (data not shown). Reovirus I/L-inoculated mice were also treated with 10% DMSO (in PBS) as a carrier control. As expected, these mice demonstrated significant pulmonary pathology similar to reovirus I/L-inoculated, untreated mice (data not shown).

Bronchoalveolar lavage (BAL)

BAL was performed in situ by injecting and withdrawing a 0.5-ml aliquot of HBSS twice through an intubation needle (21 gauge). A total of 1.5 ml of HBSS was used. BAL fluid was centrifuged at 14,000 rpm for 5 min and then was frozen at −70°C until use. Cells collected by BAL were washed three times with HBSS containing 5% FCS and 0.05% azide, and suspended at 1 × 10⁶ cells/ml for flow cytometric analysis.

Histology

Lungs were inflated in situ with 2% paraformaldehyde (Sigma-Aldrich) by intratracheal intubation, removed, and suspended in an additional 2% paraformaldehyde for 2 h at 4°C before being embedded in paraffin. H&E staining was performed on 4-μm sections. Masson’s trichrome and Sirius red staining were used to visualize collagen deposition. With Masson’s trichrome the nuclei stain a dark red/purple, muscle stains red, and connective tissue, including collagen, stains blue. With Sirius red, in bright-field microscopy, collagen is red on a pale yellow background. Nuclei, if stained, are black but may often be gray or brown. In normal or saline-inoculated lung sections, Sirius red staining is evident only within the walls of the bronchioles and arteries, which contain connective tissue including collagen, while the lung alveolar airspaces are not significantly stained. To score lung inflammation and fibrosis, lung samples were

Materials and Methods

Animals

Four- to 5-wk-old female CBA/J mice and CBA/KlJms-Fas+/−/J, which carry a point mutation in the Fas cytoplasmic region that abolishes the ability of Fas to transduce an apoptotic signal (38), were obtained from The Jackson Laboratory and maintained in microisolator cages under specific pathogen-free conditions in a BL-2 facility. Cages were housed in a high efficiency particulate air-filtered animal isolator clean room (Nuaire), and all animal manipulations were performed in class II biological safety cabinets. Virally primed mice were kept physically isolated from all other experimental and stock mice. All animal protocols were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee Board.

Virus

Reovirus I/L was originally obtained from Dr. W. Joklik (Duke University School of Medicine, Durham, NC). Third-passage gradient-purified stocks were obtained by re-cloning and amplifying parental stocks on L-929 fibroblast cells (American Type Culture Collection) (19). Following the purification of new stocks, infectious viral titers were obtained by limiting dilution on L-929 monolayers (19).

Inoculation protocol

Animals were lightly anesthetized with an i.p. injection of 0.08 cc of 20% ketamine (Vetalar, 100 mg/ml; Fort Dodge Laboratories) and 2% PromAce (acepromazine maleate, 10 mg/ml; Ayerst Laboratories) before immunization. Animals were inoculated by the i.n. application of 1 × 10⁶ PFU (BOOP) or 1 × 10⁷ PFU (ARDs) of reovirus I/L in 30 μl (15 μl in each nostril) in sterile injectable grade 0.9% NaCl (Baxter Healthcare). Control animals were inoculated with 30 μl (15 μl in each nostril) of sterile injectable grade 0.9% NaCl. After the indicated time points, animals were sacrificed with an i.p. injection of 0.2 cc sodium nembutal (50 mg/ml; Abbott Laboratories).

Caspase inhibitor administration

Mice were injected i.p. starting on day 3 postreovirus I/L inoculation and given daily until the completion of the time course with either the pan-caspase inhibitor N-benzoylcarbonyl-Val-Ala-Asp-fluoromethyl-ketone (zVAD-fmk) (Kamiya Biomedicals) or the caspase-8 (FLICE) inhibitor N-benzoyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl-ketone (zIETD-fmk) (Kamiya Biomedicals) in each nostril of sterile injectable grade 0.9% NaCl (Baxter Healthcare). Control animals were inoculated with 30 μl (15 μl in each nostril) of sterile injectable grade 0.9% NaCl. After the indicated time points, animals were sacrificed with an i.p. injection of 0.2 cc sodium nembutal (50 mg/ml; Abbott Laboratories).

Histology

Animals were obtained by re-cloning and amplifying parental stocks on L-929 fibroblast cells (American Type Culture Collection) (19). Following the purification of new stocks, infectious viral titers were obtained by limiting dilution on L-929 monolayers (19).
screened for the following three histopathological parameters: (1) deposition of extracellular matrix; (2) leukocyte infiltration (interstitial inflammation); and (3) airway obliteration due to granulation tissue formation and/or fibrosis. Each lung section was blindly evaluated and scored on a scale of 0–3 with 0 as absent (normal), 1 as mild, 2 as moderate, and 3 as severe (22, 23). Additionally, after Sirius red staining, the severity of pulmonary fibrosis was also evaluated by quantitating the amount of red stained area (connective tissue) in reovirus 1/L-induced lung samples using ImageJ software analysis (46). Results are expressed as a percentage of Sirius red content in saline-inoculated, control mice. Differences between groups were examined for statistical significance using a two-tailed Student’s t test. A p value of <0.05 was considered significant. Supplemental Fig. 1 demonstrates normal lung tissue stained with H&E, Masson’s trichrome, and Sirius red. Images from low (×20) and high (×400) magnification on an Olympus BX40 microscope were captured with a Polaroid digital microscope camera and edited using Adobe Photoshop 5.0 software.

**Hydroxyproline (HP) assay**

The extent of pulmonary fibrosis was also determined by estimating total lung collagen as reflected by the measurement of HP content of the lung. Mice were sacrificed at various time points after inoculation with reovirus 1/L and the lungs were removed, lyophillized, andweighed. Total lung HP content was assayed in duplicate as previously described (20). Differences between groups were examined for statistical significance using a two-tailed Student’s t test. A p value of <0.05 was considered significant.

**Flow cytometric analysis**

Cells were stained for cell surface marker expression as previously described except that all cells were also stained with Cy-Chrom conjugated rat anti-mouse-CD45 (35-F11, leukocyte common Ag, Ly-5; BD Pharmingen), and only anti-mouse-CD45^+/HI cells were acquired for analysis (21). The following Abs were used (in each experiment): CD4 (GK1.5, L3T4; R-PE-labeled; Caltag Laboratories), CD8a (53-6.7, PerCP-labeled; BD Pharmingen), Ly6G (RB6-8C5, Gr-1, allopolyoxycyanin-labeled; BD Pharmingen), Fas polyclonal Ab (A20, FITC-labeled; Santa Cruz Biotechnology), and FasL (Kay10, PE-labeled; BD Pharmingen). Isotype-matched controls were run for each sample (Caltag and BD-Pharmingen). Flow cytometric analysis was performed using a dual-laser FACS-Calibur flow cytometer and the CellQuest acquisition and analysis software program (BD Biosciences).

**Immunohistochemistry (IHC)**

IHC was performed on parafomaldehyde-fixed and paraffin-embedded lung tissue. Five- to 8-μm thick sections were collected on poly-l-lysine-treated slides (Sigma-Aldrich). Sections were deparaffinized in xylene and dehydrated in graded alcohol. Following deparaffinization, the tissue sections were heat treated for 10 min with a Target Retrieval Solution (S1700; Dako) following the manufacturer’s instructions. The slides were then permeabilized in 2% hydrogen peroxide to quench endogenous peroxidase for 10 min and incubated with 5% normal goat serum (Vector Laboratories) for 1 h. For IHC for Fas analysis, incubation with a rabbit anti-mouse Fas polyclonal Ab (A20, 1/100 dilution; Santa Cruz Biotechnology) for 30 min was performed followed by incubation with biotinylated anti-rabbit IgG (1/200 dilution; Santa Cruz Biotechnology) for 30 min. IHC for FasL analysis was performed using the M.O.M. Immunodetection kit PK-2200 (Vector Laboratories). Sections were incubated with M.O.M. 10% blocking reagent for 1 h to block nonspecific binding. Sections were then rinsed with PBS and incubated in M.O.M. 4% diluent, followed by incubation with mouse anti-mouse FasL mAb (Kay10, 1/50 dilution; BD Pharmingen) for 30 min. Slides were then washed and incubated with M.O.M. biotinylated anti-mouse IgG reagent (1/250 dilution; Vector Laboratories) for 30 min. IHC for caspase-8 or caspase-3, sections were incubated overnight with either a rabbit polyclonal Ab for cleaved caspase-3 (1/200 dilution; Cell Signaling Technologies) or a rabbit polyclonal Ab for active/cleaved caspase-8 (1/200 dilution; Immugen) followed by incubation with a biotinylated goat anti-rabbit IgG (1/500; Santa Cruz Biotechnology) for 1 h. Immunoreactivity was demonstrated using the ABC immunostaining system kit (ABC kit; Vector Laboratories). Sections were incubated with Fas or FasL to GAPDH. Differences between groups were examined for statistical significance using a two-tailed Student’s t test. A p value of <0.05 was considered significant.

**RNA preparation and RT-PCR analysis**

Total RNA was isolated from whole lungs by guanidium denaturation utilizing TRIzol reagent (Sigma-Aldrich). For RT-PCR analysis, cDNA was prepared by reverse transcription of isolated RNA samples using the Quigen one-step RT-PCR kit following the manufacturer’s instructions. The PCR amplifications were performed using a 20-μl reaction volume containing 4 μl of each cDNA, 6 μl of deionized water, 4 μl of 5X buffer, 4 μl of Q buffer, 0.8 μl of dNTPs, 0.8 μl of primers, and 0.4 μl of Taq polymerase. The primers used were as follows: GAPDH, Forward, 5′-CAAGCACAACAGTTGTTGCC-3′; Reverse, 5′-TCC GAC CAC GAC CACTGG-3′; Fas, forward, 5′-TCT TTT GAT CAT GAC GGG AGT GAG-3′, reverse, 5′-ATA ACA GCA CCT TGG TCA GGA-3′; DNA Damage response, forward, 5′-GAT TTC TTC TCG TTA ACC CG-3′; TUNEL analysis was quantitated by counting the number of TUNEL-positive cells in eight random 225-μm^2 fields from two independent experiments with three mice per time point in a blinded fashion. Differences between groups were examined for statistical significance using a two-tailed Student’s t test. A p value of <0.05 was considered significant.

**Western blot analysis**

For Western blotting, lungs from saline or reovirus 1/L-treated mice were removed and homogenized using a Tissue Tearor in 1.5 ml of 25 mM Tris (pH 8.0) containing 10 mM ethylene glycol bis(β-aminoethyl ether) N,N,N’N’-tetraacetic acid (EGTA), 150 mM NaCl, and 1% Nonidet P-40, pepstatin A (5 μg/ml), leupeptin (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), aprotinin (10 μg/ml), and phenylmethylsulfonyl fluoride (1 mM). The homogenates were stored at −80°C until use. Protein concentrations were determined using the bicinchoninic acid assay. Proteins were resolved by SDS-PAGE using Ready Gels Tris–HCl 8–16% (Bio-Rad). Western blotting was then accomplished using either the rabbit anti-mouse Fas polyclonal Ab (A20; Santa Cruz Biotechnology), the mouse anti-mouse FasL mAb (Kay10; BD Pharmingen), or the rabbit anti-caspase-3

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* The online version of this article contains supplemental material.
antiserum (H-277, 1/500 dilution; Santa Cruz Biotechnology) with the appropriate secondary Abs. The anti-caspase-3 recognizes the full-length pro-caspase-3 and the p17 and p20 cleaved subunits. Bands were detected using the ECL substrate kit from Pierce. The cleaved caspase-3 band intensities were compared with background controls and reported as a ratio of cleaved caspase-3 to background. Band intensities on scanned gels were analyzed using the public domain National Institutes of Health Image program. Western analysis using an anti-actin Ab (Santa Cruz Biotechnology) was also performed to demonstrate equal loading (data not shown). Differences between groups were examined for statistical significance using a two-tailed Student’s t test. A p value of <0.05 was considered significant.

Results
Apoptosis as evidenced by in situ TUNEL analysis is present in both reovirus 1/L-induced BOOP and ARDS
Apoptosis plays a major regulatory role in homeostasis by maintaining a balance between cell proliferation and cell death and is implicated in the pathogenesis of human respiratory diseases, including ARDS and other pulmonary fibrotic disorders (25–28).

With this in mind, we assessed the role of apoptosis in the reovirus 1/L-induced models of both ARDS and BOOP, which we developed in our laboratory. CBA/J mice were inoculated by the i.n. route with either 1 × 10^6 PFU (BOOP) or 1 × 10^7 PFU (ARDS) reovirus 1/L, sacrificed at the indicated time points, and apoptosis in situ was assessed by TUNEL analysis on paraffin-embedded lung sections. The extent of pulmonary fibrosis was also determined by estimating total lung collagen as reflected by the measurement of HP content of the lung (20–23). We have previously described the histological development of both reovirus 1/L-induced ARDS and BOOP (19–23). For reovirus 1/L-induced ARDS, at day 9 postinoculation, there is a severe pneumonia (peribronchiolar lesions with lymphocytic infiltration) with the presence of hyaline membranes, which are pathognomonic for human ARDS (20, 21). In addition to areas of mononuclear cell infiltration, edema, and hyaline membranes, the development of fibrotic lesions also occurs during the recovery phase of the infection. Between days 12 and 14 postinoculation, young, cellular fibrous polyps of collagenous tissue can be observed that develop into discrete fibroblastic polyps in the alveolar ducts (20, 21). Therefore, the peak acute inflammatory phase of the disease is represented at day 9 postinoculation while the peak fibrotic phase of the disease is represented by days 12–14 (20, 21). For reovirus 1/L-induced BOOP, at day 7 postinoculation, there is an inflammatory cellular infiltrate characterized by mononuclear cells and macrophages that demonstrates a typical focus of an active viral pneumonia (19). Fibrous plugs characteristic of BOOP fibrotic lesions are visible at day 14 postinoculation and well-formed BOOP fibrotic lesions (fibrous cellular plug present in alveolar ducts) peak at day 21 postinoculation (19). Therefore, these peak days, days 9 and 14 for reovirus 1/L-induced ARDS and days 7, 14, and 21 for reovirus 1/L-induced BOOP, were chosen as key time points to investigate the role of apoptosis in these models. For reovirus 1/L-induced ARDS, TUNEL analysis was evaluated on days 3, 5, 7, 9, 14, and 21 days postinoculation (Fig. 1A and data not shown). Peak apoptosis as measured by TUNEL expression was observed on day 9 postinoculation during the peak inflammatory response and declined by day 14 postinoculation when peak fibrotic lesions were observed as determined by HP content (Fig. 1A) (20, 21). Similarly, we compared the presence of apoptotic cells via TUNEL analysis on days 5, 7, 9 14, and 21 in the reovirus 1/L-induced BOOP (Fig. 1B and data not shown). Significant apoptosis as measured by TUNEL-positive cells was observed mainly at days 7–14, with a decrease in the number of TUNEL-positive cells observed on day 21, which corresponded to the peak fibrotic response as determined by HP content (Fig. 1B) (19, 22, 23). No apoptotic cells (<1%) were detected in control, saline-inoculated mice at any time point after inoculation (Fig. 1 and data not shown). Evidence of apoptosis in reovirus 1/L-induced ARDS and BOOP suggests that cell death may play an important role in lung tissue injury and apoptosis occurring during the regeneration phase of the diseases may explain the development and persistence of fibrosis.

Fas and FasL are expressed during reovirus 1/L-induced BOOP and ARDS
Apoptosis mediated by the Fas/FasL pathway has been suggested to play an important role in the development of acute lung injury and fibrosis (29–37). To determine whether the Fas/FasL pathway was activated in either reovirus 1/L-induced ARDS or BOOP, CBA/J mice were i.n. inoculated with either 1 × 10^6 PFU (BOOP) or 1 × 10^7 PFU (ARDS) reovirus 1/L and sacrificed at the indicated key time points. Lungs were paraffin embedded and stained with an Ab to either Fas or FasL. Immunohistochemical staining of lung tissue at various time points after reovirus 1/L inoculation demonstrated significant Fas expression in alveolar epithelial cells and infiltrating cells on days 7, 9, 12, and 14 postreovirus 1/L inoculation in reovirus 1/L-induced ARDS (Fig. 2A, top left and data not shown). Positive immunostaining for FasL was detected in the alveolar epithelium and infiltrating cells on days 9 and 14 postinfection in reovirus 1/L-induced ARDS (Fig. 2A, bottom left and data not shown). Similarly, Fas and FasL expression via IHC was observed in reovirus 1/L-induced BOOP with peak Fas and FasL expression being observed between days 14 and 21 after reovirus 1/L inoculation (data not shown). In control, saline-inoculated animals, constitutive, positive immunostaining for Fas was
detected only in bronchiolar epithelial cells, not in alveolar epithelial cells, while Fasl immunostaining was not observed in any cells at any time point (data not shown). To determine whether alveolar type II epithelial cells expressed the Fas Ag, two-color IHC for Fas and TTF-1 was performed on lung tissue sections obtained from reovirus 1/L-induced ARDS on day 9 postinoculation (Fig. 2A, top right and bottom right). TTF-1 binds to regulatory elements located in the promoters of a number of transcriptional targets in the lung (47, 48). In the postnatal lung, TTF-1 is most abundant in type II epithelial cells in the alveolus, where it regulates surfactant protein synthesis (47, 48). ARDS-induced lung sections stained positively with the anti-Fas Ab (brown precipitate) and TTF-1 Ab, a marker for alveolar epithelial type II cells (red/purple precipitate) (Fig. 2A, top right and bottom right), demonstrating the presence of the Fas Ag on alveolar type II epithelial cells. The TTF-1 Ag stains predominantly within the nucleus of positive cells. Fig. 2A (top right) demonstrates costaining of epithelial cells for Fas Ag in a predominantly involved section of the lung, while Fig. 2A (bottom right) demonstrates costaining of epithelial cells in a predominantly uninvolved, adjacent section of the lung. An enlarged image of an alveolar epithelial cell stained positively for both TTF-1 (predominant purple staining nucleus) and Fas (predominant brown staining cytoplasm) is shown in the indicated boxed region. B, RNA was prepared from whole lung tissue of either saline or reovirus 1/L-inoculated mice. Relative expression of Fas or Fasl on day 12 reovirus 1/L-induced ARDS or on day 21 reovirus 1/L-induced BOOP was determined by comparing the ratio of Fas or Fasl mRNA with the housekeeping gene, GAPDH. Histograms represent densitometric data from the mean ± SD autoradiogram signals from three mice per time point. * p < 0.05 compared with saline-inoculated, control mice. C, Western analysis from whole lung lysates for protein expression of Fas overtime in reovirus 1/L-induced ARDS and BOOP; representative of two independent experiments (S, saline day 9). D, Western analysis from BAL fluid for protein expression of Fasl overtime in reovirus 1/L-induced ARDS and BOOP. Representative of two independent experiments (S, saline day 9).
FIGURE 3. Fas and FasL are expressed on the surface of infiltrating cells in reovirus 1/L-induced ARDS and BOOP. CBA/J mice were i.n. inoculated with either $1 \times 10^6$ PFU (BOOP) or $1 \times 10^7$ PFU (ARDS) reovirus 1/L and cells recovered by BAL were analyzed for the coexpression of cell surface phenotype markers with surface expression of Fas and FasL. Infiltrating cells were first stained with either anti-CD4, anti-CD8, anti-Mac1, or anti-GR1. The gated positive cells were then analyzed for the expression of either Fas or FasL. A, Cells recovered from ARDS-induced mice on day 9 postinoculation. B, Cells recovered from BOOP-induced mice on day 7 postinoculation. The percentage of the total cells expressing either anti-CD4, anti-CD8, anti-Mac1, or anti-GR1 is shown in the histograms on the left. The percentage of the gated population expressing either Fas or FasL is shown in the histograms on the right. The results shown represent one of the three independent experiments demonstrating similar results.
Both inflammation and fibrosis are diminished in zIETD-fmk-treated reovirus 1/L-induced ARDS and BOOP

To demonstrate a direct role for apoptosis is the pathophysiology of reovirus 1/L-induced ARDS or BOOP, the effects of the pan-caspase inhibitor zVAD-fmk or the initiator caspase inhibitor zIETD-fmk (caspase-8 inhibitor) was determined in reovirus 1/L-induced ARDS and BOOP. Mice were inoculated i.n. with either 1 × 10^6 PFU (BOOP) or 1 × 10^7 PFU (ARDS) reovirus 1/L and were left untreated or treated with 5 mg/kg of either zVAD-fmk or zIETD-fmk daily beginning on day 3 postinoculation until sacrifice. The extent of pulmonary fibrosis was determined by estimating total lung collagen as reflected by the measurement of the HP content of the lung. Results are expressed as a percentage of HP content in saline-inoculated, control mice. Results shown are for day 14 ARDS and day 21 BOOP. Each data point represents the mean ± SD of six mice. *p < 0.05 compared with saline-inoculated, control mice (day 14); **p < 0.05 compared with reovirus 1/L-inoculated mice. B, Apoptosis in situ was assessed using TUNEL labeling on paraffin-embedded lung sections in saline (day 9 ARDS, day 14 BOOP), reovirus 1/L-induced ARDS (day 9) and reovirus 1/L-induced BOOP (day 14). TUNEL data represent mean ± SD of eight 225-mm² fields from two independent experiments with three mice per time point. *p < 0.05 compared with saline-inoculated, control mice; **p < 0.05 compared with reovirus 1/L-inoculated mice.

FIGURE 4. Both inflammation and fibrosis are diminished in zIETD-fmk-treated reovirus 1/L-induced ARDS or BOOP. CBA/J mice were i.n. inoculated with either 1 × 10^6 PFU (BOOP) or 1 × 10^7 PFU (ARDS) reovirus 1/L and were left untreated or treated with 5 mg/kg of either zVAD-fmk or zIETD-fmk daily beginning on day 3 postinoculation. A, Reovirus 1/L-induced ARDS at day 9 (H&E). B, Reovirus 1/L-induced ARDS at day 14 (Masson’s trichrome). C, Reovirus 1/L-induced BOOP at day 21 (H&E). Objective magnification, ×20. Results are representative of four independent experiments evaluating four mice per time point.
Clonal Ab for active/cleaved caspase-8 in either untreated (day 14 postinoculation) were paraffin embedded and stained with a rabbit poly-

**FIGURE 6.** The presence of both caspase-8 and caspase-3 are diminished in zIETD-fmk reovirus 1/L-induced ARDS or BOOP. CBA/J mice were i.n. inoculated with either 1 \times 10^6 PFU (BOOP) or 1 \times 10^7 PFU (ARDS) reovirus 1/L and were left untreated or treated with 5 mg/kg of either zVAD-fmk or zIETD-fmk daily beginning on day 3 postinoculation until sacrifice. A. Lungs of reovirus 1/L-induced BOOP and ARDS on day 14 postinoculation were paraffin embedded and stained with a rabbit polyclonal Ab for active/cleaved caspase-8 in either untreated (top) or zIETD-fmk-treated (bottom) reovirus 1/L-induced BOOP or ARDS. IHC for active/cleaved caspase-8 is representative of two experiments with two mice per time point. B. Western analysis from whole lung lysates for protein expression of cleaved caspase-3 was determined in reovirus 1/L-induced ARDS on day 9 postinoculation and in reovirus 1/L-induced BOOP on day 14 postinoculation. Relative expression of cleaved caspase-3 was determined by comparing the ratio of the cleaved caspase-3 band to background. Histograms represent densitometric data from the mean ± SD autoradiogram signals from three mice for the saline, ARDS-induced, and BOOP-induced mice and from at least four mice in the zVAD-fmk- and zIETD-fmk-treated groups, *p < 0.05 compared with saline-inoculated, control mice; **p < 0.05 compared with reovirus 1/L-inoculated mice.

1/L-induced ARDS (Fig. 5B) and day 14 for reovirus 1/L-induced BOOP (Fig. 5B). Treatment of both reovirus 1/L-induced ARDS (Fig. 5B) and BOOP (Fig. 5B) in vivo with zIETD-fmk significantly reduced the number of TUNEL-positive cells as compared untreated animals. An ~60% reduction in the number of TUNEL-positive cells was observed in zIETD-fmk-treated reovirus 1/L-induced ARDS (Fig. 5B) and a 75% reduction in the number of TUNEL-positive cells in reovirus 1/L-induced BOOP (Fig. 5B). While treatment of reovirus 1/L-induced ARDS or BOOP with zVAD-fmk also resulted in a reduction of TUNEL-positive cells, this reduction was less than that observed after zIETD-fmk treatment (30% in reovirus 1/L-induced ARDS (Fig. 5B) and 56% in reovirus 1/L-induced BOOP (Fig. 5B)).

Finally, we performed immunostaining for caspase-8 in untreated and zIETD-fmk-treated mice (Fig. 6A). In both zIETD-fmk-treated BOOP and ARDS on day 14 postinoculation, immunostaining for caspase-8 was significantly diminished as compared with untreated BOOP or ARDS (Fig. 6A). In control, saline-inoculated animals, positive immunostaining using the active/cleaved caspase-8 Ab was randomly detected in alveolar epithelial cells at a very low level (data not shown). Furthermore, Western analysis from whole lung lysates for protein expression of cleaved caspase-3 was determined in reovirus 1/L-induced ARDS on day 9 postinoculation and in reovirus 1/L-induced BOOP on day 14 postinoculation after treatment with either zVAD-fmk or zIETD-fmk (Fig. 6B). In reovirus 1/L-induced BOOP or ARDS, cleaved caspase-3 was significantly induced as compared with saline inoculated mice (Fig. 6B). Treatment of either reovirus 1/L-induced BOOP or ARDS with zIETD-fmk significantly reduced the induction of cleaved caspase-3 as compared with untreated reovirus 1/L-induced lungs. However, while treatment of either reovirus 1/L-induced BOOP or ARDS with zVAD-fmk reduced the expression of cleaved caspase-3, this reduction was not statistically significant. Immunostaining for cleaved caspase-3 in either zVAD-fmk- or zIETD-fmk-treated reovirus 1/L-induced BOOP and ARDS demonstrated similar results as were observed via Western analysis (data not shown). Collectively, these results confirm that treatment of mice in vivo with zIETD-fmk significantly inhibited both inflammation and fibrosis in both reovirus 1/L-induced BOOP and ARDS.

Both inflammation and fibrosis are attenuated in reovirus 1/L-induced BOOP but not in reovirus 1/L-induced ARDS in CBA/KJlms-Faslpr-cg/J mice.

To demonstrate a direct role for the Fas/FasL apoptotic pathway in either reovirus 1/L-induced ARDS or BOOP, CBA/KJlms-Faslpr-cg/J mice, which carry a point mutation in the Fas cytoplasmic region that abolishes the ability of Fas to transduce an apoptotic signal (38), were inoculated i.n. with either 1 \times 10^6 PFU (BOOP) or 1 \times 10^7 PFU (ARDS) reovirus 1/L. Animals were evaluated at key time points on days 9 and 14 postinoculation for reovirus 1/L-induced ARDS and on days 7 and 21 for reovirus 1/L-induced BOOP. Paraffin-embedded lung sections were stained with H&E, Masson’s trichrome, or Sirius red (Fig. 7). Interestingly, inoculation of CBA/KJlms-Faslpr-cg/J mice with either 1 \times 10^6 PFU (BOOP) or 1 \times 10^7 PFU (ARDS) reovirus 1/L resulted in decreased mortality. Typically, 20% mortality is observed for reovirus 1/L-induced BOOP (19). However, no mortality was observed during 21 days in CBA/KJlms-Faslpr-cg/J mice inoculated with 1 \times 10^6 PFU reovirus 1/L (BOOP). In reovirus 1/L-induced ARDS, 45–60% mortality was observed between days 9 and 14 postinoculation with 1 \times 10^7 PFU reovirus 1/L (20, 21). However, in CBA/KJlms-Faslpr-cg/J mice inoculated with 1 \times 10^7 PFU (ARDS) reovirus 1/L, only 15% of the animals died by day 14 postinoculation. All remaining animals were sacrificed on day 14 postinoculation, so that mortality after day 14 could not be assessed. In reovirus 1/L-induced ARDS, inflammation was evaluated on day 9 and fibrosis was evaluated on day 14 (Fig. 7A). As can be observed histologically, inflammation typically observed on day 9, after reovirus 1/L-induced ARDS, was attenuated in CBA/KJlms-Faslpr-cg/J mice vs CBA/J mice (Fig. 7A; CBA/J, 2.9 ± 0.2 vs CBA/KJlms-Faslpr-cg/J, 1.8 ± 0.4; p < 0.05). However, by day 14 postinoculation, CBA/KJlms-Faslpr-cg/J mice developed similar histological features (both fibrosis and inflammation) as compared with CBA/J mice (Fig. 7A; CBA/J, 2.8 ± 0.4 vs CBA/KJlms-Faslpr-cg/J, 2.8 ± 0.5) as determined by either H&E or Masson’s trichrome staining. In reovirus 1/L-induced BOOP, inflammation was evaluated on day 7 and fibrosis was evaluated on day 21 (Fig. 7B; CBA/J vs CBA/KJlms-Faslpr-cg/J). Unlike CBA/KJlms-Faslpr-cg/J mice inoculated with 1 \times 10^6 PFU (ARDS) reovirus 1/L, CBA/KJlms-Faslpr-cg/J mice inoculated with 1 \times 10^7 PFU reovirus 1/L (BOOP) demonstrated an attenuation of both inflammation (Fig. 7B; 2.1 ± 0.3 vs 1.6 ± 0.4; p < 0.05) and fibrosis (Fig. 7B; 2.6 ± 0.4 vs 1.5 ± 0.4; p < 0.05).
To more clearly demonstrate the presence of fibrotic lesions histologically in either CBA/J or CBA/KIJms-Faslpr-cg mice inoculated with 1 × 10^7 PFU (ARDS) or 1 × 10^6 PFU reovirus 1/L (BOOP), tissue sections were stained with Sirius red on day 14 for reovirus 1/L-induced ARDS and on day 21 for reovirus 1/L-induced BOOP (Fig. 7, bottom panels). With Sirius red, in bright-field microscopy, collagen is stained red on a pale yellow background. In normal or saline-immunized lung sections, Sirius red staining is evident only within the walls of the bronchioles and arterioles, which contain connective tissue including collagen, while the lung alveolar airspaces are not stained (not shown). As can be observed, significant staining for collagen via Sirius red is evident in either CBA/J or CBA/KIJms-Faslpr-cg mice inoculated with 1 × 10^2 PFU (ARDS) (Fig. 7A, bottom panel; 2.8 ± 0.8 vs 2.7 ± 0.3). However, while significant staining for collagen is observed in CBA/J mice inoculated with 1 × 10^6 PFU reovirus 1/L (BOOP), Sirius red staining is dramatically reduced in CBA/KIJms-Faslpr-cg mice inoculated with 1 × 10^6 PFU reovirus 1/L (BOOP) (Fig. 7B, bottom panel; 2.5 ± 0.5 vs 1.5 ± 0.5, p < 0.05). The severity of pulmonary fibrosis was also evaluated by quantitating the amount of Sirius red (connective tissue) staining in reovirus 1/L-induced lung samples (day 14 for reovirus 1/L-induced ARDS and day 21 for reovirus 1/L-induced BOOP) using ImageJ software analysis (Fig. 8A) (46). Results are expressed as a percentage of Sirius red staining in saline-inoculated, control mice (Fig. 8A). As shown in Fig. 8A, significant Sirius red staining was observed in both CBA/J mice and CBA/KIJms-Faslpr-cg mice inoculated with 1 × 10^7 PFU reovirus 1/L (ARDS) as compared with normal saline-inoculated control mice. In CBA/J mice inoculated with 1 × 10^6 PFU reovirus 1/L (BOOP), as expected, a significant increase in Sirius red staining over saline-inoculated mice was observed (Fig. 8A). However, while a significant increase in Sirius red staining was also observed in CBA/KIJms-Faslpr-cg mice inoculated with 1 × 10^6 PFU reovirus 1/L (BOOP) (Fig. 8A). Supplemental Fig. 1 demonstrates normal lung tissue stained with H&E, Masson’s trichrome, and Sirius red. Apoptosis in situ was also evaluated via TUNEL analysis in CBA/KIJms-Faslpr-cg mice inoculated with 1 × 10^6 PFU (BOOP) or 1 × 10^7 PFU (ARDS) reovirus 1/L (Fig. 8B). Apoptosis was significantly decreased on day 9 in CBA/KIJms-Faslpr-cg mice as compared with CBA/J mice inoculated with 1 × 10^6 PFU (ARDS) reovirus 1/L (Fig. 8B). Similarly, apoptosis was significantly decreased on day 7 in CBA/KIJms-Faslpr-cg mice as compared with CBA/J mice inoculated with 1 × 10^6 PFU (BOOP)
reovirus 1/L (Fig. 8B). However, the numbers of apoptotic cells in CBA/KJms-Fas<sup>gld</sup> or CBA/KJms-Fas<sup>lpr/lpr</sup> mice inoculated with 1 × 10<sup>7</sup> PFU (ARDS) reovirus 1/L were greater than those observed in either CBA/KJms-Fas<sup>gld</sup> or CBA/KJms-Fas<sup>lpr/lpr</sup> mice inoculated with 1 × 10<sup>6</sup> PFU (BOOP) (Fig. 8B). Taken together, these results suggest that while expression of Fas and FasL may be involved in both reovirus 1/L-induced ARDS and BOOP, a direct role for the Fas/FasL pathway is evident only in reovirus 1/L-induced BOOP.

**Discussion**

BOOP and ARDS are two clinical and histologically distinct syndromes sharing the presence of both an inflammatory infiltrate and a fibrotic component (6–17). We have previously demonstrated that CBA/J mice infected with 1 × 10<sup>6</sup> PFU reovirus 1/L develop a clinically and histopathologically severe infection with the elicitation of a nonspecific fibrotic response of the lung (BOOP) (19, 22, 23). In contrast, CBA/J mice infected with 1 × 10<sup>7</sup> PFU reovirus 1/L develop ARDS and provide a model that recapitulates both its acute exudative phase, including the formation of hyaline membranes, as well as its regenerative phase, with healing by repair, leading to intraalveolar and interstitial fibrosis (20–22). Both of these models accurately recapitulate the pathophysiology of BOOP or ARDS that is observed in human patients, and thus they provide very relevant models for deciphering common underlying cellular, biochemical, and molecular mechanisms that may alter the pulmonary environment, leading to inflammation and fibrosis. Apoptosis especially via the Fas/FasL ligand pathway has been suggested to play an important role in the development of acute lung injury and fibrosis characteristic of these and other pulmonary fibrotic syndromes (25–37). Therefore, we evaluated the role of apoptosis via the Fas/FasL pathway in the development of inflammation and pulmonary fibrosis in reovirus 1/L-induced BOOP and ARDS. Our results demonstrated the presence of apoptotic cells and Fas/FasL expression in both the alveolar epithelium and in the infiltrating cells during the fibrotic stage of ARDS (days 12–14) and BOOP (days 14–21). While treatment of mice with the pan-caspase inhibitor zVAD-fmk led to a limited reduction in fibrosis, the caspase-8/6-specific inhibitor zETD-fmk inhibited apoptosis and fibrotic lesion development in both reovirus 1/L-induced BOOP and ARDS. However, CBA/KJms-Fas<sup>gld</sup> mice, which carry a point mutation in the Fas cytoplasmic region that abolishes the ability of Fas to transduce an apoptotic signal (38), did not develop fibrotic lesions associated with reovirus 1/L-induced BOOP, but still developed fibrosis associated with reovirus 1/L-induced ARDS. Therefore, while the Fas/FasL apoptotic pathway may be involved in both reovirus 1/L-induced ARDS and BOOP, a direct role for the Fas/FasL pathway is evident only in reovirus 1/L-induced BOOP. Other alternative or redundant pathways in addition to the Fas/FasL pathway that also lead to apoptosis may be involved in the histopathological development of ARDS. Taken together, these results suggest a role for specific caspase inhibitors in the prevention of apoptosis, leading to a decrease in fibrosis, a strategy that may contribute to the prevention and treatment of pulmonary fibrotic diseases.

While apoptosis of the infiltrating cells and proliferating resident cells participate in the resolution of acute inflammation, failure to clear unwanted cells by apoptosis may prolong the inflammatory response (24–28). This prolonged and persistent inflammation may then lead to excessive apoptosis of resident epithelial cells, leading to the development of fibrosis (24–28). Recent studies have suggested that apoptosis of the alveolar epithelium via the Fas/FasL pathway may be an important determinant in the pathogenesis of pulmonary fibrosis in diseases such as IPF and in acute lung injury such as ARDS (29–34, 49, 50). In patients with acute lung injury, Fas and FasL were coexpressed on alveolar epithelial cells, on infiltrating inflammatory cells, and on sloughed epithelial cells in the alveolar spaces of patients who died (25–28, 51, 52). In patients with IPF, the expression of Fas and Fasl signaling molecules including FAS-associated death domain (FADD), a signal transducer downstream of Fas, as well as caspase-1 and caspase-3, were up-regulated in bronchiolar and alveolar epithelial cells, and FasL was expressed in the infiltrating cells (30, 53–55). Additionally, sFas and sFasL were found in the BAL fluid of patients with ARDS, IPF, and collagen vascular disease (30, 32, 36, 51, 52, 56). Significantly higher levels of soluble Fas and FasL were observed in ARDS patients with worse clinical outcomes and in those who died (32), suggesting that sFasL and sFasl released in the airspaces of patients with acute lung injury leads to activation of the Fas/FasL pathway, which contributes to the severe epithelial damage that occurs in ARDS (32, 51). In support of this hypothesis, the concentration of sFasL found in the BAL fluid of ARDS patients was sufficient to induce Fas-dependent apoptosis of primary cultures of human distal lung epithelial cells (32, 57), suggesting that sFasL is released in vivo during human disease as a “biologically active, death-inducing effector molecule capable of inducing apoptosis in Fas-susceptible target cells of the lungs” (32). In contrast, sFasl has also been found to exert an antiapoptotic effect by competing with membrane-bound Fasl for binding to Fas (58). Patients with BOOP expressed both sFas and sFasL (36, 37), suggesting that perhaps elevated levels of sFas may abrogate the cytotoxicity of sFasL in BOOP patients who respond better to therapy (36). These observations have led to the suggestion that an additional cofactor or other molecule found in the BAL fluid may be required for the effect of sFasL (36).

Apoptosis and Fas/FasL pathway expression have also been described in animal models of pulmonary fibrosis, including bleomycin-induced fibrosis (29–31, 59–61). While up-regulation of Fas and FasL and excessive apoptosis of bronchiolar and alveolar epithelial cells were demonstrated after bleomycin instillation, the role of the Fas/FasL pathway remains controversial. While it has been reported that administration of the soluble form of Fas or anti-Fasl Ab prevented the development of bleomycin-induced fibrosis (29, 30), suggesting that the Fas/FasL pathway is not a prerequisite for the development of bleomycin-induced fibrosis, the results of bleomycin-induced fibrosis as were wild-type mice (60), suggesting an essential role for the Fas/FasL pathway in the development of pulmonary fibrosis (29, 30). However, another study demonstrated that lpr and gld mice were just as susceptible to bleomycin-induced fibrosis as were wild-type mice (60), suggesting that the Fas/FasL pathway is not a prerequisite for the development of bleomycin-induced fibrosis. While a clear reason for this discrepancy was not given, it was suggested that the differences in the strain of mice used (C3H vs C57BL/6) as well as the timing of the analysis of apoptosis could be responsible for this discrepancy (29, 30, 60). In models of acute lung injury including Ig deposition, successive exposure to hemorrhage shock and cecal ligation and puncture (CLP), septic shock, and cadmium-induced lung injury, Fas was up-regulated in alveolar and inflammatory cells, Fasl-positive inflammatory cells were present in the airspaces, intratracheal administration of an anti-Fas blocking Ab attenuated lung injury, and measures of lung injury parameters were found to be reduced in both lpr and gld mice (33, 34, 62–64). Therefore, strategies to inhibit Fas-mediated epithelial apoptosis may preserve epithelial function in patients who develop acute lung injury. A recent report investigated the role of apoptotic cell suicide in lung tissues of mice with CLP-induced polymicrobial
of mice, which express a spontaneous mutation, similar critical role of epithelial cell apoptosis via activation of the Fas/FasL pathway has been also described in a model of hyperoxia-induced acute lung injury in Pneumocystis carinii-infected mice (66). These observations support the hypothesis that apoptosis of alveolar epithelial cells potentially through the Fas/FasL pathway is involved in the pathophysiology of acute lung injury and pulmonary fibrosis.

To directly investigate the role of the Fas/FasL pathway in the models of reovirus 1/L-induced BOOP and ARDS developed in our laboratory (19–23), we used the CBA/KlJms-lprcg/Faslpr-cg (38). Unlike the lpr strain, lprζ mice express full-length Fas mRNA as abundantly as do wild-type mice, but due to a point mutation in the Fas cytoplasmic region, Fas is unable to transduce an apoptotic signal (38). In the reovirus 1/L model system, we have clearly demonstrated that in both reovirus 1/L-induced ARDS and BOOP, (1) apoptosis of the alveolar epithelium is evident via TUNEL analysis; (2) Fas and FasL are up-regulated in situ in alveolar epithelium and in cells of the inflammatory infiltrate; (3) sFasL is found in the BAL fluid; (4) treatment of mice with zIETD-fmk (caspase-8 inhibitor) is more effective at attenuating inflammation and fibrosis than is treatment with the pan-caspase inhibitor zVAD-fmk; and (5) Fas-deficient animals are resistant to reovirus 1/L-induced BOOP but are still susceptible to reovirus 1/L-induced ARDS. These data would support a direct role for the Fas/FasL pathway in the development of fibrotic lesions associated with reovirus 1/L-induced BOOP but not reovirus 1/L-induced ARDS. These data are consistent with our finding that unlike reovirus 1/L-induced ARDS, reovirus 1/L-induced BOOP is dependent on the presence of T cells (22, 23), suggesting that the predominant infiltration of T cells that express Fas in reovirus 1/L-induced BOOP is responsible for damage to Fas-expressing alveolar epithelial cells, leading to fibrosis. To support this conclusion, we have previously demonstrated in reovirus 1/L-induced BOOP that depletion of either CD4+ or CD8+ T cells before reovirus 1/L infection inhibited fibrotic lesion development (23), and neonatally thymectomized CBA/J mice, which lack mature peripheral T cells, when infected with 1 × 10^7 PFU reovirus 1/L (BOOP) do not develop intraalveolar fibrosis associated with BOOP (22). In contrast, thymectomized CBA/J mice when infected with 1 × 10^7 PFU reovirus 1/L (ARDS) still develop all of the histological features of ARDS, including fibrosis (22). Therefore, excessive Fas/FasL interaction mediated through the interaction of infiltrating T cells with alveolar epithelial cells may directly lead to pulmonary fibrosis in reovirus 1/L-induced BOOP. This is supported by other studies demonstrating that intratracheal administration of FAs-activating Ab mimicking Fas/FasL, cross-linking caused alveolar epithelial apoptosis and pulmonary fibrosis, which was diminished in lpr mice (67, 68), and selective inactivation of Fas in T cells caused massive leukocyte infiltration in the lungs together with increased inflammatory cytokine production and pulmonary fibrosis resembling idiopathic pulmonary fibrosis in humans (69). Additionally, chimeric mice lacking Fas in either myeloid cells (macrophages) or nonmyeloid cells (epithelial cells) demonstrated that only animals expressing Fas in nonmyeloid cells showed significant increases in lung injury parameters, suggesting that Fas-mediated lung injury requires the expression of Fas on nonmyeloid cells of the lung (70, 71). Since reovirus 1/L can infect a variety of cell types, including epithelial cells, it was essential to determine whether epithelial cell apoptosis induced by reovirus 1/L infection contributed to the pathophysiology of reovirus 1/L-induced BOOP or ARDS. In these experiments, we did not observe significant TUNEL activity at the height of reovirus 1/L replication in vivo (days 1–3) (Fig. 1, data not shown), suggesting that epithelial cell apoptosis induced by reovirus 1/L is limited (19, 20). These data are consistent with published data on apoptosis induced by reovirus 1/L infection. Reovirus 1/L infection occurs following receptor-mediated endocytosis after the virions bind to cell surface molecules, including junctional adhesion molecule 1 (JAM1), and to other uncharacterized cell surface carbohydrate moieties, but not to sialic acid (72). Reovirus strains differ in their capacity to induce apoptosis, and the reovirus T3 prototype strain Dearing (T3D) induces apoptosis much more efficiently both in vivo and in vitro than does reovirus 1/L, since virion binding to both junctional adhesion molecule 1 and sialic acid is essential for optimal expression of apoptosis in infected cells that does not occur with reovirus 1/L (72). Additionally, it has been demonstrated that the ability of reovirus strains to infect and propagate in tissue culture cells does not correlate with its capacity to induce apoptosis (72). These observations suggest that direct apoptosis of alveolar epithelial cells due to infection with reovirus 1/L is minimal. Therefore, most apoptosis observed in both reovirus 1/L-induced ARDS and BOOP is mediated through the interaction of Fas-L expressing infiltrating cells with Fas-expressing alveolar epithelial cells or through other apoptotic pathways.

While the Fas/FasL pathway is up-regulated in reovirus 1/L-induced ARDS, it may represent only one potential mechanism leading to alveolar epithelial cell damage and eventual fibrosis in reovirus 1/L-induced ARDS. This hypothesis is consistent with our observation that CBA/KIImms-Faslpr-cg/J mice, which lack a functional Fas protein, when inoculated with 1 × 10^7 PFU reovirus 1/L still develop ARDS. We also demonstrate that the caspase-8 inhibitor zIETD-fmk, but not the caspase-3 inhibitor zVAD-fmk, significantly attenuated inflammation and fibrosis in both reovirus 1/L-induced ARDS and BOOP. This was evident through both a reduction in histopathology and in lung HP content as a measure of fibrosis. However, note that the caspase-8 inhibitor zIETD-fmk was more effective at reducing fibrosis in BOOP than in ARDS. While zIETD-fmk significantly reduced fibrosis as measured by HP content as compared with untreated reovirus 1/L-induced ARDS, the HP content in zIETD-fmk reovirus 1/L-induced ARDS was still significantly different than in control, saline-inoculated lungs. This observation is also consistent with a greater reduction in apoptotic cells in reovirus 1/L-induced BOOP after treatment with zIETD-fmk as compared with reovirus 1/L-induced ARDS. In fact, the numbers of TUNEL-positive cells in zIETD-fmk-treated reovirus 1/L-induced ARDS are greater than the numbers of TUNEL-positive cells in untreated reovirus 1/L-induced BOOP. These data, in concert with the differential response in CBA/KIImms-Faslpr-cg/J to reovirus 1/L-induced BOOP vs ARDS, suggest an essential role for the Fas/FasL pathway in reovirus 1/L-induced BOOP but not in reovirus 1/L-induced ARDS.

Multiple reports have demonstrated that administration of the angiotensin-converting enzyme inhibitor captopril, which inhibits Fas-induced apoptosis in vitro, or the caspase inhibitor zVAD-fmk inhibited apoptosis and prevented fibrotic lesion development in bleomycin-treated animals (42, 43, 59, 73) or in sepsis-induced acute lung injury (41). However, it has been suggested that a high degree of caspase-3 inhibition may be necessary to completely block apoptotic cell death in a CLP-induced sepsis model system (74). Therefore, high and persistent levels of caspase inhibition may be needed clinically, and zVAD-fmk may be a low-potency caspase-3 inhibitor (74). While treatment of reovirus 1/L-induced...
ARDS and BOOP with zVAD-fmk inhibited caspase activation, as demonstrated by a decrease in the amount of cleaved caspase-3 in treated animals, we did not observe a significant reduction in inflammation or fibrosis in either model. Perhaps the need in our model for persistent and complete caspase blockage was not achieved and that the use of a more potent caspase-3 inhibitor such as M687 may prove effective (74). In a model of liver injury, IETD-CHO, a caspase-8 inhibitor, effectively prevented hepatocellular apoptosis, hemorrhage, and liver failure after Fas receptor activation by inhibiting not only caspase-8 activation but cytochrome c release by inhibiting the activation of caspase-3 and caspase-9 (39, 40). These authors suggested that Fas may rely solely on caspase-8 activation and the mitochondria to activate caspase-3, which can process more procaspase-8 and thus propagate the amplification of the apoptotic signal (39, 40). Additionally, it was recently reported that silencing of Fas but not caspase-8 in lung epithelial cells ameliorates pulmonary apoptosis, inflammation, and neutrophil influx after hemorrhage shock and sepsis, an indirect pathway to acute lung injury, suggesting a pathophysiological role for Fas activation in nonpulmonary shock-induced acute lung injury (75). These data are not contradictory to what we present herein on reovirus 1/L-induced ARDS. In our model system i.n. inoculation of reovirus 1/L is a direct model of acute lung injury as opposed to an indirect lung injury such as that caused by extrapolunmonary insult included in some models of sepsis (75), and therefore the role of the Fas/FasL pathway may not be as significant in reovirus 1/L-induced ARDS. Therefore, other alternative or redundant pathways that also lead to apoptosis may be involved in the histopathological development of ARDS in addition to the Fas/FasL pathway.

Alternative pathways, which lead to apoptosis induction or an imbalance between apoptosis-inducible and inhibitory genes, have also been suggested to be activated in bleomycin-induced fibrosis and other models of acute lung injury (31, 76–78). These include the induction of proapoptotic cytokines such as TNF-α and TGFβ (62, 63, 77, 78). TGFβ1 and TNF-α act as enhancers of Fas-mediated apoptosis of lung epithelial cells (79–82). We have also demonstrated that TGFβ1 is up-regulated in both reovirus 1/L-induced ARDS and BOOP and that neonatally thymectomized mice that develop ARDS after reovirus 1/L inoculation express significant levels of TGFβ1 and TNF-α (21–23), suggesting that apoptotic pathways regulated by TGFβ1 and TNF-α may be activated in reovirus 1/L-induced ARDS. The proapoptotic Bcl-2 family member Bid was also shown to be required for the development of pulmonary fibrosis in bleomycin where mice lacking Bid exhibited significantly less pulmonary fibrosis despite similar levels of TGFβ in BAL fluid (76). The authors suggested that TGFβ overexpression in the lung leads to epithelial cell apoptosis and tissue fibrosis (77) through a Bax-dependent Bid-activated pathway involving matrix metalloproteinase-12 (78). Furthermore, Bak, a member of the Bcl-2 family, has recently been identified as a proapoptotic factor in the TNF-α-induced apoptotic pathway (TRAIL) in caspase 3-deficient cells, demonstrating a caspase 3-independent function of Bak in the TNF-α-induced apoptotic pathway (83). These data are consistent with our observation that while CBA/KJms-Faslpr-cg/J mice, which lack a functional Fas protein, when inoculated with 1 × 10^7 PFU reovirus 1/L develop ARDS, a significant attenuation of fibrosis is observed in reovirus 1/L-induced ARDS after treatment with the caspase-8 inhibitor zIETD-fmk. These results suggest that pathways other than the Fas/FasL pathway, which can activate caspase-8 including TRAIL apoptotic pathways or other pathways of FADD activation, such as activation of the TNF receptor I (p55), might also be responsible for the differential results observed in reovirus 1/L-induced ARDS in zVAD-fmk-treated mice vs CBA/KJms-Faslpr-cg/J mice. In support of this assumption, in ARDS patients, TRAIL levels in the BAL fluid were correlated with clinical severity and the presence of neutrophils, which are also the major infiltrating cells in reovirus 1/L-induced ARDS (20, 84). Finally, the death of epithelial cells and other cells in the lungs in acute lung injury can occur not only by regulated (apoptosis) but also in conjunction with non-regulated mechanisms of cell death, including cell necrosis (25, 85–87). In fact, the broad-spectrum caspase inhibitor zVAD-fmk has also been demonstrated to modulate the major types of cell death, where the addition of zVAD-fmk blocks apoptotic cell death, sensitizes cells to necrotic cell death, and induces autophagic cell death (88). Therefore, the inability of zVAD-fmk to inhibit the development of either reovirus 1/L-induced ARDS or BOOP may be due to the activation or enhancement of these other pathways of cell death. We are currently exploring these and other additional apoptotic pathways in the multifactorial disease process associated with reovirus 1/L-induced ARDS.

In conclusion, our data strongly support the hypothesis that apoptosis of alveolar epithelial cells potentially through the Fas/FasL pathway is involved in the pathophysiology of pulmonary fibrosis associated with reovirus 1/L-induced BOOP. Apoptosis occurring during the regeneration phase of the diseases may help explain the development and persistence of fibrosis in these model systems. While blockage of the Fas/FasL apoptotic pathway may be a useful therapeutic in the treatment of reovirus 1/L-induced BOOP, it may be less important in reovirus 1/L-induced ARDS, where the involvement of multiple apoptotic pathways may be significant. These data suggest that while the Fas/FasL pathway may play a role in eliminating injured epithelial cells that need to be replaced by epithelial renewal, the severity of lung injury may determine whether epithelial renewal is overshadowed by a fibrotic response. Therefore, the underlying insult leading to the pathophysiology of acute lung injury and fibrosis will be important in determining the pulmonary fibrotic response and its response to therapeutic interventions.

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References


gation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. Am. J. Physiol. 279: L143–L151.


gation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. Am. J. Physiol. 279: L143–L151.


gation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. Am. J. Physiol. 279: L143–L151.


