Silencing of Fas, but Not Caspase-8, in Lung Epithelial Cells Ameliorates Pulmonary Apoptosis, Inflammation, and Neutrophil Influx after Hemorrhagic Shock and Sepsis

Mario Perl,* Chun-Shiang Chung,*
Joanne Lomas-Neira,[†] Tina-Marie Rachel,*
Walter L. Biffl,[‡] William G. Cioffi,[‡] and
Alfred Ayala*

From the Shock-Trauma Research Laboratories,* Division of Surgical Research, and the Department of Surgery,[‡] Rhode Island Hospital and Brown University School of Medicine, Providence; and the Department of Cell and Molecular Biology,[†] University of Rhode Island, Kingston, Rhode Island

Apoptosis and inflammation play an important role in the pathogenesis of direct/pulmonary acute lung injury (ALI). However, the role of the Fas receptor-driven apoptotic pathway in indirect/nonpulmonary ALI is virtually unstudied. We hypothesized that if Fas or caspase-8 plays a role in the induction of indirect ALI, their local silencing using small interfering RNA (siRNA) should be protective in hemorrhage-induced septic ALI. Initially, as a proof of principle, green fluorescent protein-siRNA was administered intratracheally into transgenic mice overexpressing green fluorescent protein. Twenty-four hours after siRNA delivery, lung sections revealed a significant decrease in green fluorescence. Intratracheally administered Cy-5-labeled Fas-siRNA localized primarily in pulmonary epithelial cells. Intratracheal instillation of siRNA did not induce lung inflammation via toll-like receptor or protein kinase PKR pathways as assessed by lung tissue interferon- α , tumor necrosis factor- α , and interleukin (IL)-6 levels. Mice subjected to hemorrhagic shock and sepsis received either Fas, caspase-8-, or control-siRNA intratracheally 4 hours after hemorrhage. Fas- or caspase-8-siRNA significantly reduced lung tissue Fas or caspase-8 mRNA, respectively. Only Fas-siRNA markedly diminished lung tissue tumor necrosis factor- α , IL-6, IL-10, interferon- γ , IL-12, and caspase-3 activity. Fas-siRNA also preserved alveolar architecture and reduced lung neutrophil infiltration and pulmonary epithelial

apoptosis. These data indicate the pathophysiological significance of Fas activation in nonpulmonary/shock-induced ALI and the feasibility of intrapulmonary administration of anti-apoptotic siRNA *in vivo*. (Am J Pathol 2005, 167:1545–1559)

Acute lung injury (ALI) can be differentiated into direct ALI that evolves from direct injury to the lung, caused by pneumonia, aspiration, and lung trauma, or indirect ALI that evolves from extrapulmonary injury, caused by nonpulmonary sepsis and trauma (ie, hemorrhagic shock, fractures, and so forth).¹ So far, a substantial role for primed neutrophils in inducing indirect ALI has been indicated. Activation of neutrophils by inflammatory stimuli delays apoptosis^{2,3} and enhances their respiratory burst capacity.³ Adoptive transfer of primed neutrophils induces ALI in a septic environment³ and prevention of neutrophil priming or their immigration into the lung attenuates ALI.^{4,5}

In contrast, the process of programmed cell death in the lung has been suggested to be a major pathogenetic factor in models of direct ALI^{6,7} as well as in acute respiratory distress syndrome.^{8,9} After lipopolysaccharide-induced ALI in mice, epithelial cells display an augmented expression of Fas along with an increased migration of Fas ligand (FasL)-expressing inflammatory cells into the alveolar space.⁶ In patients, acute respiratory distress syndrome is associated with increased levels of FasL in the bronchoalveolar lavage fluid inducing

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Address reprint requests to Alfred Ayala, Ph.D., Department of Surgery, Division of Surgical Research, Rhode Island Hospital and Brown University, 593 Eddy St., Aldrich 239, Providence, RI 02903. E-mail: aayala@lifespan.org.

apoptosis in distal lung epithelial cells via the activation of the death receptor pathway.¹⁰ Similarly, high doses of Fas-activating antibody have been found to induce lung epithelial apoptosis in mice.¹¹ Interestingly, modulation of the Fas/FasL systems not only affects lung apoptosis but also results in alteration of local pulmonary inflammation.^{6,11,12}

However, so far the role of the extrinsic death (Fas) receptor pathway in mediating apoptosis and inflammation in ALI caused by extrapulmonary injury remains unclear. To address this question we attempted to shed light on the contribution of gene products of the extrinsic death (Fas) receptor pathway to the pathology of shock-induced septic ALI by using organ-specific gene knockdown. In this regard, RNA interference has become a powerful tool in selectively silencing mammalian genes. The relatively small amounts required for their gene-specific silencing abilities, as well as the longevity of silencing, are promising characteristics associated with potential *in vivo* siRNA administration. Nonetheless, there have been only a limited number of reports on siRNA administration *in vivo*. In these studies, intravenous administration of caspase-8-siRNA prevented acute liver failure in mice¹³ and RNA interference targeting Fas was protective in a murine model of fulminant hepatitis.¹⁴ In addition, *in vivo* delivery of Fas- or caspase-8-siRNA improves survival in septic mice.¹⁵ However, these approaches are not organ-specific and either require viral preparations^{13,16,17} or use a high-volume fluid injection.^{14,15,18} Thus, more localized and thereby in part organ-specific approaches have evolved, including intraperitoneal¹⁹ and intra-alveolar delivery.^{5,20,21} However, these studies 1) did not evaluate siRNA targeting of a potentially therapeutic protein,²⁰ 2) were performed as a pretreatment scenario,^{19,21} or 3) did not sufficiently address the question of which cells were the target of the administered siRNA.^{5,20,21} Thus, the feasibility of local gene silencing in the lung in a critical condition still remains to be demonstrated.

In this context, we tested the hypothesis that postshock pulmonary administration of Fas- and caspase-8-siRNA attenuates apoptosis and inflammation in indirect ALI after hemorrhagic shock and sepsis. Our data will show that organ-specific delivery of siRNA into the lung is a feasible approach. It can be performed as a posttreatment after the onset of hemorrhagic shock and is not associated with the induction of toll-like receptor or protein kinase PKR-mediated inflammation. We will demonstrate that pulmonary epithelial cells are a main target for siRNA delivered into the lung. In addition, down-regulation of Fas but not caspase-8 in these cells reduces pulmonary apoptosis and lung inflammation, decreases pulmonary neutrophil influx, and attenuates ALI, indicating the pathophysiological relevance of the Fas receptor pathway in indirect ALI.

Materials and Methods

Animals

Male transgenic C57BL/6-TgN(ACTbEGFP)1Osb mice (Jackson Laboratories, Bar Harbor, ME) expressing

green fluorescent protein (GFP) and inbred male C3H/HeN mice (Charles River, Wilmington, MA), 8 to 9 weeks old, were used. C3H/HeN mice were used in all experiments except for the initial proof of principle experiments due to our previous experience with this strain in the pathogenesis of ALI after hemorrhagic shock and sepsis.³⁻⁵ C57BL/6-TgN(ACTbEGFP)1Osb mice were used for proof of principle experiments with GFP-siRNA because mice ubiquitously expressing GFP-protein were not available on the C3H/HeN background. Animals were allowed free access to water and food before and after procedures. A day-night cycle of 12 hours was applied. Experiments described in this article were performed in accordance with National Institutes of Health guidelines and with approval from the Animal Use Committee of Rhode Island Hospital. C57BL/6-TgN(ACTbEGFP)1Osb mice were subjected to intratracheal delivery of siRNA targeting GFP mRNA (Dharmacon, Lafayette, CO), C3H/HeN mice were challenged with hemorrhagic shock, intratracheal delivery of siRNA (see below), and cecal ligation and puncture.

Animal Preparation

The hemorrhage model we have used for these experiments has been previously described.³ In brief, C3H/HeN mice were anesthetized with isoflurane, restrained in supine position, and catheters were inserted into both femoral arteries. Anesthesia was discontinued and blood pressure was continuously monitored through one catheter attached to a blood pressure analyzer (MicroMed, Louisville, KY). When fully awake, as determined by a mean blood pressure of ~110 mmHg, the mice were bled throughout a 5- to 10-minute period to a mean blood pressure of 30 mmHg (± 5 mmHg) and were kept stable for 90 minutes. Immediately after hemorrhage, mice were resuscitated (intravenously) with Ringer's lactate at four times the drawn blood volume. The catheters were then removed, the vessels were ligated, and the incisions were closed. Sham-operated controls (sham) had their femoral arteries ligated and were restrained for periods of time equal to their hemorrhaged counterparts, but no catheters were inserted and no blood was shed.

Intratracheal delivery of agents was performed as described previously.^{5,22} Four hours after resuscitation mice were anesthetized lightly with isoflurane and restrained in supine position with their head reclined. The tongue of the animals was pulled out gently to prevent swallow reflex and siRNA or placebo was administered in the oral cavity. Mice were maintained in the described position until siRNA or placebo was aspirated (usually between 5 and 10 seconds).

Polymicrobial sepsis was induced in mice by the method described previously.³ Mice were anesthetized with isoflurane, shaved at the abdomen, and scrubbed with betadine. A midline incision (~1 cm) was made below the diaphragm to expose the cecum. The cecum was ligated and punctured twice with a 22-gauge needle and gently compressed to extrude a small amount of cecal contents through the punctured holes. The cecum

was returned to the abdomen, and the incision was closed in layers with 6-0 Ethilon suture (Ethicon, Somerville, NJ). The animals were then resuscitated with 0.8 ml of lactated Ringer's solution by subcutaneous injection. This procedure usually results in ~65% mortality of the mice throughout the period of 10 days, however does not induce significant lung injury as described previously, if not preceded by hemorrhagic shock.³⁻⁵

Experimental Groups

First we evaluated, as a proof of principle, whether intratracheal delivery of siRNA was a feasible therapeutic approach and which cell type was the *in vivo* target of this delivery method. One hundred μg of GFP-siRNA in 100 μl of phosphate-buffered saline (PBS) or PBS only were administered intratracheally into male transgenic C57BL/6-TgN(ACTbEGFP)1Osb mice ($n = 3/\text{group}$). Lungs, livers, and spleens were harvested 24 hours thereafter for morphological assessment and quantification of fluorescence intensity on frozen sections. In addition, male C3H/HeN mice ($n = 3/\text{group}$) received 100 μg of Fas-siRNA labeled with a Cy5-fluorochrome in 100 μl of PBS or PBS only. The lungs of these animals were harvested 24 hours thereafter for immunohistochemistry on frozen lung sections.

To shed light on possible proinflammatory properties of *in vivo* siRNA delivery a separate set of male C3H/HeN mice were randomized into four groups ($n = 3$ to 4 each). One hundred μg of polyinosinic-polycytidylic acid sodium salt [poly(I:C)] (Sigma, St. Louis, MO) in 100 μl of PBS as a positive control, 100 μg of GFP-siRNA in 100 μl of PBS, 100 μg of caspase-8-siRNA in 100 μl of PBS, or 100 μl of PBS were administered intratracheally. For these experiments, caspase-8-siRNA was chosen over Fas-siRNA because the latter had revealed anti-inflammatory properties in preliminary experiments that could have acted as a potential confounder in the experiments. Lung, liver, and plasma were harvested 18 hours after instillation and interferon (IFN)- α , tumor necrosis factor (TNF)- α , and interleukin (IL)-6 were quantified in tissue homogenates and plasma. So far it remains unclear through which pathways short-interfering RNA signal. Thus, IFN- α was chosen as an inflammatory readout, because it is produced on activation of TLR-3²³ [recognizing double-stranded RNA and poly(I:C)] and TLR-7²⁴ [single-stranded (ss) RNA and siRNA] via IRF translocation. Alternatively, TNF- α and IL-6 were quantified because activation of TLR-3, TLR-8 (ssRNA), and TLR-9 [cytidine-phosphate-guanoside(cpg)DNA] or induction of protein kinase PKR pathways promotes genes controlled by the nuclear factor (NF)- κB transcriptional regulator.^{25,26}

Finally, we examined whether intratracheal delivery of Fas- or caspase-8-siRNA would result in an attenuation of shock/sepsis-induced pulmonary inflammation, a decrease in lung apoptosis and neutrophil influx, and/or the protection of lung histology. To do this, male C3H/HeN mice were randomized into four groups ($n = 18$ each), subjected to hemorrhagic shock, treated with 75 μg of

either Fas-, caspase-8-, or GFP(control)-siRNA in 100 μl of PBS or 100 μl of PBS, intratracheally, 4 hours thereafter and then challenged with polymicrobial sepsis 24 hours thereafter. Lung, liver, and plasma were harvested from mice 24 hours after induction of sepsis for quantification of cytokine levels, polymerase chain reaction ($n = 8/\text{group}$), determination of apoptosis ($n = 6/\text{group}$), or histological assessment of pulmonary architecture and neutrophil immigration ($n = 4/\text{group}$). The following inflammatory mediators were chosen based on our previous studies⁵ to provide some index of the nature of the inflammatory and chemotactic milieu in the lung:^{3,4} TNF- α , IL-6, IL-12, and IFN- γ were monitored as indicators of the early proinflammatory reaction, MCP-1, KC, and MIP-2 as representative chemotactic agents, and IL-10 to assess a co-existent anti-inflammatory response. Hemorrhagic shock followed by sepsis was chosen as a model of indirect/nonpulmonary-induced ALI because previous results have identified that it is potent in inducing ALI, which is at least in part a result of primed neutrophils.³⁻⁵ As opposed to cecal ligation and puncture alone, which by itself induces pulmonary inflammation only the combination of hemorrhagic shock and polymicrobial sepsis leads to pulmonary inflammation plus increased lung apoptosis, significant neutrophil apoptosis, and gross pathohistological lung alterations under the described protocol. This does not exclude that if the severity of the polymicrobial sepsis was increased or a different mouse strain was used, cecal ligation and puncture alone could not lead to such alterations per se.

Lung Fluorescent Intensity Measurements

C57BL/6-TgN(ACTbEGFP)1Osb mice, overexpressing GFP, were harvested 24 hours after intratracheal administration of GFP-siRNA. Frozen lung, liver, and spleen samples were cut into 6- μm sections at -20°C and fixed in acetone (Sigma). Tissue sections were initially incubated with 0.01% Sudan Black B (MT Biomedicals, Aurora, CO) in 70% ethanol (Sigma) for 5 minutes to reduce tissue autofluorescence. Lung, liver, and spleen slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing 6-diamidine-2'-phenylindole dihydrochloride (DAPI) for fluorescence nuclear counterstaining. A Nikon E800 microscope (Nikon Inc., Melville, NY) and a $\times 40$ Plan Apo objective were used to visually examine slides for intensity and camera settings were established based on the brightest specimen. All images were acquired at these same settings. From each lung, liver, and spleen slide of C57BL/6-TgN-GFP mice 10 randomly selected grayscale images (12 bit) were captured with a Spot II digital camera (Diagnostic Instruments, Sterling Heights, MI) using the camera's built-in green filter. Images were thresholded and analyzed for SUM intensity, defined as the sum of all pixel values within a region and MEAN intensity, defined as SUM intensity divided by the number of pixels in the region and SD of fluorescent intensity using IPLab 3.6.4 (Scanalytics Inc., Fairfax, VA). Sectioning and staining were performed by a single blinded person and

a second single blinded observer performed digital imaging and analysis. Both individuals were from the R.I. Hospital Core Research Laboratories and are not co-authors.

Lung Cytokeratin-18 and CD115 Staining

Because the cellular target of siRNA, delivered by intra-tracheal instillation, has not been established so far, we attempted to follow the uptake of this agent by dual-immunofluorescence assessment. To do this, frozen lung tissue from C3H/HeN animals having received Cy-5 labeled Fas-siRNA were cut into 6- μ m sections, fixed in acetone, and incubated with a monoclonal antibody against the epithelial cell marker cytokeratin-18 (Chemicon, Temecula, CA) or a monoclonal antibody against the macrophage CSF-1 receptor CD115 (eBioscience, San Diego, CA) for 60 minutes at a concentration of 1:100 or PBS as a negative control. After washing, a biotin-conjugated secondary goat anti-mouse (Chemicon) or mouse anti-rat antibody (BD Pharmingen, San Diego, CA), respectively, were applied at a concentration of 1:300 for 45 minutes. After a second washing step lung slides were incubated with Alexa Fluor Streptavidin Conjugate 488 (Molecular Probes, Eugene, OR) at a concentration of 1:200 for 45 minutes. After an additional washing step, slides were mounted with Vectashield mounting medium containing DAPI for fluorescence nuclear counterstaining. Specimens were fastidiously protected from light during the entire staining and postprocessing period of assessment. Cy-5 fluorescence was detected by obtaining random pictures. When Cy-5 fluorescence was detected, a picture from the exact same area was taken imaging Alexa Fluor 488 fluorescence. Subsequently, the channels were merged to identify double-stained structures. Sectioning, staining, and analysis were again performed by blinded external observers as described above.

Lung Staining of Neutrophil-Specific Myeloperoxidase (MPO) and Lung Hematoxylin and Eosin (H&E) Staining

For fluorescent staining of MPO in frozen lung-tissue sections tissue slides were fixed in 10% formaldehyde (Sigma) blocked with PBS plus 5% normal rabbit serum (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at 37°C and then slides were incubated with purified goat polyclonal antibody against a MPO peptide (MPO L-20:SC-16129, Santa Cruz Biotechnology) for 1 hour at room temperature. Slides were rinsed and incubated with a biotinylated rabbit anti-goat immunoglobulin G2b antibody (Santa Cruz Biotechnology) for 30 minutes at room temperature and then rinsed. Slides were then incubated with a streptavidin Alexa Fluor 594 conjugate (Molecular Probes) for 45 minutes, rinsed, and compared with negative-control slides (without primary). Slides were then coverslipped with a mounting medium for fluorescence microscopy with DAPI (Vector Laboratories), a DNA stain.

To establish the total number of cells per field that were neutrophils (MPO+) present in the sample, tissue sections were randomly screened (five fields/slide) at $\times 400$ (25 μ m²/field). The number of MPO-positive cells (Alexa Fluor 594) was divided by the number of DAPI-positive cells and then multiplied times 100 to derive the percentage of MPO-positive cells. H&E tissue slides were histopathologically analyzed and graded according to the following criteria: distension of alveoli, thickening of alveolar septa, perivascular and peribronchial edema, and intra-alveolar cellular infiltrates. Staining and analysis of MPO and H&E sections were again performed by blinded observers.

Sequences of siRNA

Fas-siRNA (sense sequence: 5'-P.GUGCAAGUGCAAACCAGACdTdT-3'; anti-sense sequence: 5'-P.GUCUGGUUUGCACUUGCACdTdT-3'; target sequence: 5'-AAGUGCAAGUGCAAACCAGAC-3'),¹⁴ Cy-5-labeled Fas-siRNA (sense sequence: Cy5-5'-P.GUGCAAGUGCAAACCAGACdTdT-3'; anti-sense sequence: 5'-P.GUCUGGUUUGCACUUGCACdTdT-3'; target sequence: 5'-AAGUGCAAGUGCAAACCAGAC-3'), caspase-8-siRNA (sense sequence: 5'-CCUCGGGGAUACUGUCUGAdTdT-3'; anti-sense sequence: 5'-P.UCAG ACAGUAUCCCCGAGGdTdT-3'; target sequence: AACCUCGGGGAUACUGUCUGA-3'),¹³ and GFP-siRNA (sense sequence: 5'-P.GGCUACGUCCAGAGCGCACCdTdT-3'; anti-sense sequence: 5'-P.UGCGCUCCUGGACGUAGCCUU-3'; target sequence: 5'-GGC UACGUCCAGGAGCGCACC-3')²⁷ were assembled, deprotected, duplexed, and desalted by Dharmacon.

Primer Sequences

Primer sequences for Fas (forward: 5'-GCTGCAGACATGCTGTGGATC-3'; reverse: 5'-TCACAGCCAGGAGAA-TCGAG-3'), caspase-8 (forward: 5'-TGCCCTCAAGTTCCTGTGCTTGGA-3'; reverse: 5'-GGATGCTAAGAATGTCATCTCC-3'), and GAPDH (forward: 5'-TGCA TCC-TGCACCACCAACT-3'; reverse: 5'-AACACGGAAGGC-CATGCCAG-3') were assembled by IDT, Coralville, IA.

Protein Assay

The total amount of protein was quantified using the Bradford dye binding procedure. Briefly, dye reagent concentrate (Bio-Rad, Hercules, CA) was diluted 1:5 with distilled water, and 10 μ l of sample (diluted 1:30 in distilled water) were added to 190 μ l of diluted dye reagent per well. The color was allowed to develop for 3 to 5 minutes and absorbance was read at 595 nm. Determination of protein concentration was assessed by serial dilution of a standard solution (bovine serum albumin) and plotting of a standard curve.

IFN- α and Cytokine Enzyme-Linked Immunosorbent Assays (ELISAs)

IFN- α ELISA (Antigenix America, Huntington Station, NY) on plasma, lung, and liver homogenates, was performed according to the manufacturer's recommendation. IL-6 (BD OptEIA Elisa Set, BD Pharmingen) and TNF- α (BD Pharmingen) ELISA were performed on plasma, lung, and liver tissue homogenates. Mouse KC and MIP-2 were performed on plasma, lung, and liver tissue homogenates using commercially available murine anti-cytokine antibody pairs (R&D Systems, Minneapolis, MN) as well as cytokine standards (R&D Systems) as per the manufacturer's protocol for basic sandwich ELISA. Protein assays were performed, as described above, on tissue homogenates before cytokine ELISAs. Cytokine concentration was calculated as pg per mg of protein for each sample.

Cytometric Bead Array

Mouse TNF- α , IL-6, IFN- γ , IL-12, MCP-1, and IL-10 levels were determined in plasma, lung, and liver tissue homogenates using cytometric bead array technique (BD Cytometric Bead Array Mouse Inflammation Kit; BD Biosciences, San Diego, CA) according to the manufacturer's instruction. In brief, 50 μ l of mouse inflammation capture bead suspension and 50 μ l of PE detection reagent were added to the equal amount of sample or standard dilution and incubated for 2 hours at room temperature in the dark. Subsequently, samples were washed by adding 1 ml of wash buffer and centrifuging at $200 \times g$ at room temperature for 5 minutes. Supernatant was discarded and 300 μ l of wash buffer were added. Samples were analyzed on a BD FACSArray bioanalyzer (BD Biosciences) according to the manufacturer's instruction. Protein assays were performed, as described above, on tissue homogenates before cytometric bead array. It should be noted, that the background levels of inflammatory cytokines seen in sham animals here are in line with our previous findings.^{4,5} Most likely this is due to the various sham procedures (for hemorrhagic shock and cecal ligation and puncture) these animals underwent. Naïve animals regularly display markedly lower levels. All data presented here were within the standard curve of the assay.

Caspase-3 Activity Assay

Caspase-3 activity in lung tissue was quantified as described elsewhere²⁸ with minor modifications. In brief, lung tissue was homogenized in buffer containing 25 mmol/L HEPES (Sigma), 5 mmol/L MgCl₂ (Sigma), 1 mmol/L EGTA (Sigma), 1 μ g/ml aprotinin, 0.04 mg/ml Pefabloc, and 1 μ g/ml leupeptin (all Roche, Indianapolis, IN). One hundred μ g of lung tissue (as determined by Bradford dye binding protein assay, see above) in 100 μ l of buffer were added to 100 μ l of 7-amino-4-trifluoromethyl-coumarin (AFC)-buffer containing 100 mmol/L HEPES, 2% sucrose (Sigma), 0.2% CHAPS (Sigma), 10

mmol/L dithiothreitol (Sigma), and 100 μ mol/L Ac-DEVD-AFC fluorogenic substrate stock solution (Biomol, Plymouth Meeting, PA). Extinction was read at 400-nm excitation and 505-nm emission and calculated against an AFC standard curve.

Active Caspase-3 and M-30 Immunostaining

Paraffin-embedded formaldehyde-fixed lung sections were dewaxed and rehydrated. Blocking of endogenous peroxidase was performed by incubating slides in 100% methanol including 0.3% H₂O₂. Antigen retrieval was performed in citric acid buffer (0.192% citric acid in ddH₂O) at pH 6.0 at 100°C (5 minutes for caspase-3 and 20 minutes for M-30). For caspase-3 immunostaining a serum block with 10% normal goat-serum (Pierce Biotechnology, Rockford, IL) in PBS was applied for 30 minutes at 37°C. Slides were incubated either with rabbit anti-active caspase-3 (Chemicon) at 1:50 and consecutively with goat anti-rabbit IgG, biotin-SP-conjugated (Chemicon) at 1:1000, or M-30 CytoDeath Biotin (Diapharma, Columbus, OH) at 1:50 for 45 minutes at room temperature. Slides were then incubated with Vectastain ABC kit (Vector Laboratories) for 45 minutes and then with 0.5% diaminobenzidine (Sigma) in PBS including 100 μ l of H₂O₂ (30%). Slides were counterstained for 5 minutes in 1% methyl green, pH 4.0 (Rowley Biochemical, Rowley, MA). Sections were then dehydrated and Permount (Biomed, Foster City, CA) was used to coverslip sections.

Polymerase Chain Reaction

RNA was isolated from lung samples using TriPure isolation reagent (Boehringer, Mannheim, Germany). Samples were homogenized on ice in 1000 μ l of TriPure in RNase-free microtubes. Ten μ g of glycogen (Invitrogen, Carlsbad, CA) and 200 μ l of chloroform (BCP; Molecular Research Center, Cincinnati, OH) were added to each sample and incubated for 15 minutes at room temperature. Samples were centrifuged at $12,000 \times g$ at 4°C for 10 minutes and the colorless upper phase was transferred into new tubes. Five hundred μ l of isopropanol (Sigma) were added and after incubating for 10 minutes at room temperature samples were again centrifuged at $12,000 \times g$ at 4°C for 10 minutes and supernatants were discarded. The pellet was washed in 75% ethanol (Sigma) and then reconstituted in 20 μ l of RNase-free water and RNA was quantified by reading extinction (SmartSpec 300, Bio-Rad) at 260 nm against background (320 nm).

Reverse transcriptase-polymerase chain reaction was performed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instruction. Amplification of cDNA was performed by mixing 2.5 μ l of sample from reverse transcriptase-polymerase chain reaction with 11.25 μ l of PCR-Master-Mix (Promega, Madison, WI), 11.25 μ l of nuclease-free water, and 2 μ l of forward and reverse primer equaling a final primer concentration of 2 μ mol/L each. Fifteen μ l of the resulting sample were

mixed with 3 μ l of 6 $\times$ blue/orange loading dye (Promega) and loaded into a 1.2% Agarose gel (Sigma). Densitometry was performed by a blinded observer using the Chemilmanager imaging system and Chemilmanager AlphaEase software 5.1 (AlphaInnotech Corp., San Leandro, CA).

Statistical Analysis

Results are presented as mean $\pm$ SEM. A one-way analysis of variance or a one-way analysis of variance on ranks were applicable followed by the Student-Newman-Keuls test as a post hoc test for multiple comparisons was performed to determine significant differences between experimental means. The Mann-Whitney *U*-test was used for fluorescent intensity measurements. A *P* value ≤ 0.05 was considered statistically significant.

Results

Fluorescent Intensities of Lung Sections of Transgenic C57BL/6-TgN(ACTbEGFP)1Osb Mice Treated with GFP-siRNA or PBS

Lung sections from animals having undergone intratracheal delivery of GFP-siRNA displayed a reduced fluorescent intensity (representative photograph in Figure 1, A2 and B2) when compared to sham animals (representative photograph in Figure 1, A1 and B1). To quantify these changes, SUM intensity, MEAN intensity, and SD of fluorescent intensity were assessed ($n = 10$ random pictures/mouse in $n = 3$ mice/group). For definitions see Materials and Methods section. SUM intensity [2.2 ± 0.4 versus 0.8 ± 0.1 (mean $\pm$ SEM) $\times 10^9$], MEAN intensity (2428 ± 252 versus 941 ± 49), and SD of fluorescent intensity (4078 ± 539 versus 1363 ± 328) were all markedly reduced after the transcriptional inhibition of GFP. On initial visual assessment a general decrease in lung areas from GFP-treated animals was visible. Closer analysis of sections suggested that silencing of GFP was not identical in all lung areas and that a patchy silencing pattern was evident. Alternatively, in response to intratracheal delivery of GFP-siRNA the fluorescent intensity of liver (Figure 1C2) and spleen sections (data not shown) did not display a decrease when compared to sham (Figure 1C1).

Co-Localization of Cy-5-Labeled Fas-siRNA and Cytokeratin-18 in Lungs after Intratracheal Administration of Cy-5-Fas-siRNA

At 24 hours after instillation Cy-5-labeled siRNA was detected in frozen lung sections (Figure 2A). Lung epithelial cells with positive cytokeratin-18 staining co-localized with Cy-5 fluorescence indicating the uptake of the siRNA by this cell type (Figure 2B). Interestingly, no co-localization could be detected between cells staining positive for CD115 and Cy-5 fluorescence (Figure 2C).

Lung, Plasma, and Liver IFN- α , IL-6, and TNF- α

Preliminary studies displayed that poly(I:C) caused a maximum of pulmonary activation of IRF-7 at 18 hours after intratracheal administration. Thus, this time point was chosen to elucidate possible TLR/IFN- α /NF- κ B-mediated inflammation associated with intrapulmonary delivery of siRNA. Poly(I:C) instillation significantly increased lung IFN- α (Figure 3A), IL-6 (Figure 3B), and TNF- α (Figure 3C) when compared to intratracheal delivery of GFP-siRNA, caspase-8-siRNA, or PBS (vehicle). No significant differences were observed within the GFP-siRNA, caspase-8-siRNA, or PBS treatment groups. These data were also confirmed using the ELISA technique. Neither liver tissue concentrations of IFN- α , IL-6, and TNF- α nor plasma levels of IFN- α and TNF- α changed in response to poly(I:C), GFP-siRNA, or PBS instillation (data not shown). Only IL-6 plasma levels were significantly increased in animals treated with poly(I:C) when compared to the other groups (data not shown).

Plasma IL-6, TNF- α , MCP-1, IL-10, IFN- γ , and IL-12 after Hemorrhagic Shock and Sepsis or Sham Procedures and Intratracheal siRNA Administration

Four hours after hemorrhagic shock mice underwent intratracheal instillation of siRNA, followed by cecal ligation and puncture 24 hours thereafter. Samples were harvested 24 hours after sepsis. Plasma IL-6, TNF- α , MCP-1, IL-10, and IFN- γ concentrations displayed a marked increase after hemorrhage and sepsis when compared to sham. Fas-siRNA or caspase-8-siRNA instillation did not significantly change plasma cytokine levels when compared to animals having received GFP-siRNA (data not shown).

mRNA Expression of Fas and Caspase-8 after Hemorrhagic Shock and Sepsis, Sepsis Alone, or Sham Procedures and Intratracheal siRNA Administration

Fas (Figure 4A) and caspase-8 mRNA (Figure 4B) expression displayed a significant increase in lung tissue of animals challenged with hemorrhagic shock and sepsis compared to animals having undergone cecal ligation and puncture alone or sham procedures. Fas- and caspase-8-siRNA treatment significantly reduced Fas (Figure 4A) and caspase-8 (Figure 4B) mRNA expression, respectively, to a level comparable to controls.

Lung IL-6, TNF- α , MCP-1, IL-10, IFN- γ , and IL-12 Concentrations after Hemorrhage and Sepsis or Sham Procedures and Intratracheal siRNA Administration

Lung IL-6 (Figure 5A), TNF- α (Figure 5B), MCP-1 (Figure 5C), IL-10 (Figure 6A), IFN- γ (Figure 6B), and IL-12

Figure 1. Frozen lung (A, B) and liver (C) tissue sections from transgenic mice overexpressing GFP 24 hours after intratracheal administration of PBS (A1, B1, C1) or GFP-siRNA (A2, B2, C2). Mice having received GFP-siRNA display reduced pulmonary but not hepatic green fluorescence. Nuclear counterstaining in B and C with DAPI. Original magnifications: $\times 400$ (A, B); $\times 200$ (C).

(Figure 6C) concentrations displayed markedly higher values in animals having undergone hemorrhage and sepsis when compared to sham animals. After hemorrhagic shock and sepsis lung IL-6 (Figure 5A) and TNF- α (Figure 5B) displayed significantly lower concentrations in animals treated with Fas-siRNA when

compared to mice, which received either caspase-8-siRNA or GFP-siRNA (here functioning as a nonsense control). MCP-1 (Figure 5C), KC, and MIP-2 (data not shown) also displayed a tendency toward lower values in the Fas-siRNA treatment group; however, results did not achieve statistical significance. Lung IL-10 (Figure

Figure 2. Uptake of siRNA into pulmonary epithelial cells, but not macrophages 24 hours after intratracheal instillation. Cy5-labeled Fas-siRNA (red) (**A1**), the corresponded nuclear counterstaining (blue) (**A2**), and both channels merged with differential interference contrast (**A3**) show cellular uptake of siRNA. Cy5-labeled Fas-siRNA (red) (**B1**), cyokeratin-18 staining (green) (**B2**), and both channels merged (**B3**) identify up-taking cells as pulmonary epithelium. Cy5-labeled Fas-siRNA (red) (**C1**), CD115 staining for macrophages (green) (**C2**), and both channels merged (**C3**) show that siRNA was not taken up into lung macrophages. Original magnifications, $\times 400$.

Figure 3. Lung tissue IFN- α (A), IL-6 (B), and TNF- α (C) concentrations at 18 hours after intratracheal instillation of poly(I:C), GFP-siRNA, PBS, or caspase-8-siRNA (C-8) show that administration of siRNA did not induce IRF/NF- κ B-mediated inflammation in the lung. Mediator levels were measured by ELISA (IFN- α) or cytometric bead array (TNF- α and IL-6). Values are means $\pm$ SEM of three to four animals in each group; one-way analysis of variance followed by Student-Newman-Keuls test, * P < 0.05 versus poly(I:C).

6A) was markedly diminished in the lung homogenates of Fas-siRNA-treated animals compared to the control group. IFN- γ (Figure 6B) and IL-12 (Figure 6C) levels were found to be significantly reduced in response to Fas gene silencing when compared to treatment with caspase-8-siRNA or GFP-siRNA. No significant differences could be observed between caspase-8-siRNA or GFP-siRNA treatment for all mediators. No alterations in lung IL-6, TNF- α , MCP-1, IL-10, IFN- γ , and IL-12 concentrations were found comparing GFP-siRNA versus PBS treatment groups, indicating further that GFP-siRNA served as a suitable control here (data not shown).

Figure 4. Lung tissue Fas mRNA expression and GAPDH-related integrated density values (IDTs) of this expression (A) and lung tissue caspase-8 mRNA expression and GAPDH-related IDT of this expression (B) 24 hours after polymicrobial sepsis and 52 hours after hemorrhagic shock (Hem+Sepsis), sepsis alone, or sham procedures (individual lanes represent individual animals). Animals received either Fas-siRNA (Fas) or GFP-siRNA (GFP) (here serving as a control) intratracheally. Only the combined insult of hemorrhagic shock and sepsis increased Fas and caspase-8 mRNA expression identifying them as potential therapeutic targets. Administration of Fas- or caspase-8-siRNA significantly reduced mRNA expression of Fas or caspase-8, respectively. Values for IDT are means $\pm$ SEM of three animals in each group; * P < 0.05 versus GFP-siRNA administration after sham hemorrhage and sham sepsis (Sham GFP), * P < 0.05 versus GFP-siRNA administration after sham hemorrhage and sepsis (Sepsis GFP), * P < 0.05 versus Fas siRNA (Fas) or caspase-8 (C-8) treatment after the double insult of hemorrhage and sepsis (Hem+Sepsis Fas, Hem+Sepsis C-8).

Active Caspase-3 (p17) and M-30 Immunostaining and Caspase-3 Activity after Hemorrhage and Sepsis and Intratracheal siRNA Administration

Lung caspase-3 activity was significantly increased in response to hemorrhagic shock and sepsis, when compared to mice having undergone sham procedures (Figure 7A). Fas-siRNA treatment but not caspase-8-siRNA

Figure 5. Lung tissue IL-6 (A), TNF- α (B), MCP-1 (C) concentrations per mg of total protein 24 hours after polymicrobial sepsis and 52 hours after hemorrhagic shock or sham procedures display a significant increase associated with the injury. Instillation of Fas- but not caspase-8-siRNA 4 hours after hemorrhage ameliorated this inflammation when compared to control siRNA. Mediator levels were measured by cytometric bead array. One-way analysis of variance followed by Student-Newman-Keuls test; * $P < 0.05$ versus sham, #, $P < 0.05$ versus Fas-siRNA treatment.

treatment significantly decreased caspase-3 activity (Figure 7A). Cells staining positively for active caspase-3 could only be found in considerable numbers in animals having undergone hemorrhagic shock and sepsis, and having received control-GFP-siRNA (Figure 7B1) or caspase-8-siRNA (not shown). Alternatively, sham animals (not shown) and mice after hemorrhagic shock and sepsis, and having received Fas-siRNA (Figure 7B2), exhibited virtually no positive cells. In general, only a few cells were positive for the M-30 neoepitope, as described elsewhere.²⁹ Positive cells could only be found in animals having received control siRNA (Figure 7C1) but not in

Figure 6. Lung tissue IL-10 (A), IFN- γ (B), and IL-12 (C) concentrations per mg of total protein 24 hours after polymicrobial sepsis and 52 hours after hemorrhagic shock or sham procedures display a significant increase associated with the injury. Instillation of Fas- but not caspase-8-siRNA 4 hours after hemorrhage ameliorated this inflammation when compared to control siRNA. Mediator levels were measured by cytometric bead array. Values are means $\pm$ SEM of eight animals in each group; one-way analysis of variance followed by Student-Newman-Keuls test; * $P < 0.05$ versus sham, #, $P < 0.05$ versus Fas-siRNA treatment.

those having received Fas-siRNA after shock (Figure 7C2) or in sham animals.

Alveolar Architecture, Lung Congestion, and Infiltration of Granulocytes after Hemorrhage and Sepsis and Intratracheal siRNA Administration

Hemorrhage followed by sepsis led to a significant increase in polymorphonuclear granulocytes infiltrating the

Figure 7. Caspase-3 activity in lung tissue (A) was significantly increased 24 hours after polymicrobial sepsis and 52 hours after hemorrhagic shock when compared to sham animals. A: Fas-siRNA but not caspase-8-siRNA administration 4 hours after hemorrhage significantly reduced caspase-3 activity in lung samples; *, < 0.05 versus sham, #, < 0.05 versus Fas-siRNA treatment. Lung immunostaining displayed a considerable number of cells positive for active caspase-3 (B1) as well as positive cells for M-30 (C1) in response to hemorrhagic shock and sepsis. However, in response to Fas-siRNA treatment only a few cells were positive for caspase-3 (B2) and virtually no cells for M-30 (C2). Original magnifications, $\times 400$.

lung ($12.6 \pm 0.8\%$) (Figure 8B) as determined by neutrophil-specific MPO staining when compared to sham ($3.0 \pm 0.1\%$) (Figure 8A). Fas-siRNA treatment significantly reduced the number of total neutrophils in lung tissue ($7.4 \pm 0.8\%$) (Figure 8C). Lung H&E-prepared sections displayed lung congestion, disrupted alveolar architecture, and inflammatory infiltrates (Figure 9B) in response to hemorrhage and sepsis when compared to sham (Figure 9A). Sepsis alone led to the named pulmonary alterations to a much lesser degree (not shown). Fas-siRNA treatment improved alveolar architecture and reduced lung congestion and inflammatory infiltrates (Figure 9C).

Discussion

In line with previous findings, hemorrhagic shock, especially when followed by polymicrobial sepsis, was associated with a systemic inflammation with increased plasma cytokine levels.⁴ In addition, local mediator expression was distinctly increased in the lung compartment, which is in line with reports of a pronounced pro- and anti-inflammatory response to hemorrhagic shock and sepsis in the lung.^{3,4,30}

Figure 8. Lung tissue MPO staining 24 hours after polymicrobial sepsis and 52 hours after hemorrhagic shock. Mice underwent sham procedures and administration of GFP-siRNA (A) or hemorrhage and administration of GFP-siRNA and sepsis (B) or hemorrhage and administration of Fas-siRNA and sepsis (C). Fas-siRNA treatment markedly reduced the number of MPO-stained cells (neutrophils) present in the lung in response to hemorrhagic shock and sepsis. Alexa Fluor 594-labeled MPO-stained cells (red) (A1–C1), the corresponding nuclear counterstaining (blue) (A2–C2), and both channels merged (A3–C3) display the relative change in the number of MPO-positive cells observed in a typical field. Original magnifications, $\times 400$.

Our results demonstrate that, after hemorrhagic shock and sepsis, a marked decrease of pulmonary TNF- α , IL-6, IFN- γ , IL-10, and IL-12 concentrations was present in the animals that had received Fas-siRNA when compared to control-siRNA. At this time point Fas mRNA expression in lung tissue was significantly reduced in response to the siRNA administration. Concurrently, lung caspase-3 activity and epithelial cell apoptosis were also significantly reduced as a result of Fas silencing. These findings support the hypothesis that activation of the extrinsic Fas receptor pathway in pulmonary epithelial cells is a pathophysiologically relevant event in the onset of indirect/nonpulmonary ALI. Our immunofluorescence studies displayed co-localization of Cy-5-labeled Fas-siRNA with pulmonary cells staining positive for cytokeratin-18. Together with the simultaneous decrease of Fas mRNA expression, these findings indicate that Fas-siRNA was primarily taken up by lung epithelial cells and Fas expression was silenced in this cell population. However, this does not exclude the possibility of other cell types being additional targets of intrapulmonary delivery of siRNA. Different kinetics in uptake and processing of siRNA, as well as possible alterations in cellular uptake due to the Cy-5 label might play a role here. With respect to the potential significance of these findings, the relevance of the Fas receptor pathway has been previously addressed in direct/pulmonary ALI. In this regard, ALI is associated with increased expression of Fas on lung epithelial cells.⁶ This has also been observed clinically in acute respiratory distress syndrome.³¹ Along with this increase of membrane-bound Fas, ALI also leads to an

Figure 9. Representative H&E preparation of lung tissue slides from animals 24 hours after polymicrobial sepsis and 52 hours after hemorrhagic shock. Mice underwent sham procedures and GFP-siRNA administration (**A**) or hemorrhage and GFP-siRNA administration and sepsis (**B**) or hemorrhage and administration Fas-siRNA and sepsis (**C**). Fas-siRNA administration typically reduced lung congestion and inflammatory infiltrates and improved alveolar architecture after hemorrhagic shock and sepsis (**C**). Original magnifications: $\times 200$ (**A1**, **B1**, **C1**); $\times 400$ (**A2**, **B2**, **C2**).

increase of soluble Fas ligand (sFasL)^{6,10} as well as soluble Fas.³¹ In addition, administration of Fas-activating antibody induces epithelial apoptosis,¹¹ which can be prevented by blocking Fas activation in endotoxin-induced ALI.⁶

The association of Fas up-regulation and increased local pulmonary inflammation, ie, alteration of cytokine levels, neutrophil sequestration, and so forth, is supported by various reports throughout the literature. Fas/FasL engagement on pulmonary epithelial cells resulted in protein leakage, neutrophil accumulation, and increased expression of cytokines, such as TNF- α , IL-6, and MIP-2 in the lung as early as 24 hours after the insult.¹¹ Decreased alveolar damage and MCP-1 lung concentrations after exposure to gram-negative bacteria in Fas-deficient mice have been observed.¹² Further, it has been shown, that administration of CD95 antagonists

or the use of Fas-deficient mice is associated with an ameliorated inflammatory response in lipopolysaccharide (LPS)-induced ALI.^{6,32}

In vitro experiments have also elucidated nonapoptotic proinflammatory signaling associated with the engagement of FasL and Fas. FasL-induced apoptosis in resident macrophages results in enhanced secretion of chemokines and cytokines.³³ Vascular smooth muscle cells display an increased expression of IL-1, IL-8, and MCP-1 on Fas/FADD activation.³⁴ In human macrophages ligation of Fas induces TNF- α and IL-8 secretion³⁵ and in serum-starved fibroblasts soluble Fas activates ERK-1/2 and NF- κ B and leads to IL-6 and IL-8 secretion in the absence of cell death.³⁶ Ma and colleagues³⁷ indicated that interruption of Fas-FasL engagement on human macrophages by Fas-blocking antibody significantly diminished lipopolysaccharide or IL-1 β -induced TNF- α and IL-6 release.

Administration of Fas-siRNA in our setting not only reduced local inflammatory mediators and epithelial cell apoptosis but also was associated with an improvement in alveolar architecture as well as a reduction in lung congestion and of pulmonary infiltrates. This data are supported by findings indicating that activation of the Fas/FasL systems lead to thickening of alveolar septa and neutrophil infiltration.¹¹ In addition, Fas-deficient animals displayed healthier alveolar architecture and less pulmonary inflammatory infiltrates after intranasal deposition of *Escherichia coli* LPS when compared to their genetic background.³² In addition, pretreatment of lungs with anti-Fas blocking antibody has been shown to reduce alveolar inflammation and ameliorate LPS-induced lung injury.⁶ Interestingly, the attenuation of the local inflammatory response in Fas-deficient mice has been linked to the diminished number of neutrophils immigrating into the lung,³² while this was not necessarily observed after anti-Fas treatment.⁶ Our results display that Fas-siRNA treatment was associated with a reduced number of neutrophils infiltrating the lung, probably contributing to the improvement of the described histopathological alterations. In line with these observations, the amelioration of acute respiratory distress syndrome by transfection of HSP-70 into lung epithelial cells was also associated with a marked reduction of pulmonary neutrophils.³⁸ After immune-complex-induced lung injury, Fas deficiency results in decreased lung chemokines and neutrophil accumulation in the lungs.³⁹

Serrao and colleagues⁴⁰ indicated that neutrophils in culture were capable of inducing lung epithelial cell death via the release of soluble Fas ligand, thus potentially bridging the role of activated neutrophils and the activation of the Fas receptor pathway. However, *in vivo*, this causality still remains to be established, especially as the change in neutrophil influx into the lungs seen here would seem to be more of a response to the suppression of epithelial cell apoptosis by Fas silencing. This could suggest that activation/injury of the epithelial cell may be a more proximal teleological event in this model of ALI and that neutrophils may serve more to amplify this response.

So far it also remains to be elucidated, whether the ameliorated pulmonary inflammatory response we and others^{6,39} have observed could be attributed to a separate Fas-driven inflammatory pathway. In contrast to the effective treatment using Fas-siRNA, caspase-8-siRNA administration, although leading to markedly decreased caspase-8 mRNA expression, was not associated with reduced pulmonary inflammation. In this regard, it has been suggested that the recruitment of the caspase-8 homologue cellular FLICE-like inhibitory protein (cFLIP), rather than that of procaspase-8, to the Fas-associated death domain not only inhibits procaspase-8 cleavage but results in so called nonapoptotic signaling.⁴¹ Presumably via the activation of TRAF-1, TRAF-2, RIP, and Raf-1, the NF- κ B and the ERK/AP-1 pathway are activated and proinflammatory gene expression is induced.^{42,43} Supportive data comes from the observation that exposure of GM6112 fibroblasts to soluble FasL results in a rapid phosphorylation of ERK1/2, does not affect JNK or p38

activities, and is associated with an increase in IL-6 gene expression.³⁶ Alternatively, it could be possible that the noneffectiveness of caspase-8 silencing in our model is attributable to dissimilar kinetics of Fas and caspase-8 silencing. In this regard, because silencing by siRNA is never a 100% and the time course of silencing differs between different targets, the remaining caspase-8 activity might have been sufficient to enable downstream signaling.

There is little information on the duration and degree of *in vivo* silencing of genes using siRNA. We have recently shown that after hydrodynamic delivery of siRNA, silencing in the liver lasts for up to 10 days.¹⁵ In addition, in a setting of intrapulmonary delivery of KC- and MIP-2-siRNA into the lungs of mice undergoing hemorrhagic shock and sepsis, we found silencing to be present at 48 hours after siRNA instillation.⁵ Massaro and co-workers²⁰ reported that a single intranasal instillation of 10 μ g of GAPDH-siRNA resulted in a 50% decrease in pulmonary GAPDH at day 1 (which corresponds with our findings of GFP silencing) and remained notably depressed (67%) up to day 7 after instillation.

In our experiments we have used 21-bp siRNA, which in *in vitro* experiments have raised the concern to have the capacity to up-regulate type I IFNs^{44,45} and induce inflammation. However, so far the potential signaling pathways for small interfering RNAs remain controversial. TLR-3,²³ TLR-7,²⁴ TLR-8, TLR-9, and PKR pathways^{25,26,46} are all capable of recognizing foreign nucleotides, and their activation either leads to up-regulation of type I IFNs or TNF- α , IL-6, and IL-12 via the NF- κ B transcriptional regulator.^{25,46} Based on this knowledge, we evaluated the *in vivo* capacity of siRNA to induce lung inflammation. Although intratracheal instillation of poly(I:C) induced a significant increase in pulmonary IFN- α , TNF- α , and IL-6, this was not observed after sense (caspase-8)-siRNA, nonsense (GFP)-siRNA, or PBS vehicle administration. This indicates that no significant inflammation was associated with the local intratracheal administration of naked siRNA *in vivo*. This is in line with findings by Heidel and colleagues,²⁶ who demonstrated that intravenous delivery of naked siRNA does not induce an IFN response. However, as it has been demonstrated that induction of STAT-1 in this context seems to be dose-dependent,⁴⁴ it cannot be excluded that higher doses of siRNA or using alternative siRNA carrier systems, such as microsome encapsulation, cholesterol conjugation, and so forth, might not result in activation of the IFN pathway. Furthermore, although screening for poly(I:C)-induced pulmonary IRF-7 expression within 24 hours after intratracheal delivery demonstrated a maximum at 18 hours, it seems important to investigate other time points, especially in the later course after siRNA delivery.

Our results add to the current concept of neutrophils being important effectors in indirect/nonpulmonary ALI that activation of the Fas receptor pathway in lung epithelial cells represents a significant proximal biological event after hemorrhagic shock and sepsis. To our best knowledge we are the first to demonstrate that local delivery of an anti-apoptotic siRNA construct into the lung

during the onset of an acute critical condition may be used to modify the course of inflammation and to decrease apoptosis in pulmonary epithelial cells (the major target of intratracheally administered siRNA). Furthermore, we have demonstrated that local pulmonary gene therapy using naked siRNA is safe in mice and can be performed without inducing IRF or NF- κ B-dependent inflammation. The effectiveness of Fas silencing in both lung inflammation and pulmonary apoptosis might suggest the involvement of an inflammatory nonapoptotic Fas-driven pathway in the lung. Although appreciating that further studies will be needed to optimize both preclinical and possibly clinical application, we suggest that such an approach represents a novel method to silence not only apoptotic but also other gene products involved in ALI.

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