Fas/FasL-dependent Apoptosis of Alveolar Cells after Lipopolysaccharide-induced Lung Injury in Mice

YOSHIHIRO KITAMURA, SATORU HASHIMOTO, NARUHIKO MIZUTA, ATSUKO KOBAYASHI, KUNIHIKO KOOGUCHI, IKUYA FUJIWARA, and HIROO NAKAJIMA

Department of Intensive Care and Anesthesiology and Department of Surgery, Kyoto Prefectural University of Medicine, Kyoto, Japan

To determine the possible contribution of apoptosis in the pathogenesis of acute lung injury (ALI), we investigated Fas antigen (Fas), Fas ligand (FasL), perforin, granzyme A, and granzyme B expressions in a murine model of ALI after intratracheal instillation of *Escherichia coli* lipopolysaccharide (LPS: 0.3–30 μg) into the left lung. Lung injury, examined by water-to-dry weight ratio and albumin leakage, demonstrated maximal epithelial injury 1 d after 30 μg LPS instillation. Expressions of the proapoptosis molecules' mRNA were dose-dependently up-regulated, with maximal expression in the early phase in the instilled lung and most apparent 1 d after LPS instillation. Negligible mRNA expression of proapoptosis molecules was observed in noninstilled lungs. The terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL) demonstrated positive signals in neutrophils and macrophages as well as in alveolar wall cells of the instilled lung 1 d after LPS instillation. Immunohistochemistry demonstrated that Fas was up-regulated in alveolar and inflammatory cells and FasL-positive inflammatory cells migrated into the air spaces in the LPS-instilled lung. Intratracheal administration of P2 antibody, which is an anti-Fas blocking antibody, attenuated the lung injury after 30 μg LPS instillation without attenuating mRNA expressions of proapoptosis molecules and neutrophil accumulation in the lung. In contrast, concanamycin A, which inhibits the function of perforin, did not alter the outcome after LPS instillation. These results indicate that the Fas/FasL system could be important in the pathogenesis of LPS-induced ALI, and proper regulation of the FasL/Fas system might be important for potential treatment of ARDS.

The acute respiratory distress syndrome (ARDS) is an inflammatory lung condition that is characterized by neutrophil accumulation, increased vascular permeability, increased extravascular lung water, decreased pulmonary compliance, impaired gas exchange, and a diffuse lung infiltration on a chest X-ray (1). This syndrome remains a major cause of mortality and morbidity in clinical pulmonology (1). Phagocytes, in particular macrophages and polymorphonuclear neutrophils, have played major roles in the pathogenesis of the lung injury (2–6). However, the precise mechanism of lung injury and involvement of lymphocytes remain unclear. We have previously reported up-regulation of proapoptosis molecules associated with cytotoxic T lymphocytes (CTLs) such as Fas, FasL, perforin, and granzymes in bronchoalveolar lavage (BAL) cells from patients in the acute phase of septic ARDS (7). This observation strongly suggested a role for apoptosis in the pathogenesis of acute lung injury (ALI).

Fas ligand (FasL) is a 40-kD type II membrane protein belonging to the tumor necrosis factor (TNF) family (8). It is expressed on activated T cells and is one of the effector molecules for CTL-induced cytotoxicity (9). Fas antigen (Fas), also called APO-1 or CD95, is the receptor for the FasL and is a member of the TNF/nerve growth receptor family (8). It is expressed on various cells and tissues, including thymus, liver, ovary, heart, and lung (10). Binding of FasL to Fas antigen causes apoptosis in Fas antigen-bearing cells. Although the expression of FasL was originally thought to be limited to T cells, FasL can be found in a soluble form in the circulation and on the surface of other cells such as activated neutrophils (11). CTLs have a second system to induce apoptosis and cell death in their target cells, the perforin/granzyme system (12–14). Granzymes and the pore-forming protein, perforin, induce rapid death of target cells. As a result, CTLs cause lethal damage to their target cells via two independent systems (12–14).

In this study, expressions of proapoptosis molecules in an experimental murine lung injury model of intratracheally instilled lipopolysaccharide (LPS) were investigated. Separate groups of mice were challenged with anti-mouse Fas antibodies (P2) to block the FasL/Fas system or with concanamycin A to block the perforin/granzyme system to examine the possible contribution of apoptosis in acute lung injury.

METHODS

Animals and Materials

Four hundred and eighty-five mice (6-wk-old male-specific pathogen-free ICR mice from Japan S.L.C. Co., LTD., Shizuoka, Japan) weighing 29–32 g were used for the study. The bacterial endotoxin lipopolysaccharide (LPS from *Escherichia coli* 055:B5; Difco, Detroit, MI) was used to induce lung injury. All animal procedures were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine.

Experimental Models and Protocols

To create a lung injury model, mice were anesthetized with inhaled sevoflurane. Then, LPS (0.3, 3, or 30 μg) diluted in 60 μl sterile 0.9% NaCl was slowly instilled into the left lung using a gavage needle (modified animal feeding needle, 24 gauge; Popper & Sons, Inc., New Hyde Park, NY) inserted into the left main bronchus. In negative control mice, 60 μl sterile 0.9% NaCl was instilled into the left lung using the same method. The mice recovered from the anesthesia within 2 min. After recovery, the mice were returned to their cages and allowed food and water *ad libitum.* At the time of the experiments, the mice were anesthetized with pentobarbital sodium (200 mg/kg) and exsanguinated by aortic transection and the lungs were dissected and studied according to the protocols.

Preliminary studies revealed that topical instillation of 30 μg LPS into the left lung induced consistent and maximal alveolar cell injury in the instilled lung without causing mortality. Thus, for the time-course study, separate mice were studied 1, 3, 5, 7, and 10 d after the 30 μg LPS or 0.9% NaCl instillation. As the time-course study showed that maximal lung injury was observed at 1 d after LPS instillation, all experiments other than time-course studies were carried out at 1 d after LPS or 0.9% NaCl instillation.

Anti-Fas Antigen (P2)-treated Model

An anti-Fas antibody (P2) was kindly provided by Dr. Takehiko Koji, Nagasaki University of Medicine (15). The mice were instilled with

(*Received in original form March 12, 2000 and in revised form August 22, 2000*)

The Japanese Ministry of Education (Grant 10470322 to Satoru Hashimoto) funded this work.

Correspondence and requests for reprints should be addressed to Satoru Hashimoto, M.D., Department of Intensive Care and Anesthesiology, Kyoto Prefectural University of Medicine, 465 Kajiicho, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto, 602-8566, Japan. E-mail: satoru@koto.kpu-m.ac.jp

Am J Respir Crit Care Med Vol 163. pp 762–769, 2001
Internet address: www.atsjournals.org

the P2 antibody (100 μg diluted in 60 μl sterile 0.9% NaCl) 1 h before the 30 μg LPS instillation using the method described above. A control antibody (normal goat anti-mouse IgG, 100 μg diluted in 60 μl sterile 0.9% NaCl) was instilled using the same method in a separate group of mice. Mice were examined at 1 d after the instillation.

Concanamycin A-treated Model

Concanamycin A (12 μg diluted in 60 μl sterile 0.9% NaCl) was instilled into the lungs 1 h before the 30 μg LPS instillation using the same method. Sterile 0.9 % NaCl was instilled as control for the concanamycin-instilled mice using the same method in a separate group of mice. Mice were examined at 1 d after instillation.

Analytical Protocols

RNA preparations and analysis. Total RNA was prepared from lung tissue by the use of TRIzol Reagent (Life Technologies, Gaithersburg, MD). For the polymerase chain reaction (PCR) analysis of RNA, cDNA was prepared by reverse transcription (RT) of 3 μg of each RNA sample in 20 μl reaction volume by use of SUPERSCRIPT (Life Technologies, Gaithersburg, MD). The cDNAs were diluted to 120 μl, and these cDNAs were used in all PCRs. The PCR mixture (total volume 40 μl) was comprised of 8 μl of cDNA, 2 μl of 20 mM primers, 8 μl of 10 mM dNTP, and 1 unit of *Taq* polymerase. The mixture was subjected to PCR amplification. Oligonucleotide primers for each molecule were as follows:

GAPDH
Sense: 5′-ACC ACC ATG GAG AAG GCT GC-3′
Antisense: 5′-CTC AGT GTA GCC CAG GAT GC-3′
Fas
Sense 5′-GCA CAG AAG GGA AGG AGT AC-3′
Antisense: 5′-ACT GGA GGT TCT AGA TTC AGG-3′
FasL
Sense: 5′-TAG ACA GCA GTG CCA CCA CTT CAT-3′
Antisense: 5′-AAC TCA CGG AGT TCT GCC AGT T-3′
Perforin
Sense: 5′-CAC AAG TTC GTG CCA GGT GTA-3′
Antisense: 5′-GCA TGC TCT GTG GAG CTG TTA-3′
Granzyme A
Sense: 5′-ATT CCT GAA GGA GGC TGT GAA-3′
Antisense: 5′-ATT GCA GGA GTC CTT TCC ACC AC-3′
Granzyme B
Sense: 5′-GCT GAC AGT ACA GAA GGA T-3′
Antisense: 5′-GAG CAA TCC TGG ACT CAG CT-3′

The conditions for the amplification of each molecule were as follows:

GAPDH, Fas, and perforin: 93° C for 3 min for 1 cycle, 93° C for 1 min, 64° C for 2 min, and 72° C for 3 min for 30 cycles.

FasL: 93° C for 3 min for 1 cycle, 93° C for 1 min, 64° C for 2 min, and 72° C for 3 min for 24 cycles.

Granzyme A and Granzyme B: 93° C for 3 min for 1 cycle, 93° C for 1 min, 60° C for 2 min, and 72° C for 3 min for 30 cycles.

After the PCR, 8-μl aliquots from the PCR reaction were electrophoresed on a 1.5% agarose gel stained with ethidium bromide. The gel was photographed under ultraviolet transillumination using a densitometer (ATTO, Osaka, Japan). Each signal was normalized relative to its corresponding GAPDH signal from the same RNA, and expressed as the mRNA/GAPDH ratio.

Analysis of bronchoalveolar lavage fluid (BALF). The trachea was exposed by a midline incision and cannulated with a sterile polypropylene 18-gauge catheter. The lungs were lavaged with 5 ml of cold phosphate-buffered saline without calcium (PBS[−]) supplemented with 0.6 mM EDTA in 0.2 ml increments. The total returns after lavage averaged 4–4.5 ml/mouse. BALF was centrifuged at 1,000 × *g* for 8 min at 4° C. The cell-free supernatants were stored at −80° C until further analysis. The cell pellet obtained from the centrifugation of BALF was diluted in 1.0 ml of PBS(−). The total cell number was counted with a hemocytometer after staining with gentian violet. Differential cell counts were done on cytocentrifuge preparations (Cytospin 3; Shandon Scientific, Cheshire, UK) stained with modified Giemsa stain (American Scientific, McGraw Park, IL).

Measurement of excess lung water and albumin concentration. The degree of lung injury was compared using the following two methods in mice at 1 d after instillation. Albumin concentration in seven groups (n = 10 in each group, *see* Figure 1 and its legend for more details) was determined in the cell-free BALF supernatant by dye-binding assay (Bio-Rad protein assay; Bio-Rad, Richmond, CA). The wet-to-dry weight (W/D) ratio of the instilled (left) and noninstilled (right) lungs was examined (n = 10 in each group using different mice from albumin measurement, see also Figure 1). The lungs were dissected, weighed, and then dried at 60° C for 5 d. Then the W/D ratio was calculated by dividing the wet weight by the final dry weight. The validity of these methods for assessment of the lung injury has been well established (16).

Histology of the lung. After preparation, the lungs were fixed by inflation with buffered 4% paraformaldehyde for 6 h. After embedding in paraffin, the sections were prepared and stained with hematoxylin and eosin.

DNA nick end-labeling of tissue sections. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) was carried out to detect apoptotic DNA damage using the TACS screening set (Trevigen, Inc., Gaithersburg, MD). In brief, after fixation with 4% buffered formalin, the sections (5 μm) were deparaffinized and hydrated. Then, the nick end-labeling method was applied to label the 5′-end of double-stranded DNA fragments by 3,3′-diaminobenzidine (DAB). Counterstaining was done with methyl-green solution (Wako, Osaka, Japan). Positive staining was indicated as black-brown and background staining was green.

Immunohistochemical staining for Fas and FasL. Immunohistochemical staining was carried out using an indirect, two-step labeling technique with peroxidase-conjugated immunoglobulin G (IgG). Polyclonal rabbit anti-mouse Fas (P4; 25 μg/ml) or anti-rat Fas ligand (P5; 50 μg/ml) antibodies were kindly donated by Dr. Takehiko Koji from the Nagasaki University of Medicine and were used as primary antibodies. Although they are polyclonal, the specificity of these antibodies was very high as demonstrated elsewhere (15). In brief, lung sections (5 μm thick) were deparaffinized in toluene and dehydrated by passing through a graded series of ethanol. Endogenous peroxidase activity was blocked by incubation of the slides with 0.3% H_2O_2 in methanol for 30 min at room temperature. After washing in PBS(−) three times, the slides were incubated in a solution of 500 μg/ml normal goat IgG in 1% bovine serum albumin in PBS(−) for 1 h at room temperature for blocking. The slides were then incubated overnight with P4 or P5 antibodies, and the following day incubated with a HRP goat secondary anti-rabbit IgG (100 μg/ml) antibody for 1 h at room temperature. The peroxidase reaction was developed by incubating these with H_2O_2 and 3,3′-diaminobenzidine tetrachloride. As negative control, adjacent sections were incubated with normal rabbit IgG antibody in the presence of the second antibody. Counterstaining was done with methyl green.

Data Analysis

All biochemical data are expressed as mean ± SEM. Kruskall–Wallis nonparametric analysis of variance for factorial experiments was used. Dunn's procedure was used for post hoc multicomparison analysis among the groups. We regarded the data as significant at a p value of < 0.05.

RESULTS

Analysis of Lung Injury

Albumin concentrations in the BALF at 1 d after LPS instillation increased significantly after 30 μg LPS compared with the lower doses or 0.9% NaCl-instilled lung (Figure 1, *left panel*). The lung wet-to-dry weight in the instilled lung after LPS instillation followed the same pattern as the albumin concentration (Figure 1, *right panel*). Anti-Fas P2 antibody significantly attenuated the albumin leakage and increase of the lung wet-to-dry weight ratio ($p < 0.05$ versus control IgG + LPS 30 μg). Instillation of concanamycin A did not affect these parameters.

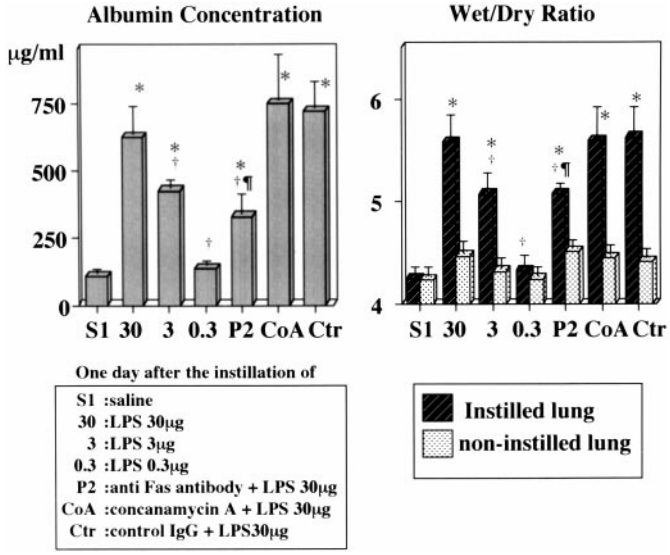

Figure 1. Albumin concentration in the BALF supernatant (*left column*) and wet/dry ratio of the instilled lung and noninstilled lung (*right column*) 1 d after the various interventions. Data shown as mean + SEM. n = 10 in each group (total 140 mice). Albumin concentration and wet/dry ratio were studied using separate mice. *$p < 0.05$ versus negative control (0.9% NaCl instillation depicted as saline in the figure); †$p < 0.05$ versus positive control (30 μg LPS instillation); ¶$p < 0.05$ versus control IgG + 30 μg LPS.

BALF Cell Number

A large number of neutrophils and a small number of lymphocytes migrated into the alveolar spaces 1 d after the high LPS dose (30 μg). The number of neutrophils and lymphocytes subsequently decreased after LPS instillation (Figure 2, *left panel*). At the lower LPS doses (0.3 or 3 μg), the influxes of neutrophils and lymphocytes were lower and the number of macrophages was similar to the 30 μg LPS group (Figure 2, *right panel*). Neither anti-Fas antibody (P2) nor concanamycin A affected the migration of neutrophils and lymphocytes after the high dose of LPS (Figure 2, *right panel*).

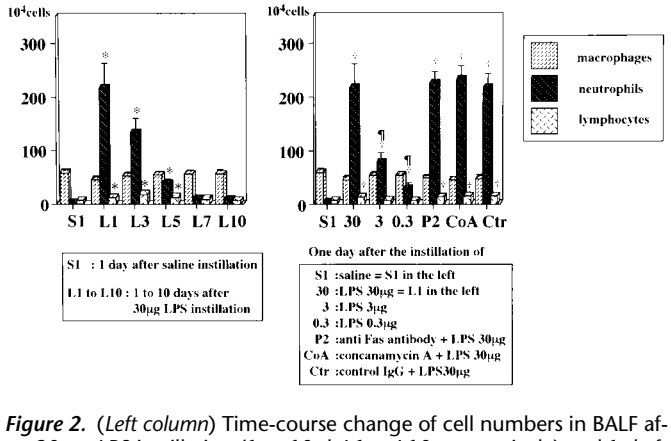

Figure 2. (*Left column*) Time-course change of cell numbers in BALF after 30 μg LPS instillation (1 to 10 d, L1 to L10, respectively) and 1 d after 0.9% NaCl (saline) instillation (S1). Mice instilled with 0.9% NaCl were also studied for 10 d (S3, S5, S7, and S10) and the statistical comparison (respective neutrophils, lymphocytes, and macrophages) was carried out on the same day. Only data from 1 d after 0.9% NaCl instillation (S1) are showed here, as no changes were noted during the study for 10 d after 0.9% NaCl instillation. *$p < 0.05$ versus those after 0.9% NaCl instillation on the same days. (*Right column*) Cell numbers in BALF 1 d after the various interventions as depicted. Note that P2 anti-Fas antibody did not alter neutrophil infiltration into the air space. †$p < 0.05$ versus negative control (0.9% NaCl instillation, S1); ¶$p < 0.05$ versus positive control (30 μg LPS instillation). Data shown as mean + SEM. n = 10 in each group (total 150 mice).

mRNA Expressions of Proapoptotic Molecules

mRNA expressions of all proapoptosis molecules (Fas, FasL, perforin, and granzymes A and B) were significantly up-regulated in the instilled lungs at 1 to 3 d after 30 μg LPS instillation and were subdued over the time course of the study (Figures 3A and 3C). The expressions were low or negligible in the noninstilled lungs at any time point. Instillation of 0.9% NaCl (expressed as saline in the figures) did not cause significant mRNA expression of these molecules at any point of the study over 10 d. At the lower LPS dose (0.3 or 3 μg), lower expressions of the apoptosis molecules' mRNAs were observed dose dependently in the instilled lung (Figures 3B and 3D). Neither administration of the P2 antibody, irrelevant IgG, nor concanamycin A inhibited mRNA expressions of proapoptosis molecules with the 30 μg LPS instillation. These observations were confirmed by the calculation of the densitometric ratio for each mRNA expression (Figures 3C and 3D).

Histology

Significant numbers of inflammatory cells migrated into the alveolar spaces and the alveolar walls showed destructive changes at 1 d after 30 μg LPS instillation (Figure 4B). No inflammatory changes were observed in the control, 0.9% NaCl-instilled, lung (Figure 4A). In the TUNEL assay, only a few macrophages were positive in the control lung (Figure 5A), whereas in the LPS-instilled lung a large number of positive cells were characterized as inflammatory cells or alveolar epithelial and endothelial cells (Figure 5B). Semiquantitative counting of these cells showed that LPS instillation resulted in about 30% of alveolar wall cells being TUNEL positive, whereas about 70% of the inflammatory cells were apoptotic in the injured lung at 1 d after the 30 μg LPS instillation (Figure 6). With P2 treatment, the incidence of apoptosis decreased both in alveolar wall cells and inflammatory cells.

In the immunohistochemical assay, the expressions of Fas were up-regulated in alveolar wall cells (epithelial and endothelial cells) and inflammatory cells (macrophages and polymorphonuclear neutrophils) and the expressions of FasL were highly up-regulated in some of the inflammatory cells (mainly macrophages) in the LPS-instilled lung (Figures 7 and 8). Parallel to the result of hematoxylin and eosin staining and TUNEL assay, no positive signals for Fas or FasL were detected except for a few macrophages in the 0.9% NaCl-instilled lungs. The expressions of the proapoptotic molecules in the noninstilled lung were limited even with 30 μg LPS instillation (data not shown). Also, no significant signals were detected in the injured lungs after LPS instillation stained with irrelevant rabbit IgG antibody in the presence of the second antibody (data not shown).

When comparing the findings of LPS-instilled lungs with those of the P2 or concanamycin A pretreated lungs, the number of inflammatory cells did not show any difference. However, in the P2 pretreated mice, the TUNEL-positive cells were significantly reduced (Figure 5C) and the lung showed intense but less alveolar inflammation (Figure 4C) compared with the LPS-instilled lung (Figure 4B). Concanamycin A, however, failed to show any protective effects against acute lung injury caused by LPS instillation (Figures 4D and 5D). Also, neither P2 nor concanamycin A altered the expression of Fas or FasL in the instilled lung after 30 μg LPS instillation (data not shown).

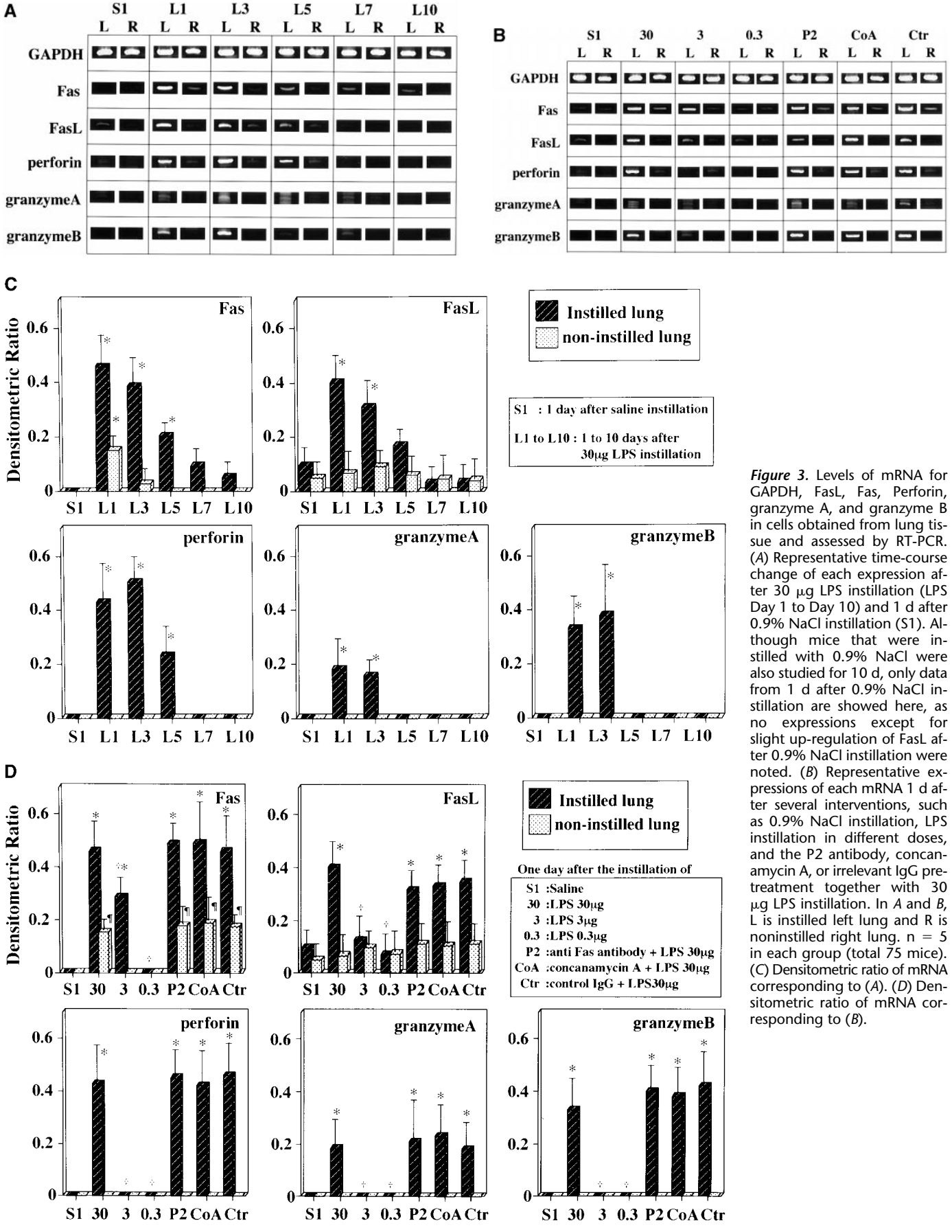

Figure 3. Levels of mRNA for GAPDH, FasL, Fas, Perforin, granzyme A, and granzyme B in cells obtained from lung tissue and assessed by RT-PCR. (*A*) Representative time-course change of each expression after 30 μg LPS instillation (LPS Day 1 to Day 10) and 1 d after 0.9% NaCl instillation (S1). Although mice that were instilled with 0.9% NaCl were also studied for 10 d, only data from 1 d after 0.9% NaCl instillation are showed here, as no expressions except for slight up-regulation of FasL after 0.9% NaCl instillation were noted. (*B*) Representative expressions of each mRNA 1 d after several interventions, such as 0.9% NaCl instillation, LPS instillation in different doses, and the P2 antibody, concanamycin A, or irrelevant IgG pretreatment together with 30 μg LPS instillation. In *A* and *B*, L is instilled left lung and R is noninstilled right lung. n = 5 in each group (total 75 mice). (*C*) Densitometric ratio of mRNA corresponding to (*A*). (*D*) Densitometric ratio of mRNA corresponding to (*B*).

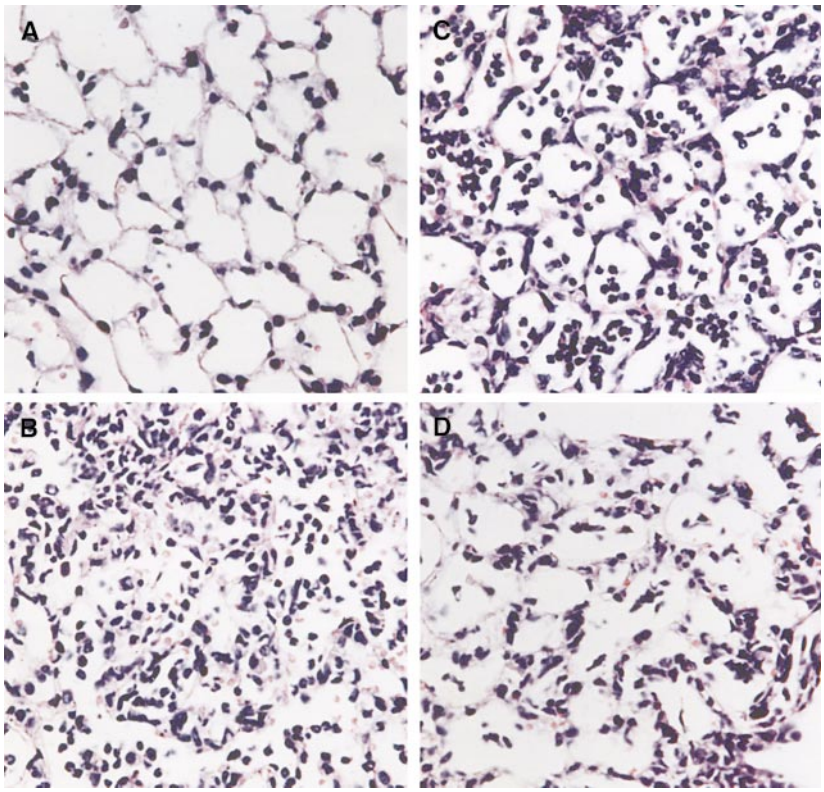

Figure 4. Hematoxylin and eosin staining of instilled lungs 1 d after instillation of 0.9% NaCl (*A*), 30 μg LPS (*B*), anti Fas antibody + 30 μg LPS (*C*), and concanamycin A + 30 μg LPS (*D*). Note the amelioration of lung injury with anti-Fas treatment while neutrophil infiltration into the air spaces was noted. Five mice were examined in each group. Original magnification: ×100.

DISCUSSION

Perforin and Fas-based cytotoxic pathways are the two major mechanisms of cell-mediated cytotoxicity (12–14). Recently, we showed that both FasL/Fas and perforin/granzyme were significantly up-regulated in the acute phase of ARDS following sepsis (7). However, in that report, we could not investigate the specific mechanisms of apoptosis adequately on lung injury because of the evident restrictions of a clinical study.

Although the intratracheal LPS instillation murine model is a model of self-limited lung inflammation, it reproduces many features of sepsis-induced acute lung injury (17, 18). We

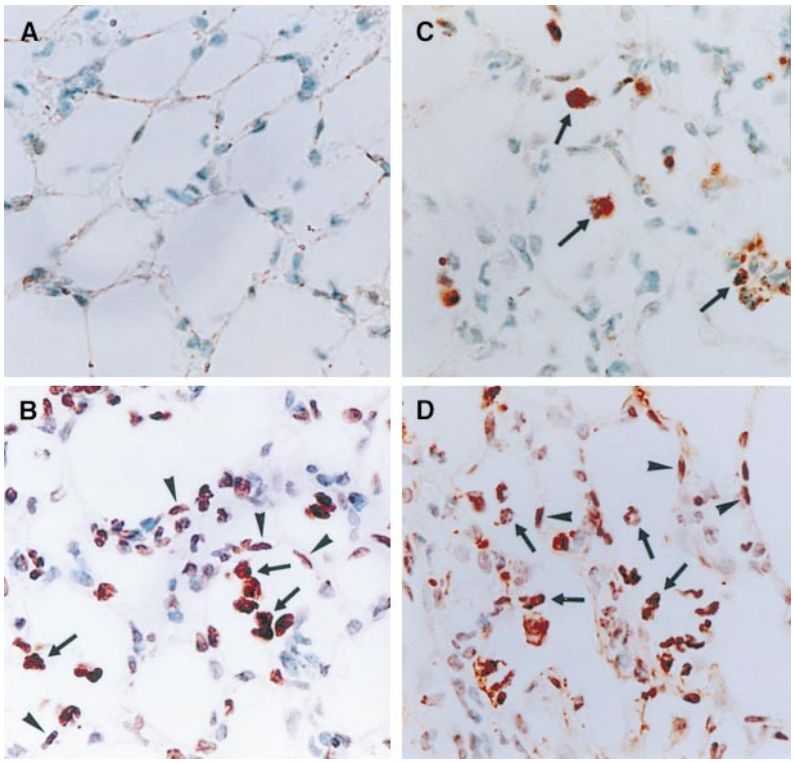

Figure 5. TUNEL staining of instilled lungs 1 d after instillation of 0.9% NaCl (*A*), 30 μg LPS (*B*), anti-Fas antibody + 30 μg LPS (*C*), and concanamycin A + 30 μg LPS (*D*). Positive staining is indicated by *black-brown* and the contrast background staining is *green*. Note that both alveolar wall cells and infiltrating inflammatory cells are positive in *B* and *D*, whereas only inflammatory cells are positive in *C*. *Arrows* with bars indicate inflammatory cells and *arrows* without bars indicate alveolar wall cells. Five mice were examined in each group. Original magnification: ×200.

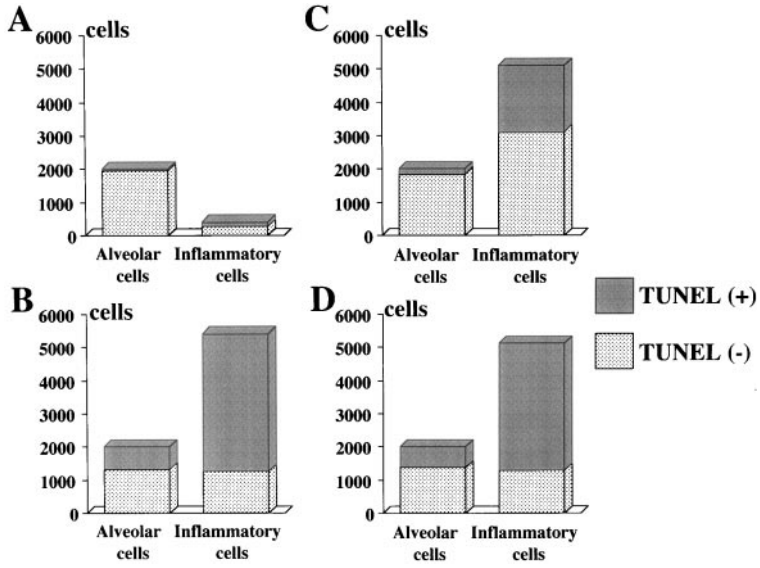

Figure 6. Semiquantitative measurement of apoptosis was done by counting TUNEL positive and negative alveolar wall cells and inflammatory cells. A pathologist who was blinded to the diagnosis of the slides counted the cells randomly using 15 to 20 fields (×400) until reaching a total number of 2,000 alveolar wall cells. (*A, B, C, D*) The same as in Figure 5.

therefore decided to carry out an experimental study using this murine model of acute lung injury. Several previous reports have indicated that lung injury following LPS instillation is initiated by a rapid influx of neutrophils into the air spaces within 24 h followed by excessive inflammation. There are several candidates to reduce lung injury, such as anti-tumor necrosis factor-α (TNF-α) antibodies or neutrophil elastase inhibitors, and most of the causative candidates for lung injury were thought to be neutrophils or alveolar macrophages (2–6). Involvement of proapoptosis molecules that are basically related to lymphocytes has only begun to become apparent.

In the present study, using the murine model of LPS-induced lung injury, we studied the expressions of FasL/Fas and perforin/granzyme in the alveolar cells and infiltrating inflammatory cells. Especially in the early stage, alveolar wall cells, namely epithelial type I and II cells and endothelial cells, are involved and showed a significant apoptotic change, which was demonstrated by TUNEL staining in this study. Unfortunately, so far we are unable to differentiate these cells properly because of the lack of appropriate antibodies for mice. We found no apoptotic changes in the noninstilled lungs that we used as control lungs. Thus, the data strongly suggest that LPS instillation caused a topical inflammatory response that was restricted to the exposed lung and that these processes induced apoptosis of epithelial type I and type II cells as well as endothelial cells in the instilled lung only.

Recently, Kataoka and colleagues (19) demonstrated that an inhibitor of the vascular H^+-ATPase, concanamycin A, inhibits perforin-based cytotoxic activity, mostly due to accelerated degradation of perforin by an increased pH of the lytic granules. Concanamycin A is a selective apoptosis inhibitor and is unable to inhibit the Fas-linked cytotoxic pathway (19). In this study, concanamycin A lacked protective effects in the acute phase of lung injury. However, the anti-Fas blocking antibody (P2) attenuated the lung injury. Consequently, the results suggested that between these two cytotoxic T cell-mediated apoptosis systems, the FasL/Fas system was involved in the pathogenesis of LPS-induced lung injury in mice. However, not *in vitro* but *in vivo*, the two pathways are complexly linked in the apoptosis changes of the target cells (20). The injured lung consequently showed strong mRNA expression of not only FasL/Fas but also of perforin/granzyme. So, from these results alone, we cannot conclude that the perforin/granzyme system was not active in LPS-induced lung injury in mice. Furthermore, P2 antibody treatment did not reduce mRNA and protein expressions of either Fas and FasL or neutrophil influx into the air spaces. It reduced the apoptosis only of inflammatory cells and alveolar wall cells. These observations

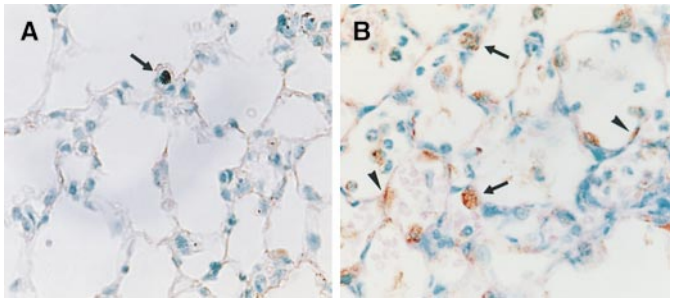

Figure 7. Immunohistochemical staining for Fas after instillation of 0.9% NaCl (*A*) and 30 μg LPS (*B*). Five mice were examined in each group in addition to five negative control mice (data not shown). Neither P2 (n = 5) nor concanamycin A (n = 5) altered the expression of Fas in the instilled lung after 30 μg LPS instillation (data not shown). Arrows with bars indicate inflammatory cells and arrows without bar indicate alveolar wall cells. Original magnification: ×200.

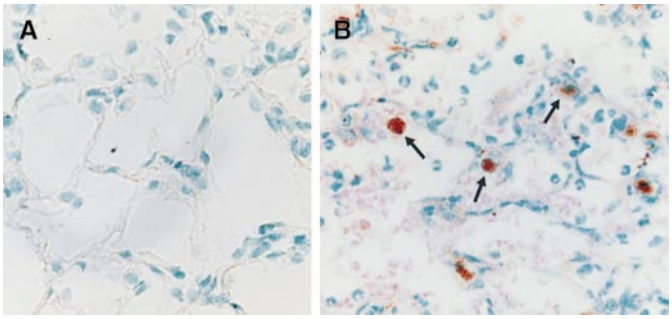

Figure 8. Immunohistochemical staining for FasL after the instillation of 0.9% NaCl (*A*) and 30 μg LPS (*B*). All positive cells are inflammatory cells. Five mice were examined in each group in addition to five negative control mice (data not shown). Neither P2 (n = 5) nor concanamycin A (n = 5) altered the expression of FasL in the instilled lung after 30 μg LPS instillation (data not shown). Original magnification: ×200.

indicate that the P2 antibody acts by blocking the final stage of apoptosis in the target cells.

As proapoptotic molecules are capable of inducing apoptosis in their target cells, they could play a role in the pathogenesis of the alveolar epithelial and endothelial cell injury seen in the acute phase of ALI. It is interesting to note that Fujita and colleagues (21) reported endothelial cell apoptosis in lung after intravenous administration of LPS in mice. In contrast, Hagimoto and colleagues (22) indicated that inhalation of a Jo2 antibody, which accelerated the FasL/Fas system, caused significant epithelial cell injury. Wiener-Kronish and colleagues (23) reported that simultaneous intravenous and intratracheal LPS administration was needed to produce a widespread destruction of epithelial and endothelial cells. The present study partly confirmed these findings in acute lung injury from several causes and indicated that injury could be related to apoptotic cell death in the air space. The immunohistochemical analysis showed that the expression of Fas was seen in the alveolar wall cells as well as in the infiltrating neutrophils and alveolar macrophages, whereas expression of FasL was limited to the macrophage-like cells in the air space. These findings and the P2 antibody study indicate that positive TUNEL signals both in the alveolar wall cells and inflammatory cells are closely related to the Fas/FasL system. Although several investigators suggested that FasL was expressed in several tissues other than CTLs (11, 24, 25), there has been no reports concerning the expression of FasL in macrophages. It is possible that some FasL-positive cells in the injured lungs may be lymphocytes. However, most of the FasL-positive cells were larger than neutrophils. Further investigations concerning this issue should be conducted.

The role of apoptosis remains unclear in the development of ALI and especially in the late process. However, some reports suggest that elimination of activated neutrophils was hindered in ARDS, which resulted in accumulation of neutrophils in the air spaces and destruction of the alveolar epithelial lining (26). This suggests that an appropriate expression of apoptosis molecules would be essential for resolution of lung injury. Hagimoto and colleagues (27) showed that Fas was expressed on alveolar epithelial cells and FasL was expressed on infiltrating lymphocytes in the early and late phase in lung fibrosis induced by bleomycin. They speculated that these effector molecules play an important role not only in damaging tissues by overexpression in the early phase, but also in remodeling of the cell layer lining in the alveolar spaces in the late phase of lung injury (27).

Moreover, an intact alveolar epithelium is of critical importance for the resolution of pulmonary edema and ARDS. Matthay and colleagues (28) demonstrated that clinical resolution of pulmonary edema was linked to an intact alveolar epithelium, that is, patients with permeability type pulmonary edema showed a less favorable prognosis for recovery than those with an intact alveolar epithelium. If significant apoptosis would be induced in the alveolar epithelium with accompanying cell death, this may affect the recovery and outcome of ARDS. Whether the expression of proapoptosis molecules affects the outcome in both experimental and clinical settings would certainly be a subject for further investigation.

In conclusion, we have shown that the two major independent pathways of apoptosis, the FasL/Fas system and the perforin/granzyme system, were involved in a murine model of LPS-induced acute lung injury. Inhibition of the FasL/Fas system, but not the perforin/granzyme system, attenuated acute lung injury seen after LPS instillation without affecting the mRNA/protein expressions nor neutrophil accumulation in the injured lungs. Similar up-regulations of proapoptosis pathways were also observed in our earlier clinical study of septic ARDS patients (7). The result here suggests that excessive apoptosis might be a causative factor for LPS-induced acute lung injury. To clinically control expression of apoptosis in ALI may prove important for the outcome of lung injury.

Acknowledgment: The authors thank Dr. Hans G. Folkesson (Department of Physiology at the Northeastern Ohio Universities College of Medicine, Rootstown, OH), Dr. Yoshifumi Tanaka from our department, and Dr. Yoji Urata (Department of Pathology at our university) for their useful comments in preparing this manuscript.

References

1. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, Legall JR, Morris A, Spragg R. The American-European Consensus Conference on ARDS: definitions, mechanisms, relevant outcomes, and clinical trial coordination. *Am J Respir Crit Care Med* 1994;149:818–824.
2. Weiland JE, Davis WB, Holter JF, Mohammed JR, Dorinsky PM, Gadek JE. Lung neutrophils in the adult respiratory distress syndrome: clinical and pathophysiologic significance. *Am Rev Respir Dis* 1986;133:218–225.
3. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am Rev Respir Dis* 1990;141:471–501.
4. Xing Z, Kirpalani H, Torry D, Jordana D, Gauldie J. Polymorphonuclear leukocytes as a significant source of tumor necrosis factor-alpha in endotoxin-challenged lung tissue. *Am J Pathol* 1993;143:1009–1015.
5. Goodman RB, Strieter RM, Martin DP, Steinberg KP, Milberg JA, Maunder RJ, Kunkel SL, Walz A, Hudson LD, Martin TR. Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1996;154:602–611.
6. Kooguchi K, Hashimoto S, Kobayashi A, Kitamura Y, Kudoh I, Wiener-Kronish JP, Sawa T. Role of alveolar macrophages in initiation and regulation of inflammation in *Pseudomonas aeruginosa* pneumonia. *Infect Immun* 1998;66:3164–3169.
7. Hashimoto S, Kobayashi A, Kooguchi K, Kitamura Y, Onodera H, Nakajima H. Upregulation of two death pathways of perforin/granzyme and FasL/Fas in septic acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2000;161:237–243.
8. Nagata S, Golstein P. The Fas death factor. *Science* 1995;267:1449–1456.
9. Suda T, Okazaki T, Naito Y, Yokota T, Arai N, Ozaki S, Nakao K, Nagata S. Expression of the Fas ligand in cells of T cell lineage. *J Immunol* 1995;154:3806–3813.
10. Watanabe-Fukunaga R, Brannan CI, Itoh N, Yonehara S, Copeland NG, Jenkins NA, Nagata S. The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. *J Immunol* 1992;148:1274–1279.
11. Liles WC, Kiener PA, Ledbetter JA, Aruffo A, Klebanoff SJ. Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes: implications for the regulation of apoptosis in neutrophils. *J Exp Med* 1996;184:429–440.
12. Lowin B, Hahne M, Mattmann C, Tschopp J. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* 1994;370:650–652.
13. Kägi D, Vignaux F, Ledermann B, Bürki K, Depraetere V, Nagata S, Hengartner H, Golstein P. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 1994;265:528–530.
14. Kojima H, Shinohara N, Hanaoka S, Someya Shirota Y, Takagaki Y, Ohno H, Saito T, Katayama T, Yagita H, Okumura K, *et al.* Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. *Immunity* 1994;1:357–364.
15. Hakuno N, Koji T, Yano T, Kobayashi N, Tsutsumi O, Taketani Y, Nakane PK. Fas/APO-1/CD95 system as a mediator of granulosa cell apoptosis in ovarian follicle atresia. *Endocrinology* 1996;137:1938–1948.
16. Folkesson HG, Weström BR, Pierzynowski SG, Karlsson BW. Lung to blood passage of different-sized molecules during lung inflammation in the rat. *J Appl Physiol* 1991;71:1106–1111.
17. Frevert CW, Huang S, Danaee H, Paulauskis JD, Kobzik L. Functional characterization of the rat chemokine KC and its importance in neutrophil recruitment in a rat model of pulmonary inflammation. *J Immunol* 1995;154:335–344.
18. Ulich TR, Yin S, Guo K, Yi ES, Remick D, del Castillo J. Intratracheal injection of endotoxin and cytokines: II. Interleukin-6 and transforming growth factor beta inhibit acute inflammation. *Am J Pathol* 1991;138:1097–1101.

19. Kataoka T, Shinohara N, Takayama H, Takaku K, Kondo S, Yonehara S, Nagai K. Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. *J Immunol* 1996;156:3678–3686.
20. Nakajima H, Park HL, Henkart PA. Synergistic roles of granzymes A and B in mediating target cell death by rat basophilic leukemia mast cell tumors also expressing cytolysin/perforin. *J Exp Med* 1995;181: 1037–1046.
21. Fujita M, Kuwano K, Kunitake R, Hagimoto N, Miyazaki H, Kaneko Y, Kawasaki M, Maeyama T, Hara N. Endothelial cell apoptosis in lipopolysaccharide-induced lung injury in mice. *Int Arch Allergy Immunol* 1998;117:202–208.
22. Hagimoto N, Kuwano K, Miyazaki H, Kunitake R, Fujita M, Kawasaki M, Kaneko Y, Hara N. Induction of apoptosis and pulmonary fibrosis in mice in response to ligation of Fas antigen. *Am J Respir Cell Mol Biol* 1997;17:272–278.
23. Wiener-Kronish JP, Albertine KH, Matthay MA. Differential responses of the endothelial and epithelial barriers of the lung in sheep to *Escherichia coli* endotoxin. *J Clin Invest* 1991;88:864–875.
24. Kuwano K, Miyazaki H, Hagimoto N, Kawasaki M, Fujita M, Kunitake R, Kaneko Y, Hara N. The involvement of Fas-Fas ligand pathway in fibrosing lung diseases. *Am J Respir Cell Mol Biol* 1999;20:53–60.
25. French LE, Hahne M, Viard I, Radlgruber G, Zanone R, Becker K, Muller C, Tschopp J. Fas and Fas ligand in embryos and adult mice: ligand expression in several immune-privileged tissues and coexpression in adult tissues characterized by apoptotic cell turnover. *J Cell Biol* 1996;133:335–343.
26. Matute-Bello G, Liles WC, Radella II F, Steinberg KP, Ruzinski JT, Jonas M, Chi EY, Hudson LD, Martin TR. Neutrophil apoptosis in the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1997; 156:1969–1977.
27. Hagimoto N, Kuwano K, Nomoto Y, Kunitake R, Hara N. Apoptosis and expression of Fas/Fas ligand mRNA in bleomycin-induced pulmonary fibrosis in mice. *Am J Respir Cell Mol Biol* 1997;16:91–101.
28. Matthay MA, Wiener-Kronish JP. Intact epithelial barrier function is critical for the resolution of alveolar edema in humans. *Am Rev Respir Dis* 1990;142:1250–1257.