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Relationship of Acute Lung Inflammatory Injury to Fas/FasL System

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There is mounting evidence that apoptosis plays a significant role in tissue damage during acute lung injury. To evaluate the role of the apoptosis mediators Fas and FasL in acute lung injury, Fas (*lpr*)- or FasL (*gld*)-deficient and wild-type mice were challenged with intrapulmonary deposition of IgG immune complexes. Lung injury parameters (¹²⁵I-albumin leak, accumulation of myeloperoxidase, and wet lung weights) were measured and found to be consistently reduced in both *lpr* and *gld* mice. In wild-type mice, lung injury was associated with a marked increase in Fas protein in lung. Inflamed lungs of wild-type mice showed striking evidence of activated caspase-3, which was much diminished in inflamed lungs from *lpr* mice. Intratracheal administration of a monoclonal Fas-activating antibody (Jo2) in wild-type mice induced MIP-2 and KC production in bronchoalveolar lavage fluids, and a murine alveolar macrophage cell line (MH-S) showed significantly increased MIP-2 production after incubation with this antibody. Bronchoalveolar lavage fluid content of MIP-2 and KC was substantially reduced in *lpr* mice after lung injury when compared to levels in wild-type mice. These data suggest that the Fas/FasL system regulates the acute lung inflammatory response by positively affecting CXC-chemokine production, ultimately leading to enhanced neutrophil influx and tissue damage. (*Am J Pathol* 2005, 166:685–694)

Acute lung injury and the acute respiratory distress syndrome as first described 30 years ago by Ashbaugh and

colleagues,¹ remain leading causes of morbidity and mortality in clinical settings.^{2,3} Fatality rates may exceed 50% and range from 10 to 90%, depending on etiology, severity, definition of acute respiratory distress syndrome, and the presence of pre-existing diseases.^{4–8} The pathogenesis of the inflammatory tissue injury in general and lung injury in particular is complex and inadequately understood. According to traditional concepts, acute lung injury is usually accompanied by extensive accumulation of activated inflammatory cells, including a predominance of neutrophils and progressive damage of the lung parenchyma accompanied by oxidative stress, resulting in edema and hemorrhage, and, in some instances progressive development of pulmonary fibrosis. However, there is increasing evidence that apoptosis (programmed cell death), known to be essential for the physiological process of cell turnover, may also play a key role in events related to cell death, removal of dead cells, and tissue damage.^{9–15} Fas-mediated apoptosis of alveolar epithelial cells may be important in fibrotic lung diseases.^{16–18} High levels of soluble Fas and FasL in bronchoalveolar lavage (BAL) fluids from acute lung injury/acute respiratory distress syndrome patients have been reported to correlate with increased mortality.^{10,15} Many inflammatory mediators such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-2, IL-6, IL-10, and G-CSF have been implicated in the regulation of apoptosis. Fas, a 48-kd type I-membrane protein that belongs to the TNF-receptor superfamily is expressed in various cells and tissues including neutrophils, alveolar macrophages, and lung tissue.^{19–22} The natural ligand, FasL, exists in a soluble and in a membrane-bound form as a 37-kd type II-membrane protein, which when bound to Fas is able to trigger the apoptotic cascade via activation of caspase-8.^{22–24}

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In this study, we have used the animal model of IgG immune complex-induced lung injury to evaluate the role of Fas and FasL in acute lung inflammation. By challenging lungs of mutant mice defective in Fas (*lpr*) or FasL (*gld*),²⁵ we found evidence that a defective Fas/FasL system in this model of acute lung injury was associated with an attenuated lung inflammatory response together with decreased CXC-chemokine content in lung, the consequence being reduced neutrophil recruitment and injury. These data suggest that the Fas/FasL system regulates the acute inflammatory response in lung.

Materials and Methods

Animals

For all experiments, specific pathogen-free male wild-type mice (C57BL/6), mutant mice defective in Fas receptor (*lpr*) or Fas ligand (*gld*) were obtained from Jackson Laboratory (Bar Harbor, ME). All strains had a C57BL/6 background. The mutations *lpr* and *gld* are autosomal recessive loss-of-function mutations located on chromosomes 19 and 1, respectively.²⁵ Mice were allowed free access to water and food. They were used at the age of 8 to 10 weeks (weighing 20 to 30 g). All experiments were done with Institutional Animal Care and Utilization Committee approval.

IgG Immune Complex-Induced Lung Injury

Mice were anesthetized intraperitoneally with ketamine, the trachea was surgically exposed by a midline incision, and 120 μ g of rabbit IgG antibody to bovine serum albumin ((BSA) (ICN Pharmaceuticals, Inc., Aurora, OH) in 40 μ l of phosphate-buffered saline (PBS) were administered intratracheally by tracheal puncture with a 30-gauge needle. The incision was closed by two surgical clips and 1 mg of BSA in a volume of 200 μ l was injected intravenously immediately thereafter.

Permeability Index

For permeability index measurements, BSA was labeled with ¹²⁵I by the chloramine T method. A trace amount of ¹²⁵I-BSA (specific activity 5 μ Ci/ μ g) was added to unlabeled BSA (5 mg/ml in PBS), and 200 μ l of this solution was injected intravenously to induce the IgG immune complex lung injury as described above. Four hours later, mice were euthanized with ketamine (given intraperitoneally) and blood was collected from the inferior vena cava. The thorax was opened, left atrium incised, and the lung was perfused *in situ* with PBS via the pulmonary artery. The flushed lungs were removed and permeability index (indicating the extent of pulmonary leakage) was determined by using a gamma counter and expressed as the ratio of counts per min (cpm) in the whole lung versus radioactivity in 100 μ l of blood.

Caspase Inhibitor Treatment

The pan-caspase inhibitor [Z-VAD(Ome)-FMK] and its inactive derivative (Z-FA-FMK) were purchased from Enzyme Systems Products (Livermore, CA). IgG immune complex lung injury was induced as described above. Either the caspase inhibitor or its inactive derivative (at a dose of 8 mg/kg body weight) was injected intravenously together with the radiolabeled BSA. Four hours later, the lung permeability index was determined.

Intratracheal Administration of Activating Anti-Fas Antibody (Jo2)

Either a monoclonal activating hamster anti-mouse Fas antibody (Jo2) or hamster IgG1 isotype control immunoglobulin (both purified, no azide, low endotoxin; BD Pharmingen, San Diego, CA) at a dose of 2 μ g/g was administered intratracheally by tracheal puncture. Animals were sacrificed after 4 hours, and lungs were lavaged with 0.8 ml of sterile, ice-cold PBS. The recovered BAL fluids were centrifuged (450 $\times$ g for 6 minutes) and the cell-free supernatant was used for determination of CXC-chemokine production by enzyme-linked immunosorbent assay (ELISA) (details described below).

Cell Culture

A murine alveolar macrophage cell line (MH-S) was purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (ATCC) containing 5% fetal calf serum, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 1% nonessential amino acids. Cells were seeded in plastic 48-well plates (2×10^5 cells/well) and incubated at 37°C in 5% CO₂ overnight and grown to 80% confluence before being used for further experiments. Cells were then washed once with RPMI medium and incubated with activating anti-mouse Fas antibody (Jo2) or isotype control IgG1 (both purified, no azide, low endotoxin; BD Pharmingen) at concentrations of 0.1, 0.25, and 0.5 μ g/ml in RPMI. After 6 hours of incubation, supernatants were aspirated, clarified by centrifugation, and analyzed by ELISA for chemokine production. To assure intact cell viability at the Fas-activating antibody concentrations tested, an MTT-assay was performed. The yellow tetrazolium MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] is thereby reduced by metabolically active cells. The resulting intracellular purple formazan was solubilized and quantified by spectrophotometric means at 560 nm.

Myeloperoxidase (MPO) Assay

Lungs were removed and snap-frozen in liquid nitrogen 4 hours after induction of lung injury. Lungs were then homogenized in 50 mmol/L potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide and 5 mmol/L ethylenediaminetetraacetic acid, and the aliquots were briefly sonicated at 4°C. After cen-

trifugation at $12,000 \times g$ for 10 minutes at 4°C , the supernatant fluids were incubated in 50 mmol/L potassium phosphate buffer containing the substrate H_2O_2 . The enzymatic activity was determined in the presence of *o*-dianisidine dihydrochloride by measuring the kinetics of absorbance at 460 nm throughout 3 minutes.

Assessment of Wet Lung Weight

Lungs were removed and lung weights determined using an electronic balance (sensitivity, 0.0001 g).

Histology of the Lungs

After sacrifice of mice, lungs were instilled with 0.9 ml of 3.7% buffered formaldehyde in PBS (pH 7.4) via a tracheal cannula, removed from the thorax, and immersed in 3.7% buffered formaldehyde for 24 hours before paraffin embedding and routine histological processing. Six- μm -thick sections of lungs were placed on standard microscope slides and stained with hematoxylin and eosin (H&E).

BAL Fluid Collection, Total and Differential White Blood Cell Counts, and Chemokine/Cytokine ELISAs

Four hours after initiation of the acute lung injury, the thorax was opened and 0.8 ml of ice-cold, sterile PBS was instilled into the lung via a tracheal incision. The recovered lavage fluid (BAL) was centrifuged at $450 \times g$ for 6 minutes and the cell-free supernatants were stored at -20°C . Cell pellets were resuspended in 1 ml of Hanks' balanced salt solution containing 0.5% BSA and differential cell analyses were performed by Diff-Quik-stained cytopspin preparations (Dade, Duedingen, Switzerland) counting a total of 300 cells per slide in randomly selected high-powered fields ($\times 1000$). The supernatant was used for chemokine and cytokine measurements by sandwich ELISA. ELISA plates were coated overnight at 4°C with 5 $\mu\text{g}/\text{ml}$ of capture antibody per well. After blocking with 3% BSA in PBS, the samples were added to the 96-well plates and incubated for 2 hours, followed by incubation with the biotinylated secondary antibody (2 $\mu\text{g}/\text{ml}$) for 1 hour. After washing, peroxidase-conjugated streptavidin was added for 30 minutes followed by incubation with *o*-phenylenediamine dihydrochloride (peroxidase substrate) for 10 minutes, and the reaction was stopped with 0.5 mol/L sulfuric acid. Optical density was measured at 490 nm.

Western Blot Analysis

Whole lung extracts were obtained 4 hours after IgG immune complex deposition. Protein concentrations in lung lysates were determined by bicinchoninic acid assay with trichloroacetic acid precipitation using BSA as a reference standard (Pierce Biotechnology, Rockford, IL). Samples containing 25 μg of protein were electropho-

resed in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and then transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with Tris-buffered saline plus Tween (TBST; 40 mmol/L Tris, pH 7.6, 300 mmol/L NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 12 hours at 4°C . Membranes were incubated with the following antibodies: rabbit polyclonal IgG to activated (cleaved) caspase-3 (Cell Signaling Technology, Inc., Beverly, MA) at a 1:1000 dilution, or mouse monoclonal Fas antibody (clone 5F9; Kamiya Biomedical Co., Seattle, WA) at a 1:333 dilution at 4°C overnight. After five washes in TBST, membranes were incubated at a 1:10,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG or sheep anti-mouse IgG, respectively (Amersham Biosciences Corp, Piscataway, NJ). The membranes were developed by enhanced chemiluminescence technique according to the manufacturer's protocol (Amersham).

Statistical Analysis

Results were expressed as mean $\pm$ SEM. Two-way analysis of variance with posthoc Bonferroni's multiple comparison test and *t*-test were used to detect significant differences between means. All statistical analyses were performed using Prism 2.0 (GraphPad Software, San Diego, CA). *P* values less than 0.05 were considered significant.

Results

Lung Permeability, MPO Activity, and Wet Lung Weights in Wild-Type and Fas-Deficient (*lpr*) Mice after Acute Lung Injury

IgG immune complex-induced lung injury was evaluated in Fas-deficient mice and C57BL/6 wild-type mice 4 hours after deposition of IgG immune complexes. As shown in Figure 1, lung vascular leak of albumin (permeability index) (Figure 1A), MPO content (Figure 1B), and wet lung weights (Figure 1C) after deposition of IgG immune complexes were significantly increased in inflamed lungs of wild-type and *lpr* mice when compared to the corresponding uninjured animals ($*P < 0.001$). In the lung injury groups, *lpr* mice had significant attenuation of the permeability index (decreased by 61%, $**P < 0.001$), MPO activity (decreased by 35%, $**P < 0.01$) and wet lung weights (decreased by 9%, $**P < 0.05$) when compared to wild-type mice. As expected, lungs from uninjured wild-type and *lpr* mice showed much lower values and did not reveal differences between groups in any of these parameters.

Total and Differential White Blood Cell Counts in BAL Fluids of Wild-Type and Fas-Deficient (*lpr*) Mice after Lung Injury

Cell content of BAL fluids from wild-type and Fas-deficient mice was evaluated 4 hours after IgG immune com-

Figure 1. Lung injury parameters permeability index (A), MPO content (B), and wet lung weights (C) 4 hours after IgG immune complex deposition in wild-type (WT IC) and *lpr* mice (lpr IC). For each vertical bar, $n \geq 5$ animals. Two-way analysis of variance: factor 1 (condition), factor 2 (strain), and the interaction between the factors showed significant effects on the permeability index and MPO content. For wet lung weights the effect of factor 1 was significant, factor 2 not quite significant ($P = 0.0541$), and the interaction was not significant. Bonferroni's post tests: *, $P < 0.001$ when compared to the corresponding uninjured animals (WT Ctrl and lpr Ctrl); **, $P < 0.001$ (A); **, $P < 0.01$ (B); and **, $P < 0.05$ (C) when compared to immediately preceding vertical bar.

plex deposition. As shown in Figure 2A, the total number of white blood cells increased by approximately ninefold in mice with injury (WT IC and *lpr* IC) when compared to control animals ($*P < 0.001$). The cells in BAL fluids from noninjured lungs were uniformly mononuclear macrophages. In injured lungs from wild-type and *lpr* mice the most cells were neutrophils (70% and 80%) followed by macrophages (30% and 20%) (Figure 2B). Other cell

types were present in insignificant numbers. Neither total nor differential cell counts were statistically different between wild-type and *lpr* mice with injury.

Histopathology of Lung Tissue in Mice after Lung Injury

Morphological changes were evaluated 4 hours after the onset of acute lung injury in *lpr* mice and wild-type mice. Shown in Figure 3 are lung sections from uninjured wild-type (Figure 3A) and *lpr* mice (Figure 3C) as well as from wild-type (Figure 3B) and *lpr* mice (Figure 3D) after immune complex deposition. In the presence of IgG immune complexes, lungs from wild-type mice showed extensive neutrophil accumulation, intra-alveolar hemorrhage, and fibrin deposits (Figure 3B). In *lpr* animals, all of these features were attenuated (Figure 3D).

Chemokine/Cytokine Levels in BAL Fluids from Lungs

In Figure 4, BAL fluids from lungs of C57BL/6 wild-type mice and *lpr* mice 4 hours after deposition of IgG immune complexes (IC) showed increased content of the CXC-chemokines KC and MIP-2 (Figure 4, A and B) when compared to corresponding control BAL fluids (Ctrl) from lungs ($*P < 0.001$). However, the levels of both chemokines were significantly reduced by 34% (Figure 4A) and by 37% (Figure 4B) ($**P < 0.001$) in *lpr* mice when compared to BAL fluids from injured wild-type mice. MCP-1 and TNF- α levels were also elevated in wild-type and *lpr* mice ($*P < 0.001$), but lower than those of KC and MIP-2 and not different in WT and *lpr* mice (Figure 4, C and D). Baseline BAL content (Ctrl) was barely measurable and did not differ between wild-type and *lpr* animals for all four chemokines.

Fas Protein Expression after IgG Immune Complex Deposition in the Lungs of Wild-Type Mice

Lung homogenates were evaluated by Western blot analysis for the presence of Fas in control lungs and in lungs of wild-type mice 4 hours after deposition of IgG immune complexes. The results, shown in Figure 5, revealed little if any detectable Fas in uninjured (Ctrl) lungs obtained from wild-type mice. However, there was a dramatic up-regulation of Fas protein in lungs of mice after deposition of immune complexes. This suggests that immune complex deposition in lungs of wild-type mice causes induction of Fas.

Activated Caspase-3 in Wild-Type and Fas-Deficient (lpr) Lungs after Injury

Activation of cellular caspase-3 is a key event leading to apoptosis. Its activation requires proteolytic processing of the caspase-3 zymogen into activated p17 and p12

Figure 2. Total cell counts (**A**) in BAL fluids 4 hours after onset of lung injury. Each vertical bar represents $n \geq 5$ animals of noninjured wild-type mice (Ctrl), injured wild-type (WT IC), and injured *lpr* (*lpr* IC) mice. *, $P < 0.001$ when compared to noninjured mice (Ctrl); no statistical significant differences (n.s.) between injured wild-type and *lpr* animals (WT IC versus *lpr* IC). Differential white blood cell counts (**B**) in the same BAL fluids. No significant differences (n.s.) in neutrophil and mononuclear cell counts are found between the injured animal groups (WT IC and *lpr* IC); n.d., not detectable.

subunits. A polyclonal antibody was used to detect activated caspase-3 (p17) in lung lysates by Western blotting (Figure 6). Homogenates from injured wild-type and *lpr* mouse lungs showed the presence of the 17-kd cleaved

form of caspase-3 protein 4 hours after initiation of the IgG immune complex injury, whereas none was found in uninjured (Ctrl) wild-type lungs. In comparison to lungs from injured wild-type mice, the amount of the activated

Figure 3. H&E-stained paraffin-embedded lung sections (6 μ m) from wild-type (**A**, **B**) and *lpr* mice (**C**, **D**). **A** and **C**: Noninjured lungs. **B** and **D**: Lungs 4 hours after intrapulmonary deposition of IgG immune complexes. Shown are representative sections of at least three animals for each condition. Original magnifications, $\times 400$.

Figure 4. BAL fluid content of KC (A), MIP-2 (B), MCP-1 (C), and TNF- α (D) in wild-type (WT) and Fas-deficient (*lpr*) animals from noninjured (Ctrl) and injured (IC) lungs 4 hours after onset of injury. For each vertical bar, $n \geq 5$. Two-way analysis of variance: factor 1 (condition), factor 2 (strain), and the interaction between the factors showed significant effects on content of KC and MIP-2. For MCP-1 and TNF- α only the effect of factor 1 was significant, whereas factor 2 and the interaction were not significant. Bonferroni's post tests: *, $P < 0.001$ when compared to the corresponding noninjured lungs; **, $P < 0.001$ (A and B) relative to inflamed WT lungs.

caspase-3 fragment was lower in the Fas-deficient animal group. These findings indicate that caspase-3 activation is attenuated in IgG immune complex-injured lungs from *lpr* mice.

Effects of the Caspase Inhibitor Z-VAD-FMK on Lung Permeability, MPO Activity, and Wet Lung Weight in Injured Lungs

Lung permeability in wild-type and *lpr* mice after immune complex deposition was evaluated after treatment with

Figure 5. Western blot analysis for Fas protein in lung homogenates from normal (WT Ctrl) lungs and 4 hours after onset of injury (WT IC). Equivalent amounts of protein (25 μ g) were loaded in each lane. For each group, $n = 2$. Lower frame is relative density.

Figure 6. Western blot analysis of lung homogenates for activated (cleaved) caspase-3 (17 kD) in noninjured (WT Ctrl) lungs and injured lungs from wild-type (WT IC) and *lpr* mice (*lpr* IC), 4 hours after IgG immune complex deposition. Equal amounts of protein (25 μ g) were loaded in each lane. For each group, $n = 2$. Lower frame is the corresponding relative density. Data are representative of three separate and independent experiments.

the pan-caspase inhibitor Z-VAD-FMK (Figure 7A). As expected, IgG immune complex deposition caused a dramatic increase in permeability index in both wild-type and *lpr* mice (IC) when compared to the corresponding negative control animals (Ctrl) (* $P < 0.001$). In wild-type mice, intravenous injection of the caspase inhibitor Z-VAD at the onset of lung injury (IC+Z-VAD) resulted in significant attenuation of the lung permeability index (by 36%, ** $P < 0.05$). Intravenous administration of the inactive derivative of Z-VAD, Z-FA, into mice undergoing acute lung injury failed to show inhibitory effects (IC+Z-FA). In *lpr* animals, the lung permeability index after immune complex deposition (IC) increased to a lower extent than in wild-type mice (as expected; see Figure 1A). Treatment of the *lpr* animals with Z-VAD did not further suppress lung permeability.

MPO content (Figure 7B) and wet lung weights (Figure 7C) did not show any significant differences between wild-type mice with immune-complex deposition only and injured mice that had in addition either the caspase inhibitor Z-VAD or its inactive derivative Z-FA injected. However, a statistically significant difference in lung weight (Figure 7C) was detectable between injured mice plus Z-VAD treatment compared to mice injected with Z-FA (** $P < 0.05$). This decrease in lung weight by Z-VAD (compared to the treatment with the inactive derivative) is in keeping with the reduced permeability index seen in these mice. All injured animal groups showed a significant increase for both parameters in comparison to control mice (* $P < 0.01$).

Reduced Lung Inflammatory Response in Fas Ligand-Deficient (*gld*) Mice

Experiments described above using Fas-deficient (*lpr*) mice (Figure 1) were repeated in mice deficient for FasL (*gld*) and compared to results in wild-type mice (Figure 8). Similar to the findings in *lpr* animals, the lung injury parameters (permeability index, MPO, and wet lung weight) after deposition of IgG immune complexes were

Figure 7. Lung permeability (**A**) in wild-type and *lpr* noninjured mice (Ctrl), injured (IC) mice, injured mice treated with the pan-caspase inhibitor (IC+Z-VAD), or its inactive derivative (IC+Z-FA) 4 hours after induction of lung injury. *, $P < 0.01$ when compared to the noninjured control (Ctrl) lungs; **, $P < 0.05$ when compared to immediately preceding and following vertical bar; n.s., no significant difference. MPO (**B**) and wet lung weights (**C**) in wild-type mice under the same conditions as in **A** (Ctrl, IC, IC+Z-VAD, and IC+Z-FA). *, $P < 0.001$ when compared to the noninjured control (Ctrl) lungs (**B** and **C**); **, $P < 0.05$ when compared to immediately preceding vertical bar (**C**); n.s., no significant difference; for each vertical bar, $n \geq 5$.

significantly increased in wild-type and *gld* mice [$*P < 0.001$ (Figure 8, A and B) and $*P < 0.01$ (Figure 8C)] when compared to the corresponding noninjured mice. In the lung injured groups, the permeability index (Figure 8A), lung MPO content (Figure 8B), and wet lung weights (Figure 8C) were significantly reduced in *gld* mice, by 35% ($**P < 0.05$), 34% ($**P < 0.001$), and 13% ($**P < 0.05$), respectively, when compared to injured wild-type animals. These data are consistent with those obtained in *lpr* mice (Figure 1).

Fas-Induced CXC-Chemokine Production in Vivo and in Vitro

To assess if activating anti-Fas IgG can induce mediator release, the Fas-activating monoclonal antibody (mAb) Jo2 was used both *in vivo* and *in vitro*. As shown in Figure

Figure 8. Lung injury parameters permeability index (**A**), MPO content (**B**), and wet lung weights (**C**) 4 hours after IgG immune complex deposition in wild-type (WT IC) and *gld* mice (*gld* IC). For each vertical bar, $n \geq 5$ animals. Two-way analysis of variance: factor 1 (condition), factor 2 (strain), and the interaction between the factors showed significant effects on permeability index and MPO content. For wet lung weights the effect of factor 1 and factor 2 were significant, but not the interaction between the factors. Bonferroni's post tests: *, $P < 0.001$ (**A** and **B**) and *, $P < 0.01$ (**C**) when compared to the corresponding noninjured animals (WT Ctrl and *gld* Ctrl); **, $P < 0.05$ (**A** and **C**) or **, $P < 0.001$ (**B**) when compared to the immediately preceding vertical bar.

9, intratracheal administration of the Jo2-antibody in wild-type mice resulted in the induction of both MIP-2 and KC in BAL fluids when compared to animals treated with the isotype control IgG ($*P < 0.05$) (Figure 9A). *In vitro*, the Fas-activating mAb induced MIP-2 release in a murine alveolar macrophage cell line (MH-S) (Figure 9B). A concentration-dependent, significant increase was measured at concentrations of 0.25 and 0.5 $\mu\text{g/ml}$ of anti-Fas mAb ($*P < 0.01$). No KC production occurred in these

Figure 9. **A:** MIP-2 and KC levels in BAL fluids of wild-type mice 4 hours after intratracheal administration of Fas-activating monoclonal antibody (mAb) Jo2 or isotype control IgG. *, $P < 0.05$ when compared to isotype control IgG; for each condition, $n = 6$; n.d., not detectable. **B:** MIP-2 content in cell culture supernatants of murine alveolar cell line macrophages (MH-S) treated with different doses of either anti-Fas mAb (Jo2) or isotype control IgG for 6 hours. Shown are representative data from two separate and independent experiments; for each condition, $n = 3$. Two-way analysis of variance: factor 1 (dose) and factor 2 (antibody) showed significant effects on MIP-2 content whereas the interaction between the two factors was not significant. Bonferroni's post tests: *, $P < 0.01$ when compared to the corresponding isotype IgG control.

cells (data not shown). Cell viability was preserved under the different conditions (determined by MTT assay, data not shown).

Discussion

The main goal of this study was to determine whether the Fas/Fas ligand system might affect the outcome of lung inflammation caused by IgG immune complex deposition in lung. This model has been extensively characterized in terms of the pathophysiological events involved. According to traditional concepts, IgG immune complex-in-

duced lung injury appears to be associated with interactions between inflammatory cells (neutrophils and macrophages), alveolar epithelial cells, and endothelial cells.²⁶ Early response cytokines such as TNF- α and IL-1 β together with the complement activation product, C5a, initiate a proinflammatory cascade resulting in up-regulation of adhesion molecules, transmigration of neutrophils into the alveolar compartment and lung interstitium, and release of proteases and reactive oxygen radicals, which are linked to tissue damage.^{27–32}

Beyond the integrated interplay of the complement, the cytokine/chemokine systems, adhesion molecules, and oxidants, a critical role for apoptosis in the pathogenesis of various lung inflammatory processes has been suggested.^{10,16,17,33} Intratracheal administration of a Fas-blocking antibody resulted in attenuated lipopolysaccharide-induced lung injury in mice,¹³ and intranasal instillation of a Fas-activating antibody (Jo2) in *lpr* mice did not lead to acute lung inflammation, whereas wild-type mice developed acute alveolar epithelial injury.³⁴ Fas/FasL-dependent apoptosis of epithelial and endothelial cells has also been demonstrated in a murine model of lipopolysaccharide-induced lung injury.^{13,14} Furthermore, mutant mice defective in Fas (*lpr*) or FasL (*gld*) showed after challenge with IL-12 decreased endothelial damage in liver and spleen but not in the lungs.⁹ In contrast to these studies suggesting a proinflammatory role of Fas activation, it has also been reported that Fas-mediated apoptosis may be protective after intranasal challenge with *Pseudomonas aeruginosa*. Inoculation of normal mice led to a rapid induction of apoptosis in epithelial cells of small bronchi, whereas lungs from infected *lpr* or *gld* mice did not show apoptotic cells. Interestingly, intranasal bacterial challenge of the *lpr* and *gld* mice resulted in severe sepsis and 100% of the animals died, in striking contrast to results in wild-type mice in which the mortality rate was only 10%.³⁵ The authors suggest a beneficial role of early cell apoptosis of bronchial epithelial cells in effective host defense during intranasal *P. aeruginosa* infection.

We have demonstrated for the first time in the highly neutrophil-dependent IgG immune complex-induced lung injury model that *lpr* mice (with functional loss of Fas) as well as *gld* animals (deficient in FasL) have significantly reduced lung inflammatory responses and reduced lung injury after immune complex deposition. This was indicated by reduced permeability index, wet lung weights, and MPO content (Figures 1 and 8). The Fas transcript was up-regulated in lungs of wild-type and *lpr* mice with IgG immune complex-induced lung injury (data not shown), even though the Fas receptor is known to be nonfunctional in the mutant mouse. Furthermore, Fas protein was up-regulated in this model of acute lung inflammation (Figure 5). Because ligation of the Fas ligand to Fas ultimately leads to endonuclear DNA degradation through activation of caspase-3 and the specific DNase, CAD,³⁶ Western blot analysis revealed activated (cleaved) caspase-3 in lungs after IgG immune complex deposition, but at a clearly lower extent in *lpr* mice (Figure 6). Involvement of caspase activation in this inflammatory process was further supported by the finding that intra-

venous injection into wild-type mice of a pan-caspase inhibitor (Z-VAD-FMK) was protective, as indicated by a decreased lung permeability index and lung weights (Figure 7, A and C). Administering the pan-caspase inhibitor into *lpr* animals did not demonstrate any further protective effect (Figure 7A). Interestingly, MPO content did not change with Z-VAD treatment. Studies by Rowe and colleagues³⁷ have shown that neutrophils from caspase-1-deficient mice have delayed apoptosis. It seems possible that Z-VAD may also protect infiltrated neutrophils from undergoing apoptosis and thus increase their life span in the lung. The longer-lived neutrophils in the lung tissue may counterbalance the protective effects of Z-VAD in lung injury involving endothelial and epithelial cells. The overall net effect, however, would still be an attenuated injury (indicated by reduced permeability index and lung weight) after Z-VAD administration as shown in Figure 7, A (left) and C. Whether the reduced lung inflammatory response in Fas- and FasL-deficient mice has any relevance to reduced apoptosis *in vivo* is not currently known. What seems to be clear is that, in mice with Fas or FasL deficiency, there is reduced CXC-chemokine production. The reasons for this are not known.

Neutrophil transmigration into the alveolar compartment and lung interstitium plays a crucial role in the development of acute lung inflammation. It is assumed that the production of chemoattractant chemokines precedes neutrophil influx. The CXC-chemokines, MIP-2 and KC, are among the most potent chemoattractants for rodent neutrophils.³² Blocking studies with MIP-2 and KC antibodies in lungs significantly decreased MPO buildup, reflective of reduced neutrophil accumulation and less lung injury in the IgG immune complex model.³⁸ Interestingly, in the current study we demonstrated a significantly lower MPO content in *lpr* and *gld* mice, indicating a role for the Fas/FasL system in neutrophil accumulation in the lungs. However, total cells and neutrophil counts in BAL fluids were similar in wild-type and Fas-deficient (*lpr*) mice (Figure 2). This suggests that a small population of neutrophils rapidly migrates into the alveolar compartment, while most neutrophils are in the interstitial compartment and are clearly reduced in number in *lpr* lungs. Fas ligation has been reported earlier to stimulate production of various inflammatory mediators in different cell types^{39–42} including bronchiolar epithelial cells.⁴³ In a murine macrophage cell line, Fas-mediated TNF- α secretion has been observed,⁴⁴ and Fas-induced IL-1 β production has been found in normal murine peritoneal macrophages.⁴⁵ TNF- α and IL-8 release by human monocytes and monocyte-derived macrophages has been reported after Fas ligation.⁴⁶ In the present study, we found significantly reduced levels of the neutrophil attracting chemokines, MIP-2 and KC, in the lungs of injured *lpr* mice 4 hours after onset of injury (Figure 4), suggesting a direct role of the Fas/FasL system in neutrophil recruitment to the lung through expression of CXC-chemokines. Intratracheal administration of the Jo2-antibody in wild-type mice also led to a significant stimulation of MIP-2 and KC release (Figure 9A). *In vitro*, alveolar mouse macrophages (MH-S cells) incubated with a Fas-

activating monoclonal (Jo2) antibody showed a significant increase in MIP-2 production (Figure 9B). These experiments are confirmative for a linkage between Fas-induced, apoptosis-independent CXC-chemokine production, neutrophil recruitment, and lung inflammation.

In summary, we present evidence that an intact Fas/FasL system is required for the full development of IgG immune complex-induced lung inflammatory injury in mice. Mutant mice defective in Fas (*lpr*) or FasL (*gld*) were significantly protected from acute lung inflammation and lung edema formation after intrapulmonary deposition of IgG immune complexes. Two mechanisms appear to be involved. First, direct activation of the Fas/FasL signaling pathway occurred, as evidenced by up-regulation of Fas protein and activated caspase-3, a key protein in the apoptotic signal transduction, in the lungs of IgG immune complex-challenged animals. Second, CXC-chemokine production (MIP-2 and KC) is somehow linked to Fas and FasL and ultimately contributes to neutrophil accumulation in lung during inflammation. This CXC-chemokine response was diminished in Fas-deficient (*lpr*) mice. To our knowledge, this is the first reported *in vivo* and *in vitro* evidence about Fas-induced production of the neutrophil attractant chemokines MIP-2 and KC in an acute lung inflammatory animal model.

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