

## Sustained Lipopolysaccharide-Induced Lung Inflammation in Mice Is Attenuated by Functional Deficiency of the Fas/Fas Ligand System

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Received 17 March 2003/Returned for modification 31 October 2003/Accepted 26 November 2003

**To determine whether the Fas/Fas ligand (FasL) (CD95/CD178) system contributes to the development of an inflammatory response in vivo, 2.5 µg of bacterial lipopolysaccharide (LPS; endotoxin) per g was administered intranasally to healthy mice (C57BL/6) and mutant mice deficient in either Fas (*lpr* mice) or FasL (*gld* mice). Sustained LPS-induced neutrophilic inflammation in the lungs was attenuated in both *lpr* and *gld* mice. These observations provide further evidence of a proinflammatory role for the Fas/FasL system in the lungs.**

The Fas/Fas ligand (FasL) (CD95/CD178) system is recognized for its importance in the regulation of apoptosis in a wide variety of tissues. As a “death receptor,” Fas is generally considered to exclusively transmit a death signal, resulting in a biologically silent, noninflammatory mechanism for the elimination of cells, especially during the regulation and resolution of immune and inflammatory responses (6, 13). However, accumulating evidence indicates that the Fas/FasL system may also preferentially signal cellular proinflammatory responses associated with tissue injury under certain circumstances (1, 4, 14). Data from both animals and humans implicating the Fas/FasL system as a potential mediator of acute inflammatory lung injury have previously been reported (7–10).

A growing body of literature suggests that a complex interaction exists between bacterial lipopolysaccharide (LPS; endotoxin)-induced inflammatory responses and the Fas/FasL system. Experimental evidence suggests that LPS may induce apoptosis via Fas-associated death domain (FADD)-dependent mechanisms (3). LPS has been reported to initiate caspase activation and apoptosis in endothelial cell lines via a CD14- and FADD-dependent mechanism (3). In another study, mice developed lung endothelial injury, as well as evidence of apoptosis in neutrophils (polymorphonuclear leukocytes [PMN]), macrophages, and cells of the alveolar walls, within 24 h of intratracheal instillation of LPS (5). These changes were attenuated by the administration of a Fas inhibitory antibody, suggesting that the Fas/FasL system participates in apoptotic lung injury induced by intratracheal LPS in mice. In a third study, it was shown that FADD modulates proinflammatory responses in endothelial cells. Specifically, the overexpression of FADD blocked the activation of NF-κB

following LPS exposure in an endothelial cell line (2). Furthermore, LPS-induced expression of the cytokines interleukin-6 and KC was enhanced in FADD-deficient murine fibroblasts (2).

The present study was conducted to determine whether the Fas/FasL system plays a role in the development of an inflammatory response to LPS in vivo. The response to intranasal instillation of LPS (2.5 µg/g) was examined in healthy mice (C57BL/6) and mutant mice deficient in either Fas (*lpr* mice) or FasL (*gld* mice).

### MATERIALS AND METHODS

The animal protocol was approved by the Animal Care Committee of the University of Washington, Seattle. Briefly, male mice weighing 20 to 30 g were anesthetized with inhaled halothane. The mice were either C57BL/6 mice (B&K Universal, Seattle, Wash.) or naturally occurring mutant mice lacking the Fas receptor (*lpr* mice) or FasL (*gld* mice) (Jackson Laboratories) on the C57BL/6 background. Each mouse received *Escherichia coli* O111:B4 LPS (Sigma, St. Louis, Mo.) at a dose of 2.5 µg/g by intranasal instillation as previously described (11). The animals were allowed to recover from anesthesia, returned to their cages, and given free access to water and food. After 6, 24, or 48 h, the animals were euthanized with ketamine and xylazine, their thoraxes were rapidly opened, and the animals were exsanguinated by direct cardiac puncture. The lungs were dissected free, and the tracheas were cannulated in order to perform bronchoalveolar lavage (BAL) or to fix the lungs. BAL was performed by instilling 0.9% NaCl containing 0.6 mM EDTA in two separate 0.5-ml aliquots. The BAL fluid (BALF) was recovered by gentle suction and placed on ice for immediate processing. The lungs were fixed by inflation with 10% neutral buffered formalin at a transpulmonary pressure of 15 cm H<sub>2</sub>O and embedded in paraffin. An aliquot of BALF was processed immediately for total and differential cell counts. Total cells were counted with a hemacytometer, and differential cell counts were obtained from cytospin preparations stained with modified Wright-Giemsa stain (Diff-Quik; American Scientific Products, McGaw Park, Ill.). The remainder of the lavage fluid was spun at 200 × g for 30 min, and the supernatant was removed aseptically and stored in individual aliquots at –70°C. The total protein concentration in BALF was measured by using the bicinchoninic acid method (Pierce Co., Rockford, Ill.). The cytokines KC and tumor necrosis factor alpha (TNF-α) were measured in BALF by using commercially available immunoassays (R&D Systems, Minneapolis, Minn.). Comparisons among groups were made with factorial analysis of variance and the Tukey post hoc test with GraphPad InStat software (San Diego, Calif.). A *P* value of <0.05 was considered significant.

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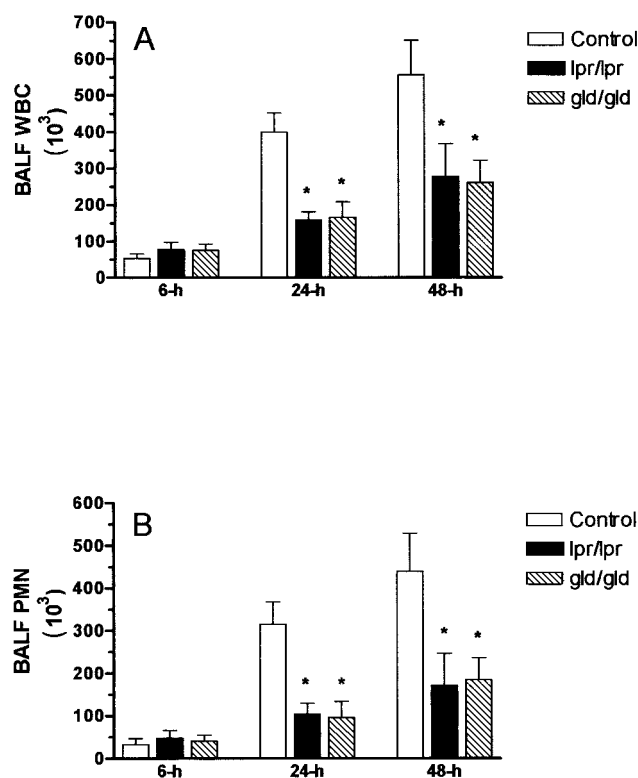


FIG. 1. Total numbers of WBC (A) and PMN (B) in BALF from wild-type C57BL/6, Fas-deficient *lpr*, and FasL-deficient *gld* mice 6, 24, and 48 h after intranasal administration of *E. coli* LPS (2.5  $\mu$ g/g). Asterisk,  $P < 0.05$ .

## RESULTS

All of the animals survived for the duration of the experiments. Six hours after intranasal instillation of LPS, the numbers of leukocytes in BALF specimens were similar for the three groups of mice (Fig. 1). In contrast, at 24 and 48 h, BALF from the *lpr* and *gld* animals contained significantly fewer white blood cells (WBC) than BALF from wild-type C57BL/6 animals ( $P < 0.05$ ) (Fig. 1A). The difference in numbers of WBC was due to the presence of lower numbers of PMN in the BALF from *lpr* animals [PMN at 24 h,  $(104 \pm 25) \times 10^3$ ; PMN at 48 h,  $(171 \pm 75) \times 10^3$ ] and *gld* animals [PMN at 24 h,  $(96 \pm 37) \times 10^3$ ; PMN at 48 h,  $(184 \pm 52) \times 10^3$ ] than in wild-type C57BL/6 animals [PMN at 24 h,  $(315 \pm 53) \times 10^3$ ; at 48 h,  $(439 \pm 90) \times 10^3$ ] ( $P < 0.05$ ) (Fig. 1B). The numbers of mononuclear cells remained similar in all three groups of animals at all times examined (data not shown).

Concentrations of the proinflammatory cytokine TNF- $\alpha$  and the murine chemokine KC were measured in the BALF from animals euthanized 24 and 48 h after the administration of LPS. The concentrations of KC in BALF were not significantly different among the groups of animals at the times examined. In contrast, the concentration of TNF- $\alpha$  was significantly decreased in BALF from *lpr* mice ( $53 \pm 28$  ng/ml [mean  $\pm$  standard error of the mean]) compared to that in wild-type C57BL/6 mice ( $186 \pm 98$  ng/ml) at the 48-h time point following LPS administration ( $P < 0.05$ ) (Fig. 2).

Representative histopathological samples from animals euthanized 24 h after receiving intranasal LPS are shown in Fig. 3. Dense neutrophilic infiltrates and poor expansion were present in lungs from wild-type C57BL/6 mice (Fig. 3A and B). In contrast, only moderate neutrophilic infiltration was present in lungs from *lpr* (Fig. 3C and D) and *gld* (Fig. 3E and F) mice.

## DISCUSSION

These results demonstrate that the Fas/FasL system mediates, at least in part, sustained pulmonary neutrophilic inflammation in response to LPS administration in mice. The lung neutrophilic inflammatory response was impaired in both Fas-deficient *lpr* mice and FasL-deficient *gld* mice at 24 and 48 h following the administration of LPS. Decreased recruitment of PMN to the airspaces resulting from functional impairment of the Fas/FasL system might account for the differences in responses observed in *lpr* and *gld* mice compared to that in wild-type C57BL/6 mice. The Fas/FasL system might play a direct role in PMN recruitment to the lung airspaces, as suggested by previous reports of soluble FasL serving as a PMN chemoattractant (11, 15). However, we have been unable to confirm that FasL functions directly as a chemotactic factor for neutrophils (12). Another possibility is that the Fas/FasL system may induce the production of chemotactic cytokines from resident tissue cells, such as macrophages (12). Interestingly, the decreased neutrophilic inflammatory responses observed in *lpr* and *gld* mice were not associated with reduced concentrations of the potent murine PMN chemokine, KC, in the BALF from *lpr* and *gld* mice compared to that from wild-type C57BL/6 mice, although there were lower concentrations of TNF- $\alpha$  in the *lpr* mice at the 48-h time point. A third possibility, given previous findings suggesting that the Fas/FasL system may play an important role in the pathogenesis of acute lung injury in both animals and humans (7–10), is that Fas/FasL-mediated disruption of the alveolar-endothelial barrier is required to allow chemotactic cytokines to reach the intraluminal surface for presentation to and recruitment of circulating PMN (5).

In summary, our results demonstrate that the acute inflammatory response to LPS is blunted in the lungs of mice functionally deficient in either Fas or FasL. To our knowledge,

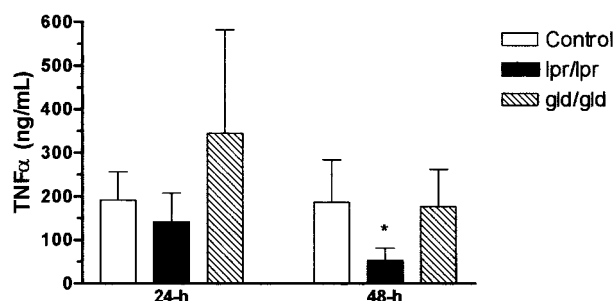


FIG. 2. Concentrations of TNF- $\alpha$  in the BALF of wild-type C57BL/6, Fas-deficient *lpr*, and FasL-deficient *gld* mice 24 and 48 h after intranasal administration of *E. coli* LPS (2.5  $\mu$ g/g). Asterisk,  $P < 0.05$ .

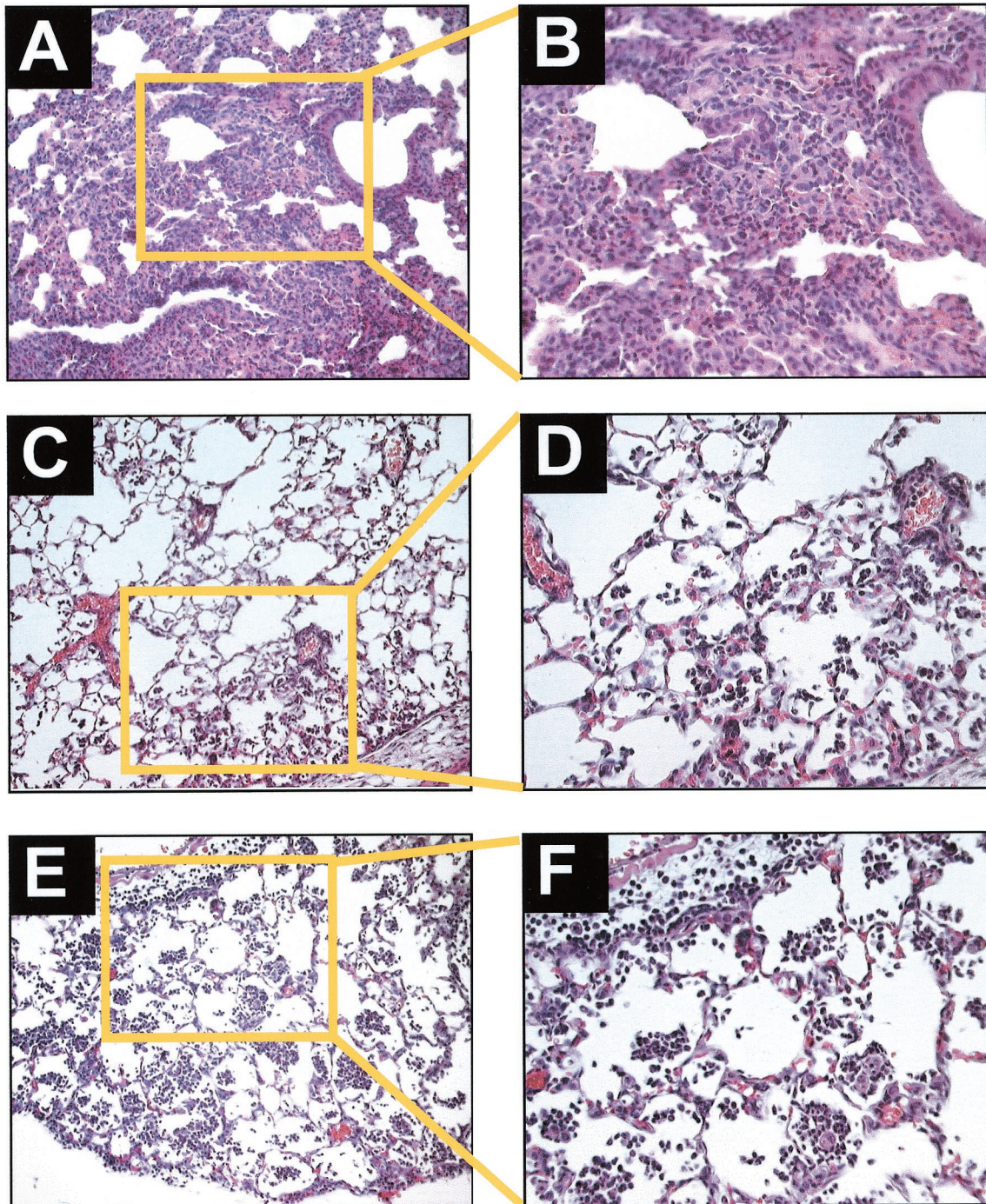


FIG. 3. Lung tissue sections stained with hematoxylin-eosin from wild-type C57BL/6 (A and B), Fas-deficient *lpr* (C and D), and FasL-deficient *gld* (E and F) mice 24 h after intranasal administration of *E. coli* LPS (2.5  $\mu\text{g/g}$ ). Lungs from C57BL/6 mice show dense neutrophilic alveolar infiltrates and poor inflation (magnifications,  $\times 100$  [A] and  $\times 400$  [B]). Lungs from *lpr* (magnifications,  $\times 100$  [C] and  $\times 400$  [D]) and *gld* (magnifications,  $\times 100$  [E] and  $\times 400$  [F]) mice show healthy alveolar walls and moderate neutrophilic infiltrates without intra-alveolar protein deposition.

these findings represent the first reported evidence indicating that the Fas/FasL system plays a role in LPS-induced inflammation *in vivo*. Furthermore, these observations provide further evidence of a proinflammatory role for the Fas/FasL system in the lungs.

#### ACKNOWLEDGMENTS

This work was supported in part by the Medical Research Service of the Seattle VAMC and by Public Health Service grants HL70840-01 (G.M.-B.), HL65892 (T.R.M.), and HL62995 (W.C.L.) from the National Institutes of Health.

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