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# Acute Lung Injury Induced by Lipopolysaccharide Is Independent of Complement Activation<sup>1</sup>

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and Peter A. Ward<sup>2\*</sup>

Although acute lung injury (ALI) is an important problem in humans, its pathogenesis is poorly understood. Airway instillation of bacterial LPS, a known complement activator, represents a frequently used model of ALI. In the present study, pathways in the immunopathogenesis of ALI were evaluated. ALI was induced in wild-type, C3<sup>-/-</sup>, and C5<sup>-/-</sup> mice by airway deposition of LPS. To assess the relevant inflammatory mediators, bronchoalveolar lavage fluids were evaluated by ELISA analyses and various neutralizing Abs and receptor antagonists were administered in vivo. LPS-induced ALI was neutrophil-dependent, but it was not associated with generation of C5a in the lung and was independent of C3, C5, or C5a. Instead, LPS injury was associated with robust generation of macrophage migration inhibitory factor (MIF), leukotriene B<sub>4</sub> (LTB<sub>4</sub>), and high mobility group box 1 protein (HMGB1) and required engagement of receptors for both MIF and LTB<sub>4</sub>. Neutralization of MIF or blockade of the MIF receptor and/or LTB<sub>4</sub> receptor resulted in protection from LPS-induced ALI. These findings indicate that the MIF and LTB<sub>4</sub> mediator pathways are involved in the immunopathogenesis of LPS-induced experimental ALI. Most strikingly, complement activation does not contribute to the development of ALI in the LPS model. *The Journal of Immunology*, 2008, 180: 7664–7672.

To investigate the molecular mechanisms of acute lung injury (ALI),<sup>3</sup> which is a major problem in humans, various experimental models of ALI have been used, the most common being the endotoxin (bacterial LPS) model. In experimental ALI, the lung parenchyma is damaged by the generation and release of proteases and reactive oxygen and nitrogen species produced by activated lung macrophages and transmigrated neutrophils in the interstitial and alveolar compartments. The end results are microvascular injury and diffuse alveolar damage with intrapulmonary hemorrhage, edema, and fibrin deposition (1, 2), which are also features in patients with ALI and the acute respiratory distress syndrome (ARDS) (3, 4).

Clinical studies (5, 6) as well as experimental studies (4, 7) have suggested an important role for complement activation products in the pathophysiology of ALI/ARDS. For the full development of

injury in other experimental ALI models (e.g., intrapulmonary IgG immune complex deposition), local activation of complement is usually required (7). In particular, generation of C5a amplifies production of proinflammatory cytokines (7–10), leading to intrapulmonary accumulation and activation of neutrophils and macrophages.

However, in the LPS-induced model of ALI, it is not clear to what extent activation of the complement system contributes to the development of lung injury, even though LPS is known to be an activator of the complement system via the classical and the alternative pathways (11–13). As a so-called pathogen-associated molecular pattern, LPS is recognized by TLR4, which is up-regulated on bronchial epithelial cells and lung macrophages during LPS-induced ALI and is considered to play a crucial role in innate immune responses (14, 15). The interaction of LPS with TLR4 ultimately leads to release of proinflammatory mediators and the subsequent recruitment of leukocytes into lungs (3, 10, 14, 16, 17).

Because the role of complement activation and its contribution to the development of experimental ALI after LPS challenge is unclear, the immunopathogenesis of the LPS model of ALI was investigated for specific mediator pathways involved in events leading to lung injury. LPS-induced ALI was neutrophil-dependent and required participation of macrophage migration inhibitory factor (MIF) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>). Unexpectedly, the development of ALI after LPS administration was independent of complement activation.

## Materials and Methods

### Animals

Adult male (22–25 g) specific pathogen-free C57BL/6 mice were used in these studies. Additionally, lung injury was used in C3<sup>-/-</sup> (on a C57BL/6 genetic background) (18), C5<sup>+/+</sup>, and C5<sup>-/-</sup> mice (congenic strains B10.D2/oSn and B10.D2/nSn, respectively) (19). All studies were done in accordance with the University of Michigan committee on the use and care of animals.

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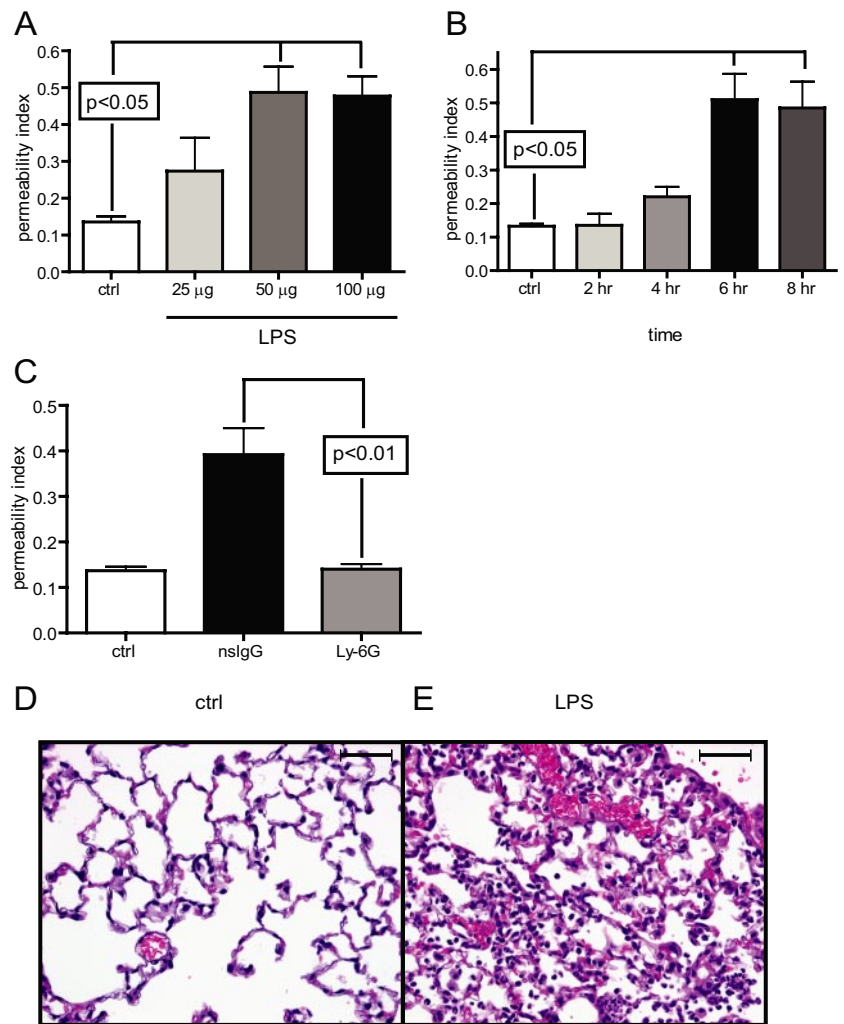
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<sup>3</sup> Abbreviations used in this paper: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; HMGB1, high mobility group box 1 protein; KC, CXCL1; LIX, LPS-induced CXC chemokine; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; MIF, migration inhibitory factor; MPO, myeloperoxidase; WT, wild type.

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**FIGURE 1.** Parameters of acute lung injury as reflected by leak of  $^{125}\text{I}$ -albumin into lung parenchyma (permeability index) as a function of the dose (25–100  $\mu\text{g}$ ) of LPS used (A) or as a function of time (0–8 h) after LPS administration (B). C, Effects of systemic neutrophil depletion on LPS-induced ALI. For each bar,  $n \geq 5$  mice. Histologic features in control lung (D) and lungs injured by deposition of LPS (E). H&E,  $\times 40$  (scale bar = 100  $\mu\text{m}$ ).

### LPS lung injury

For LPS lung injury, unless otherwise indicated, 50  $\mu\text{g}$  LPS from *Escherichia coli* (serotype O111:B4; Sigma-Aldrich) in 40  $\mu\text{l}$  PBS was given intratracheally. Sham-operated animals underwent the same procedure with intratracheal injection of PBS. Permeability index as a quantitative marker for vascular leakage was determined as described elsewhere (20). For retrieval of bronchoalveolar lavage (BAL) fluids, airways were flushed with 0.8 ml PBS. If not otherwise noted, the permeability index was determined and BAL fluids were collected 6 h after lung injury induction.

### Neutrophil depletion

Neutropenia was induced using monoclonal anti-mouse Ly-6G Ab (RB6–8C5; eBioscience). Control animals received injections of nonspecific ChromPure Rat IgG (nsIgG; Jackson ImmunoResearch Laboratories). Mice were given a single injection of 25  $\mu\text{g}$  Ly-6G Ab or nsIgG in 100  $\mu\text{l}$  of sterile saline i.v. 24 h before lung injury induction (21).

### Lung myeloperoxidase (MPO) activity in lung extracts

After 6 h, mouse lungs were perfused through the right ventricle with 2 ml PBS, snap frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ . To measure MPO activity, whole lungs were homogenized in 50 mmol/L potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide and 5 mmol/L EDTA. After centrifugation at  $12,000 \times g$  for 10 min at  $4^\circ\text{C}$ , the supernatant fluids were incubated in a 50 mmol/L potassium phosphate buffer containing the substrate,  $\text{H}_2\text{O}_2$  (1.5 mol/L) and *o*-dianisidine dihydrochloride (167  $\mu\text{g}/\text{ml}$ ; Sigma-Aldrich). The enzymatic activity was determined spectrophotometrically by measuring the change in absorbance at 460 nm over 3 min (Molecular Devices) (10).

### Leukocyte count in BAL fluids

Immediately after collection of BAL fluids, RBC were lysed with 1% acetic acid and total white cell count of each BAL sample was determined

using a Neubauer hemocytometer (Hauser Scientific). Cell differentials were analyzed (300 cells for each experimental condition) after cytopsin centrifugation ( $500 \times g$ , 3 min), methanol fixation (10 min), and Papanheim's staining.

### Anti-C5a treatment

Rabbit anti-rat C5a IgG (40  $\mu\text{g}$ ) (22) or nonspecific rabbit IgG (40  $\mu\text{g}$ ) (Jackson ImmunoResearch Laboratories) was given intratracheally together with LPS.

### Blockade of MIF or MIF receptor

Neutralizing mAb against mouse MIF was purified from mouse ascites fluids (ImmunoPure IgG purification kit, Pierce). Ten to 80  $\mu\text{g}$  anti-MIF mAb (IgG1) or irrelevant mouse IgG1 (Jackson ImmunoResearch Laboratories) was mixed with LPS. For blockade of the MIF receptor, mice were treated with ISO-1 (35 mg/kg body weight i.p.; Calbiochem) or vehicle (aqueous 5% DMSO) 30 min before lung injury induction.

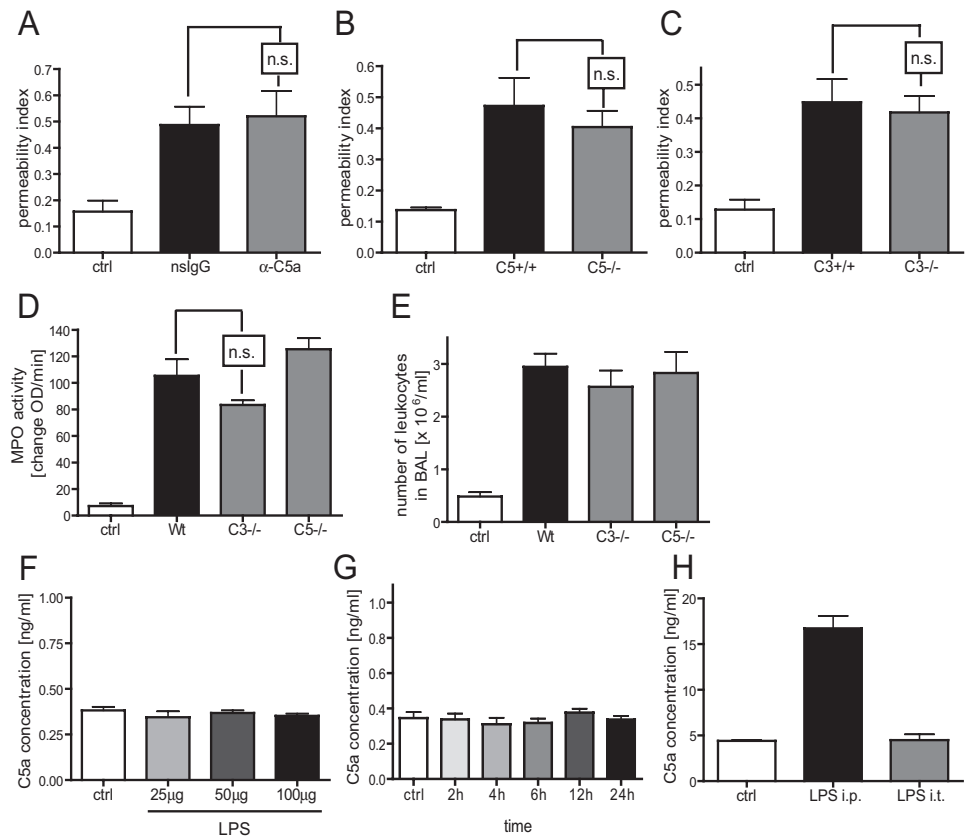
### Blockade of LTB4 receptor

For in vivo blockade of the LTB4 receptor BLT1, the synthetic receptor antagonist U-75302 (BIOMOL) or vehicle (DMSO) was given intratracheally together with endotoxin (23).

### ELISA for mouse C5a and C3a

To measure the concentration of mouse C5a in BAL fluids, ELISA plates were coated with purified monoclonal anti-mouse C5a IgG (BD Pharmingen, capture Ab, 5  $\mu\text{g}/\text{ml}$ ). After blocking, BAL fluids and recombinant mouse C5a (as standards) were applied and biotinylated monoclonal anti-mouse C5a Ab was used subsequently (BD Pharmingen, detection Ab, 500 ng/ml) followed by incubation with streptavidin-peroxidase (400 ng/ml). *O*-phenylenediamine dihydrochloride was then added, the color reaction

**FIGURE 2.** A, Effects of anti-C5a (40  $\mu$ g IgG) administered intratracheally on ALI in the LPS model (50  $\mu$ g). Lung injury induced by LPS in C5<sup>-/-</sup> mice (B) or C3<sup>-/-</sup> mice (C). Lung MPO content in WT and C3- and C5-deficient mice (D). E, Number of leukocytes in BAL from C3<sup>-/-</sup> and C5<sup>-/-</sup> mice in comparison to WT animals with ALI. F, BAL C5a content in LPS-injured lungs as a function of dose of LPS, as measured at 6 h. G, Time-course for BAL C5a content (determined by ELISA) after airway deposition of 50  $\mu$ g LPS. H, Plasma C5a levels after intraperitoneal vs intratracheal administration of LPS. For each bar,  $n \geq 5$  mice.



was stopped with 3 M sulfuric acid, and the absorbance was read at 490 nm (24).

**ELISA for mouse IL-6, TNF- $\alpha$ , LPS-induced CXC chemokine (LIX), CXCL1 (KC), MIP-2, high mobility group box 1 protein (HMGB1), MIF, and LTB4**

For measurement of IL-6, TNF- $\alpha$ , MIP-2, LIX, and KC in BAL fluids, ELISA kits (DuoSet, R&D Systems) were used according to the manufacturer's protocol. For quantification of HMGB1 in BAL fluids, a commercially available ELISA assay (Shino-Test) was used. Measurement of MIF was done using purified rabbit anti-MIF IgG (5  $\mu$ g/ml, Cell Sciences). As detection Ab, purified rabbit anti-MIF IgG was biotinylated using the EZ-link NHS-PEO solid-phase biotinylation kit (Pierce). After washing and blocking of wells, BAL fluids or standards (recombinant mouse MIF, R&D Systems) were applied in various dilutions. Biotinylated detection Ab (500 ng/ml) was added and development was performed as described above (ELISAs for C5a and C3a). LTB4 concentrations in BAL fluids were determined by using a commercially available ELISA kit (Cayman Chemical).

**Statistical analysis**

All values were expressed as means  $\pm$  SEM. Data sets were analyzed by one-way ANOVA followed by Tukey multiple comparison test with GraphPad Prism 4 software (GraphPad Software). Results were considered statistically significant when  $p < 0.05$ .

**Results**

**Characterization of lung injury after deposition of LPS**

Lung injury as defined by the permeability index (albumin leak) was studied as a function of dose of LPS, which was administered directly into the airways of mice. As shown in Fig. 1A, the dose of the inflammatory stimulus was related to an increase in the permeability index. There was no significant difference in the permeability index between the 50  $\mu$ g and the 100  $\mu$ g dose. In all subsequent experiments, unless otherwise indicated, the dose of LPS used was 50  $\mu$ g. The injury peaked 6 h after LPS administration (Fig. 1B). In the current study we used the mAb Ly-6G to induce >95% depletion of blood neutrophils (without affecting the number of blood monocytes), as recently described (21). The permeability index rose to a level of  $0.39 \pm 0.06$  6 h after intratracheal instillation of 50  $\mu$ g LPS, as contrasted to a value of  $0.14 \pm 0.01$  in the uninjured lungs (Fig. 1C). In neutrophil-depleted mice (Ly-6G), the permeability index remained at baseline ( $0.14 \pm 0.01$ ), indicating that for LPS to induce lung injury blood neutrophils must be available (Fig. 1C).

The histological patterns of ALI due to LPS are shown in Fig. 1, D and E. Fig. 1D depicts a control lung, in which PBS was

Table I. Cytokine/chemokine concentrations in BAL fluids (pg/ml)<sup>a</sup>

Cytokine/Chemokine	Control PBS	WT LPS	C3 <sup>-/-</sup> LPS <sup>b</sup>	C5 <sup>-/-</sup> LPS <sup>b</sup>
IL-6	69.12 $\pm$ 11.63	1200 $\pm$ 161.4	1491 $\pm$ 195.1	1797 $\pm$ 242.2
TNF- $\alpha$	396.2 $\pm$ 41.65	5028 $\pm$ 472.6	3763 $\pm$ 332.2	5404 $\pm$ 728.5
MIP-2	15.44 $\pm$ 1.577	764.3 $\pm$ 191.0	1124 $\pm$ 207.5	927.9 $\pm$ 385.1
KC	87.7 $\pm$ 12.1	4671 $\pm$ 1609	7583 $\pm$ 832.1	2158 $\pm$ 668.8
LIX	300.8 $\pm$ 10.3	1262 $\pm$ 73	953.9 $\pm$ 67.84	1132 $\pm$ 234.8

<sup>a</sup>  $n \geq 5$  for each group.

<sup>b</sup> All values in column are not significant when compared with WT LPS data.

Table II. Mediator concentrations in BAL fluids<sup>a</sup>

Mediator	Control PBS	LPS	Unit
HMGB1	48.4 ± 1.3	141.3 ± 22.7*	ng/ml
MIF	0.6 ± 0.2	4.6 ± 0.8*	μg/ml
LTB4	n.d.	426.2 ± 38.5*	pg/ml

<sup>a</sup>  $n \geq 5$  for each group. All  $p$  values are based on comparison to control PBS data. \*,  $p < 0.05$ . n.d., Not detectable.

administered intratracheally. The lung was essentially normal in appearance. In Fig. 1E, LPS-induced ALI was characterized by interstitial and intraalveolar deposits of neutrophils and fibrin, prominence of alveolar macrophages, and intraalveolar hemorrhage.

#### Complement activation is not required in the LPS model of ALI

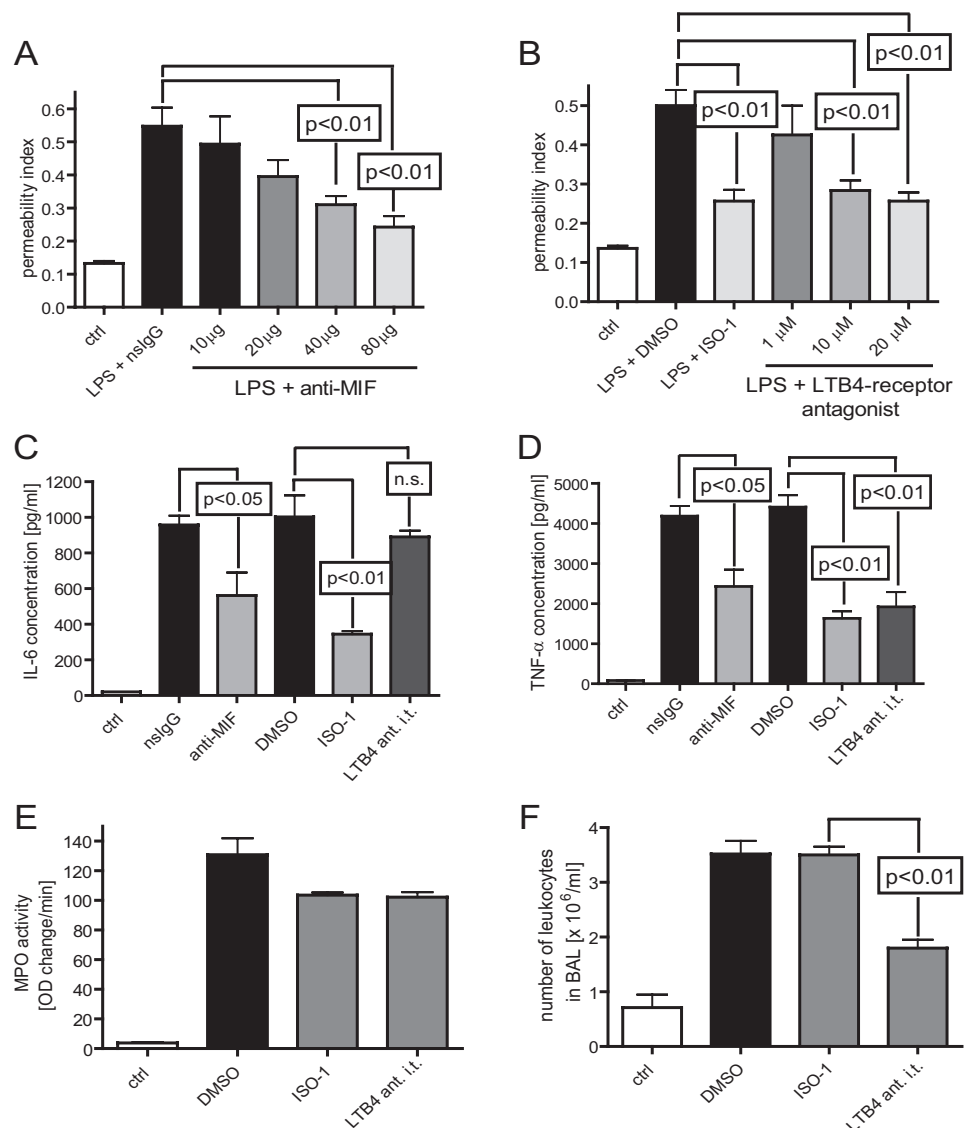
Previous studies have shown a requirement for C5 for the full expression of lung injury following deposition of IgGIC in mouse lung (24). Contrarily, in LPS-induced ALI blockade of C5a with 40 μg anti-C5a-IgG, which was given together with the LPS, did not suppress lung injury (Fig. 2A), nor was there reduced injury in the absence of C5 (Fig. 2B) or C3 (Fig. 2C). In accord, the absence

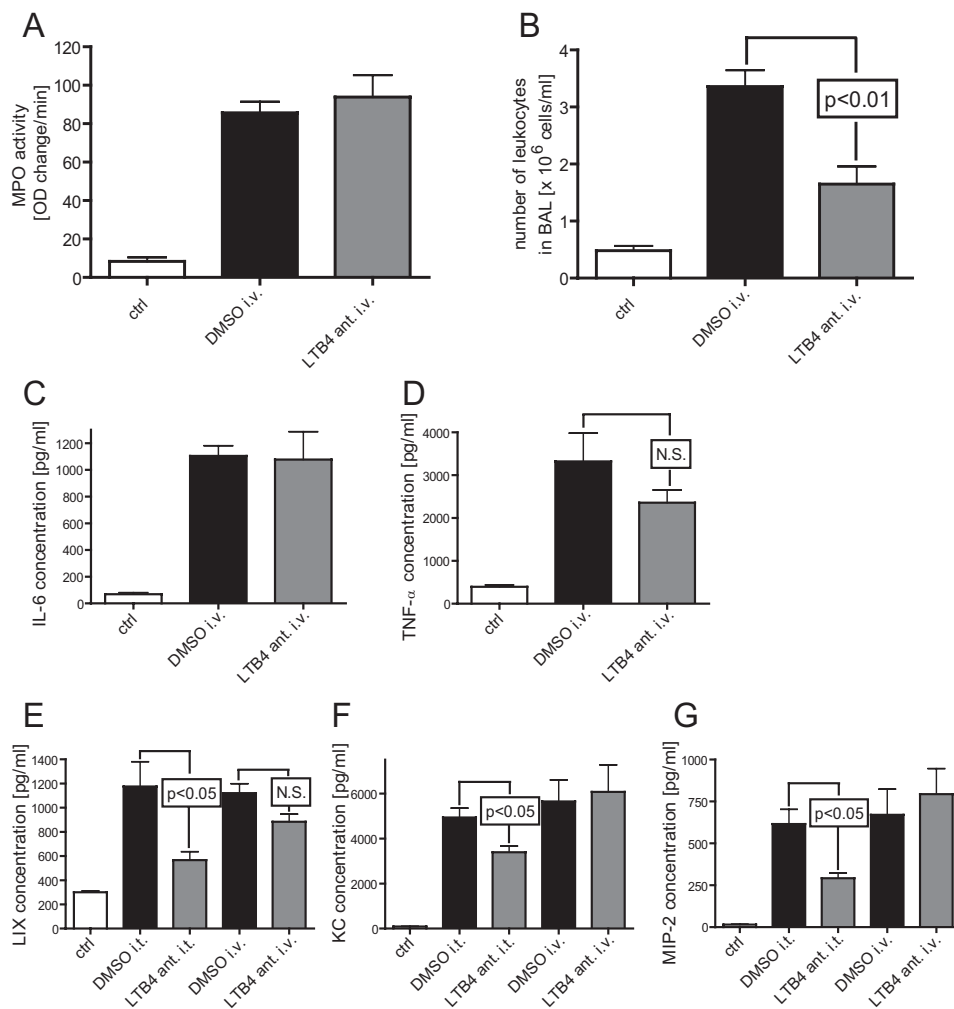
of C3 or C5 neither affected accumulation of leukocytes in lung as indicated by assessment of lung myeloperoxidase activity (Fig. 2D) nor their appearance in the alveolar space (Fig. 2E). Using a self-developed ELISA with which C5a has been detected in mouse BAL fluids (24), no increase of C5a in BAL was found as a function of the dose (25–100 μg) of LPS used (Fig. 2F) or as a function of time (0–24 h) after LPS administration (Fig. 2G). Additionally, there was no increase in C5a plasma levels after intratracheal injection of LPS, indicating that systemic complement activation in terms of a possible LPS clearance mechanism does not occur during ALI (Fig. 2H). In contrast, in wild-type mice (WT) injected i.p. with 10 μg LPS in 200 μl PBS, C5a plasma levels rose 4-fold as compared with WT mice not given LPS (Fig. 2H), indicating that the LPS used in the present study is capable of activating mouse complement if given i.p. Collectively, these data suggest that ALI following airway deposition of LPS is complement-independent.

#### Appearance of proinflammatory mediators in experimental ALI

The levels of proinflammatory mediators were quantitated in BAL fluids after airway deposition of LPS. The lung cytokines IL-6 and TNF-α and the chemokines MIP-2, KC, and LIX, which are chiefly derived from lung macrophages in a NF-κB-dependent fashion, are known to play important roles in ALI (25, 26). It has

**FIGURE 3.** A, Intensity of lung injury (measured as permeability index) in the LPS model with isotype-matched IgG1 or with anti-MIF mAb (each at 40 μg mixed with LPS). B, Effects of ISO-1 (the MIF receptor antagonist, 35 mg/kg body weight, administered i.p. 30 min before lung injury induction) or the LTB4 receptor antagonist (1–20 μM) after admixture with the intratracheally administered LPS. Effects of MIF or LTB4 blockade on the buildup of IL-6 (C) and TNF-α (D). For the interventions indicated in C and D, 40 μg neutralizing mAb to mouse MIF or 20 μM of the LTB4 receptor antagonist was mixed with the LPS; when the MIF receptor antagonist (ISO-1) was used, 35 mg/kg body weight was injected i.p. 30 min before intratracheal administration of 50 μg LPS. E, Lung MPO content in LPS-injured lungs of the LPS-treated animals of the ISO-1 (35 mg/kg body weight) or the LTB4 receptor antagonist (20 μM). F, Effects of MIF or LTB4 blockade on the total white cell count in BAL fluids. For each bar,  $n \geq 5$  mice.





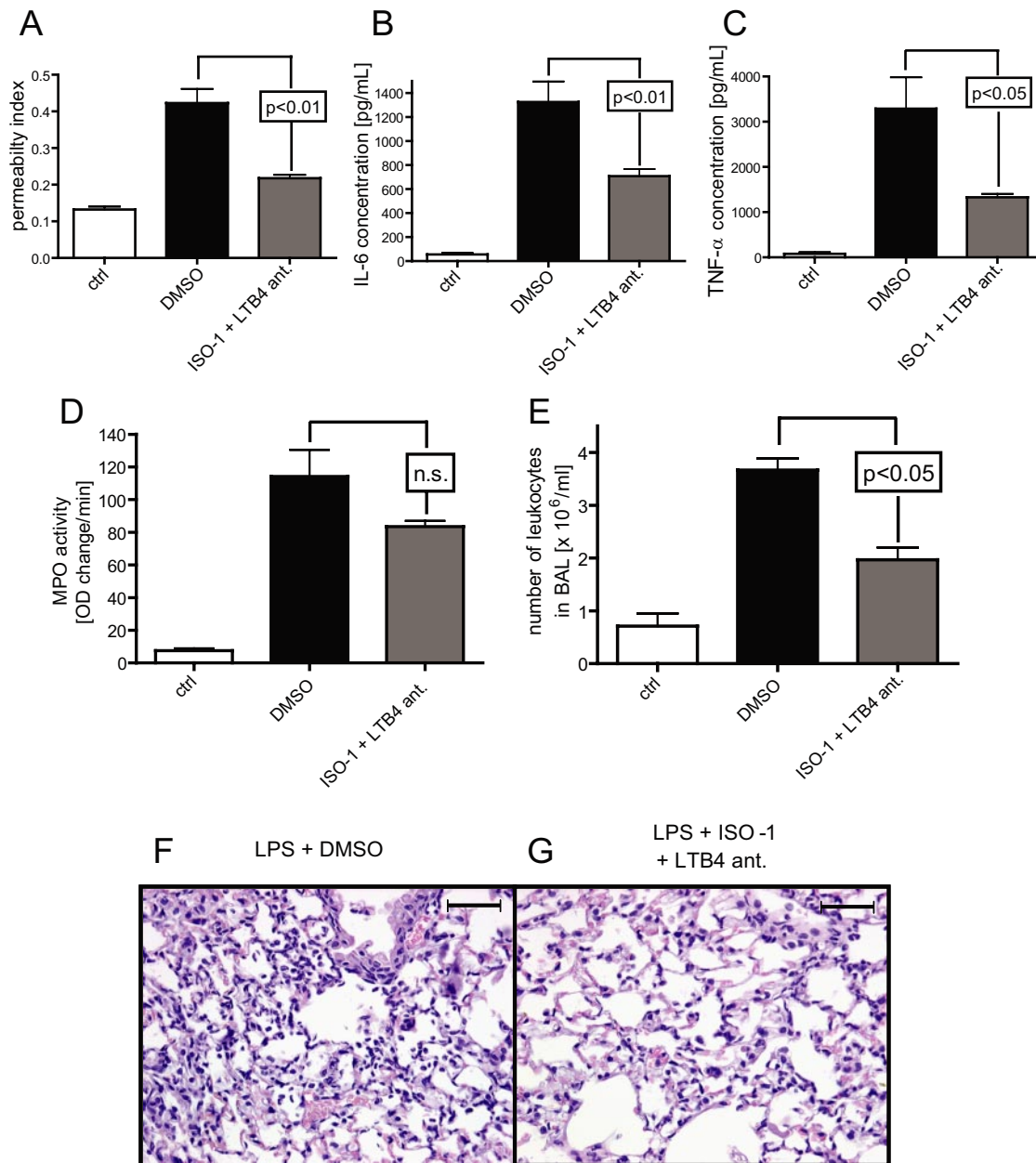
**FIGURE 4.** Effects of LTB4 blockade by the LTB4 antagonist when administered i.v. in comparison to intratracheal administration on lung MPO content (A) and appearance of leukocytes in BAL fluids (B). Levels of IL-6 (C) and TNF- $\alpha$  (D) in BAL fluids after i.v. application of the LTB4 antagonist. Concentrations of the chemoattractants LIX (E), KC (F), and MIP-2 (G) in BAL fluids after i.v. vs intratracheal administration of the LTB4 antagonist in lung injury. For each bar,  $n \geq 5$  mice.

been shown that PMN recruitment is mediated by neutrophil chemoattractants such as KC, MIP-2, and LIX. In particular, PMN infiltration into lung following exposure to LPS is dependent on interaction of CXCR2 with its ligands (e.g., KC, MIP-2) (26). As shown in Table I, the cytokine/chemokine levels were substantially elevated in BAL fluids of LPS-injured lungs of WT mice. As is evident, there was no difference in cytokine/chemokine levels in BAL fluids from C3<sup>-/-</sup> or C5<sup>-/-</sup> mice when compared with WT mice with lung injury. Consistent with the findings described above (Fig. 2), these data support the conclusion that complement is not required for the full inflammatory response during LPS-induced ALI.

#### Requirements for MIF and LTB4 in the LPS model of ALI

BAL fluids from WT mice after LPS-induced lung injury were screened for the mediators HMGB1, MIF, and LTB4, which have been described to play important roles in the pulmonary inflammatory response (16, 27, 28). HMGB1, which is known to be a distal mediator in ALI and the blockade of which has shown protective effects in the LPS model (16), showed a robust increase in BAL fluids after LPS administration (Table II). MIF was also found at increased levels in BAL fluids from LPS-injured lungs as compared with the PBS controls (Table II). Finally, measurement of LTB4 in BAL fluids revealed no detectable levels in controls, but readily detectable levels of LTB4 in LPS-injured lungs (Table II), suggesting that LTB4, HMGB1, and MIF contribute to the development of LPS-induced lung injury.

Because no complement requirement could be demonstrated in the LPS model (Fig. 2) and MIF and LTB4 levels were robustly elevated in LPS-induced ALI (Table II), the possible roles of these mediators in the development of ALI were evaluated. In particular, we were interested in whether these mediators might be required for PMN recruitment, which was found to be necessary for the development of lung injury (Fig. 1C). As shown in Fig. 3, neutralization of MIF (10–80  $\mu$ g anti-MIF IgG intratracheally) reduced the index of lung injury as a function of dose (Fig. 3A), while intraperitoneal administration of the MIF receptor antagonist ISO-1 also significantly suppressed injury (Fig. 3B). When the LTB4 receptor antagonist (1–20  $\mu$ M) was administered intratracheally together with LPS, there was a dose-dependent reduction in the permeability index (Fig. 3B). We assessed how neutralization of MIF, blockade of the MIF receptor, or blockade of the LTB4 receptor (20  $\mu$ M) might affect production of IL-6 and TNF- $\alpha$ . In Fig. 3C, protective interventions with anti-MIF or ISO-1 reduced the BAL levels of IL-6 by 48 and 71%, respectively. In contrast, the use of the LTB4 receptor antagonist did not significantly reduce the levels of IL-6. When BAL levels of TNF- $\alpha$  were determined, using the same BAL samples used for IL-6 assays, similar data were obtained, except for mice treated with the LTB4 receptor antagonist in which TNF- $\alpha$  levels were also significantly suppressed (Fig. 3D). In summary, these data suggest that MIF and LTB4 promote production of proinflammatory cytokines during ALI induced by LPS.



**FIGURE 5.** A, Effects of a dual blockade of MIF (ISO-1; 35 mg/kg body weight) and LTB4 (receptor antagonist; 20  $\mu$ M) on LPS-induced ALI. Intensity of lung injury as determined by lung permeability. Levels of proinflammatory cytokines IL-6 (B) and TNF- $\alpha$  (C) in the combined presence of ISO-1 and the LTB4 antagonist. D, Buildup of lung MPO after intratracheal LPS administration and ISO-1 + LTB4 antagonist. E, Total leukocyte count in BAL fluids. For each bar,  $n \geq 5$  mice. Effect of the LTB4 antagonist + ISO-1 on lung histology (G) in comparison to deposition of LPS + DMSO (F). H&E,  $\times 40$  (scale bar = 100  $\mu$ m).

Finally, lung MPO levels after LPS administration were assessed (Fig. 3E). When the protective interventions (ISO-1, LTB4 receptor antagonist) were used with the doses described above, minor reductions in MPO content were found, but these were not significantly different from MPO content in LPS lungs that were not otherwise manipulated. However, the number of leukocytes in BAL fluids was significantly reduced in the presence of the LTB4 antagonist, but not when ISO-1 was administered (Fig. 3F). In all groups treated with LPS, there was no difference in cell differentials, with predominantly PMNs and 10–15% macrophages (data not shown). Collectively, these data suggest that protective interventions directed at receptors for MIF or LTB4 reduce the development of ALI but do not interfere with neutrophil accumulation in lung after administration of LPS. LTB4 appears to promote

transmigration of PMNs into the alveolar space, while MIF seems to accentuate proinflammatory mediator release from macrophages and neutrophils rather than interfering with their migration.

Similar to the results displayed in Fig. 3, E and F, where the LTB4 antagonist was given intratracheally, i.v. administration of the antagonist did not alter buildup of lung MPO (Fig. 4A), but resulted in reduced numbers of leukocytes present in the airway compartment (Fig. 4B). In accord with Fig. 3C (intratracheal application), i.v. treatment with the LTB4 antagonist also did not affect the secretion of IL-6 (Fig. 4C). TNF- $\alpha$  levels in BAL fluids were not substantially reduced by LTB4 blockade when the antagonist was injected i.v. (Fig. 4D), although intratracheal administration significantly suppressed TNF- $\alpha$  production (Fig. 3D). A similar pattern was found for the chemokines LIX, KC, and MIP-2,

with the latter two being ligands for CXCR2, which is known to mediate PMN recruitment into lungs. Only intratracheal administration of the LTB4 antagonist resulted in decreased chemokine levels in BAL fluids of LPS-injured mice, whereas its i.v. application had no effect on local chemokine production (Fig. 4E–G). In summary, these data suggest that LTB4 might trigger the release of chemoattractants from alveolar macrophages rather than directly recruiting PMNs into the lung. However, leukocyte transmigration into the alveolar space seems to depend on direct interaction of LTB4 with the recruited cells because the number of leukocytes was significantly reduced, regardless of whether the LTB4 antagonist was administered via the intratracheal or the i.v. route.

#### *Dual inhibition of LTB4 and MIF in the LPS model of ALI*

When blockade of MIF was used together with inhibition of LTB4 (Fig. 5), the intensity of lung injury was reduced to an extent similar to that found when either antagonist was administered alone (70% reduction vs 67% by ISO-1 or LTB4 antagonist, respectively) (Fig. 5A). Moreover, concentrations of IL-6 (Fig. 5B) and TNF- $\alpha$  (Fig. 5C) in BAL fluids were significantly suppressed in the presence of ISO-1 and the LTB4 antagonist, but they were still elevated in comparison to control animals. Dual inhibition of MIF and LTB4 failed to significantly reduce the buildup of lung MPO (Fig. 5D). In contrast, as in the case of the single inhibition of LTB4 (Fig. 3F), the number of leukocytes in BAL fluids was clearly lower when the LTB4 antagonist + ISO-1 were given (Fig. 5E), but without any further accentuation of this effect by the additional blockade of MIF. These findings were underpinned by lung histology. In the presence of the LTB4 antagonist and ISO-1, the bulk of PMNs only accumulated in the lung interstitium (Fig. 5G), whereas in LPS-injured lungs without inhibitors, PMNs were also present in the alveolar compartment (Fig. 5F). Taken together, these data suggest that MIF and LTB4 promote lung injury via different mechanisms and that dual inhibition of both mediators does not result in a synergistic effect on the attenuation of inflammatory response.

## Discussion

ALI and ARDS, as well as chronic obstructive pulmonary disease, represent fundamentally disordered inflammation in the lung and continue to be prevalent clinical problems (2). Despite extensive efforts in both the clinical and laboratory settings, the molecular mechanisms of these inflammatory disorders are poorly understood. LPS and related products are known to be present in BAL fluids from patients with ARDS (29). Therefore, lung injury induction by LPS in rodents represents a frequently used ALI model, mimicking many features of ALI/ARDS in humans. In this study, the LPS model was evaluated for immunopathological events leading to lung injury.

It is well established that following the intrapulmonary deposition of IgG immune complexes, generation of C5a plays an important role in the pathogenesis of ALI (7, 24). However, activation of the complement system was not required for the full development of LPS-induced lung injury suggesting that there is differential regulation of local complement activation in the lung depending on the nature of the inflammatory stimulus. In the present study, neither the blockade of C5a nor the absence of C5 or C3 influenced the intensity of lung injury in the LPS model, and no increase of BAL C5a was found after LPS administration (Fig. 2). This is in accord with an earlier report (30), but contrasts with a recent publication that describes altered complement levels and expression in LPS-induced ALI (31). However, in the latter report, only the expression of nonactivated complement proteins was presented, making it far from certain if activation of the complement

cascade actually occurred. In another conflicting report, C3 and C5b-9 deposition on the endothelium of pulmonary vessels has been reported, but complement depletion had no effect on lung MPO activity and TNF- $\alpha$  levels, which again raises the question of whether complement activation had effectively proceeded (32). In various studies, patients with ARDS showed evidence for complement activation, the extent of which correlated with the degree and outcome of ARDS (5, 6, 33). In contrast, in another study published some years ago, C5a could not be detected in BAL fluids from patients with ARDS using the methods available at that time (34). Although hepatic production is the main source for complement proteins, virtually all complement proteins can be locally synthesized in the lung by type II alveolar pneumocytes, alveolar macrophages, and lung fibroblasts (35, 36). However, while the total pulmonary complement protein concentration is comparable to levels found in serum, its activity in normal lung is markedly reduced due to the complement-inhibitory activity of surfactant protein A and C1 inhibitor, both being abundantly present and active in the lung (37, 38). Reduced lung activity of both surfactant protein A and C1 inhibitor can be related to the development of ARDS in humans (39, 40). In other words, pulmonary activation of the complement system underlies a complex and distinct regulation. Therefore, presence of complement proteins in the lung does not necessarily imply their local activation.

There is also diverse information regarding the role of the complement system in the setting of endotoxemia. The febrile response induced by infusion of LPS seems related to generation of C5a, and endotoxic C5<sup>-/-</sup> mice had reduced evidence for organ failure when compared with C5<sup>+/+</sup> mice (41, 42). In contrast, C3- and C4-deficient mice infused with LPS showed greatly increased mortality, suggesting that systemic *in vivo* clearance of LPS requires C3 and C4 (43). In line with the above-mentioned observations, in this study robust complement activation (as indicated by increased plasma levels of C5a) only occurred when LPS was injected i.p., but not when it was administered via the intratracheal route (Fig. 2F–H). These observations suggest that complement may be necessary for systemic clearance of LPS from the blood compartment *in vivo*, but not in the local setting of the lung as described in this report, where LPS failed to induce activation of the complement system. In other words, different mechanisms of endotoxin clearance might be involved that are dependent on the entry route of LPS.

Finally, although our data strongly suggest that the complement activation does not contribute in the development of ALI after LPS exposure, we were able to identify MIF and LTB4 as key mediators in the pathogenesis of LPS-induced ALI. MIF has been found in BAL fluids from humans with ARDS and may play a role in sustaining the pulmonary inflammatory response (27). In the present study we sought to evaluate the role of MIF in experimental ALI. MIF functions as a pleiotropic proinflammatory protein and plays a key role in systemic and local inflammatory responses (44). It is abundantly produced by monocytes/macrophages, can induce and enhance the production of other cytokines, and regulates apoptosis of leukocytes (44–46). Previous studies suggest the participation of MIF in neutrophil accumulation in lung after intraperitoneal injection of LPS (47). Intratracheal administration of neutralizing mAb to MIF or use of the MIF receptor antagonist ISO-1 attenuated the capillary leak and tissue damage in ALI. Blockade of MIF suppressed proinflammatory cytokine release in LPS-induced ALI, but did not interfere with PMN accumulation or their transmigration into the alveolar space. The chief effects of MIF in experimental ALI may be enhancement of the proinflammatory response (27), up-regulation of TLR4 (48), glucocorticoid antagonism (27), or a combination of all the above.



Because of its importance in the pathogenesis of airway hyper-responsiveness, the role of LTB<sub>4</sub> and interaction with its receptor (BLT1) in acute lung injury was evaluated (28). LTB<sub>4</sub> is a chemotactic factor for neutrophils and appears to be responsible for neutrophil accumulation in lung tissue during acute asthmatic exacerbation (49, 50). Additionally, LTB<sub>4</sub> enhances the release of active oxygen species and the respiratory burst of neutrophils via its priming effects (51, 52). In the present LPS model, LTB<sub>4</sub> levels in BAL fluids were substantially elevated when compared with noninjured lungs. Furthermore, blockade of the LTB<sub>4</sub> receptor BLT1 strikingly reduced lung injury. Interestingly, these protective effects in the LPS model were not linked to altered accumulation of neutrophils based on lung MPO content, which does not distinguish between interstitial and intraalveolar PMNs. However, the transmigration of PMNs from the interstitium to the airway compartment seems to be LTB<sub>4</sub>-dependent (Figs. 3 and 4). These findings are consistent with previous studies describing that intratracheal instillation of LTB<sub>4</sub>, which is also a major product of alveolar macrophages, can recruit active PMNs into airspace (53, 54). Although the precise mechanisms involved are largely unknown, reactive oxygen species and the expression of neutrophil elastase seem to be involved in the regulation of transepithelial migration of PMNs into the alveolar compartment in response to LTB<sub>4</sub> (54, 55).

In summary, the immunopathogenesis of LPS-induced ALI underlies a complex regulation regarding mediators and pathways involved in neutrophil mobilization and priming. Most strikingly, LPS-induced ALI is independent of activation of the complement system and, instead, is orchestrated by MIF and LTB<sub>4</sub>.

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## Disclosures

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