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*J Immunol* 2001; 167:7060-7068; doi: 10.4049/jimmunol.167.12.7060
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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Role of IL-18 in Acute Lung Inflammation


We have examined the role of IL-18 after acute lung inflammation in rats caused by intrapulmonary deposition of IgG immune complexes. Constitutive IL-18 mRNA and protein expression (precursor form, 26 kDa) were found in normal rat lung, whereas in inflamed lungs, IL-18 mRNA was up-regulated; in bronchoalveolar (BAL) fluids, the 26-kDa protein form of IL-18 was increased at 2–4 h in inflamed lungs and remained elevated at 24 h, and the “mature” protein form of IL-18 (18 kDa) appeared in BAL fluids 1–8 h after onset of inflammation. ELISA studies confirmed induction of IL-18 in inflamed lungs (in lung homogenates and in BAL fluids). Prominent immunostaining for IL-18 was found in alveolar macrophages from inflamed lungs. When rat lung macrophages, fibroblasts, type II cells, and endothelial cells were cultured in vitro with LPS, only the first two produced IL-18. Intratracheal administration of rat recombinant IL-18 in the lung model caused significant increases in lung vascular permeability and in BAL content of neutrophils and in BAL content of TNF-α, IL-1β, and cytokine-induced neutrophil chemoattractant, whereas intratracheal instillation of anti-IL-18 greatly reduced these changes and prevented increases in BAL content of IFN-γ. Intratracheal administration of the natural antagonist of IL-18, IL-18 binding protein, resulted in suppressed lung vascular permeability and decreased BAL content of neutrophils, cytokines, and chemokines. These findings suggest that endogenous IL-18 functions as a proinflammatory cytokine in this model of acute lung inflammation, serving as an autocrine activator to bring about expression of other inflammatory mediators.

Interferon-γ production by CD4+ helper T cells, CD8+ cytotoxic T cells, and NK cells plays an important role in the immune response, especially in the Th1 pathway. IFN-γ has the ability to activate macrophages, enhance NK cell activity, increase cytokine production, and protect cells from viral replication (1, 2). Only a few cytokines have been shown to secrete IL-18, including epidermal keratinocytes, intestinal epithelial cells, adrenal cortical cells, and osteoblasts (3–11). The three-dimensional structure of IL-18 indicates a relationship to IL-1α (12%) and IL-1β (19%). Like IL-1β, IL-18 is synthesized in precursor form with an unusual signal peptide that allows secretion from the cell. The precursor form of IL-1β has a very low biological activity (defined by the induction of IFN-γ), whereas the precursor form of IL-18 is totally devoid of activity (12). IL-1β-converting enzyme (ICE; also called caspase 1) is responsible for cleaving IL-1β and IL-18 to generate active, mature forms of these proteins. Studies have shown that ICE-deficient mice have reduced levels of IFN-γ and mature IL-18 (13, 14).

In the present study, we investigated the in vivo role of IL-18 in acute lung inflammation in rats caused by deposition of IgG immune complexes. This model is associated with production of TNF-α and IL-1, up-regulation of vascular adhesion molecules, and CXC/CC chemokine expression, all of which facilitate the recruitment of blood neutrophils, activation of lung macrophages, and the release of oxidants and proteases (15, 16). The role of IL-18 after acute lung inflammation caused by IgG immune complex deposition has not been explored previously. The current studies also explore the effects of IL-18 binding protein (IL-18bp), a natural IL-18 antagonist (17).

Materials and Methods

Reagents

Except where noted, all reagents were purchased from Sigma (St. Louis, MO). The anti-IL-18 was affinity-purified rabbit IgG directed against murine IL-18. This Ab neutralizes the biological activity of IL-18 (R&D Systems, Minneapolis, MN).

IgG immune complex-mediated alveolitis

Adult male Long-Evans rats weighing 275–300 g were used in all studies (specific pathogen-free). All experimental protocols have been approved by the University of Michigan Institutional Animal Committee for the Use and Care of Animals. Ketamine was administered i.p. for sedation and anesthesia. Rabbit polyclonal IgG (1.5 or 2.5 mg) rich in Ab to BSA (anti-BSA) was intratracheally instilled in a volume of 300 μl of PBS (pH 7.4) during inspiration. This was followed by the i.v. injection of BSA (10 mg) with trace amounts of 125I-labeled BSA as a quantitative marker of permeability. Rats were sacrificed 4 h later, the pulmonary circulation flushed, and lung injury quantified by increased vascular permeability. It has been shown elsewhere that inflammation and mediator presence in lung peak at this point at approximately 3–6 h (18), although at this time point it was shown that the IgG immune complex deposition was still in progress.

Received for publication June 1, 2001. Accepted for publication September 28, 2001.

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1 This work was supported by National Institutes of Health Grant HL-31963.
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4 Abbreviations used in this paper: ICE, IL-1β-converting enzyme; IL-18bp, IL-18 binding protein; (rr)IL-18, rat recombinant IL-18; MIP, macrophage-inflammatory protein; CINC, cytokine-induced neutrophil chemoattractant.
time point (18). The permeability index was determined by measuring the amount of radioactivity ([3H]-labeled BSA) remaining in the lungs compared with the radioactivity present in 1.0 ml of blood obtained from the inferior vena cava. Sensitivity control animals were intratracheally instilled with PBS only. In all experiments in which this model was used, n = ≥ 6 animals unless otherwise indicated.

Cloning of rat IL-18

Previously, it has been shown that IL-18 is strongly induced in the adrenal gland of rats after reserpine administration (10). In this study, we cloned rat IL-18 after inflammatory challenge. Rat lungs were injured by IgG immune complex deposition as described above. Four hours later, whole adrenal glands (cortex and medulla) were removed and frozen in liquid nitrogen. Total RNA was extracted by using the guanidinium-isothiocyanate method (TRizol; Life Technologies, Grand Island, NY). First-strand cDNA was constructed by reverse-transcribing 10 μg of total RNA with an oligo(dT) primer. cDNA amplification of rat IL-18 was performed with the above RT products and the following rat primers: 5′-5′-AACAATGGCTGCCAT GTGAC-3′ and 3′-5′-AGTGAAATTACAGATTTCC-3′. These primers result in a long 566-bp and a shorter 510-bp PCR band. The PCR products were ligated into a PCR 2.1 vector (Invitrogen, Carlsbad, CA), sequenced, and compared with the GenBank database. After confirmation of the sequence of both transcripts with the published rat IL-18 sequence, the longer sequence was used as a cDNA probe for Northern blot analysis.

Recombinant rat (rr)IL-18, anti-mouse IL-18 polyclonal Ab, and IL-18bp

(rr)IL-18 was purchased from R&D Systems. The protein was expressed in Escherichia coli and was determined by SDS-PAGE to be >97% pure. The endotoxin level was <0.1 ng per 1 μg of IL-18, and activity was confirmed by its ability to induce mouse IFN-γ production by activated mouse T cells. Where indicated, 1.0 or 2.0 μg of rat IL-18 was coinstilled intratracheally (constant volume of 10 μl) alone or with anti-BSA (in a final volume of 300 μl) at the commencement of injury. Polyclonal goat anti-mouse IL-18 Ab was derived from immunized goats. The endotoxin level in the IgG was <0.1 ng per 1.0 mg of the Ab. Cross-reactivity with rat IL-18 was determined by Western blotting. The Ab was reconstituted from the lyophilized form with sterile PBS to a concentration of 100 μg/ml. Our studies, shown below, indicate that the anti-IL-18 Ab, when absorbed with (rr)IL-18, loses its ability to detect IL-18 in BAL fluids from inflamed lungs. Human recombinant IL-18bp (1.5, 6, or 18 μg) was coinstilled intratracheally with anti-BSA at the commencement of inflammation. Human IL-18bp was expressed in mammalian cells and purified by HPLC, and shown to inhibit LPS-induced IFN-γ production in rat lungs (G. Senaldio, unpublished observations).

Northern Blot Analysis

Total RNA was extracted (TRizol Reagent; Life Technologies) from whole lungs of rats injured with IgG immune complexes. The pulmonary circulation was flushed with 10 ml of PBS, and the lungs were surgically reamed lungs. Human recombinant IL-18bp was produced in mammalian cells and purified by HPLC, and shown to inhibit LPS-induced IFN-γ production in rat lungs (G. Senaldio, unpublished observations).

ELISA for rat IL-18

Anti-IL-18 Ab, biotinylated Ab, and (rr)IL-18 protein were purchased from R&D Systems. A-sandwich ELISA was performed with 100 μl of 1 μl/ml of anti-IL-18 Ab (primary Ab) in borate buffer to coat ELISA plates. A 1% BSA solution in Dulbecco’s PBS was used to block nonspecific binding. After washing, samples and standards were added to individual wells and incubated for 2 h at 37°C. This was followed by washing and incubation with 100 μl of biotinylated Ab (1 μg/ml) for 1 h at 37°C. Subsequently, after washing, samples were added to a streptavidin-HRP conjugate and the developing reaction was stopped by 3 M H2SO4. The plate was read on an ELISA plate reader at 492 nm.

Western blot analysis

BAL fluids from IgG immune complex-injured animals were analyzed for rat IL-18 immunoreactive protein. Five milliliters of PBS was instilled into the lungs and collected three times, and cellular contents were removed by centrifugation. BAL fluids were then concentrated (~5-fold) to 500 μl by using a centrifuge (Amicon, Beverly, MA). Concentrated BAL fluids were subjected to SDS-PAGE (15%) according to the method of Laemmli. Equivalent amounts of protein (100 μg) were added to each lane. The acrylamide gels then were transblotted to nitrocellulose (0.1 μm) for 2 h at 12 V. The membrane was blocked with TweenTris-buffered saline containing 5% milk for 1 h at room temperature. Polyclonal goat anti-mouse IL-18 (100 μg/ml) then was added at a dilution of 1:1000, and the membrane was incubated overnight at 4°C. After washing, peroxidase-conjugated donkey anti-goat IgG (800 μg/ml) was added (1:10,000), and the membrane was incubated for 1 h at room temperature. The membrane was developed with an ECL technique (Amersham Life Science, Piscataway, NJ). (rr)IL-18 was used as a positive control, and a m.w. marker was used to estimate the size of the immunoreactive bands. Intensity of the protein band was determined as above. For experiments where indicated, the anti-IL-18 Ab was preabsorbed by the addition of (rr)-IL-18 at a final concentration of 1 μg/ml.

BAL fluid analysis and cytokine and chemokine content

BAL fluid was collected from IgG immune complex-injured rats 4 h after injury, and when used for Western blot analysis, was concentrated 5-fold by using Centricron filters with a cut-off of 3 kDa (Amicon). Briefly, 5 ml of PBS was intratracheally instilled three times into the lungs. Cell counts were normalized to the volume of BAL fluid recovered per rat. Cytokine activity was determined using a standard ELISA assay. BAL concentrations of macrophage-inflammatory protein (MIP)-2 and cytokine-induced neutrophil chemoattractant (CINC) were determined by ELISA as reported previously (19). The ELISA kits for detection of rat IL-18 and IFN-γ were purchased from R&D Systems.

Immunostaining of alveolar macrophages

BAL fluids from control and injured animals were collected and centrifuged at 450 × g for 10 min and were layered onto Ficoll-Paque to remove RBCs and neutrophils (450 × g for 30 min). The upper layer containing alveolar macrophages was removed and resuspended in PBS containing 1% BSA to a concentration of 250,000 cells/ml. Slides were prepared by adding 100 μl of this cell suspension to a Petri dish and centrifuging at 450 × g for 7 min. Cytospin slides were fixed in 100% methanol and stored at −20°C. For immunostaining, slides were washed in PBS and incubated with anti-IL-18 (1 μg/ml) for 1 h in a humidified chamber. Slides then were washed two times and incubated for 1 h with biotinylated anti-goat IgG (1 μg/ml). After washing in PBS, the slides were incubated for 30 min with streptavidin-HRP (1:3000). After a final wash, slides were reacted with diaminobenzidine reagent for 10 min and later counterstained with hematoxylin.

Isolation and culture of rat lung cells

Alveolar macrophages were isolated from BAL fluids from normal rat lungs as described (20). Cells were pelleted and then resuspended in DMEM supplemented with 10% FBS and penicillin-streptomycin (DMEM-FBS). After allowing cells to adhere to the plate (5 × 105 cells per well in 24-well tissue culture plates; Corning, Corning, NY), nonadherent cells were removed with two washes. Cell monolayers then were stimulated with the appropriate agonists suspended in DMEM-FBS in a 5% CO2 humidified incubator at 37°C for 24 h. Rat alveolar fibroblasts were isolated from normal lungs, lungs were removed, minced into small plates (Corning) containing DMEM-FBS. Cells were stimulated with appropriate agonists suspended in DMEM-FBS in a 5% CO2 humidified incubator at 37°C and allowed to grow for 24 h. Type II alveolar epithelial cells were isolated from normal rat lung using elastase cell dispersion and IgG panning by the method of Warner and associates (21). Cells were plated in DMEM-FBS at 5 × 106 cells per well in 24-well tissue culture plates and incubated at 37°C in 5% CO2. Cells were stimulated with appropriate agonists suspended in DMEM-FBS and allowed to grow for 24 h. Microvascular endothelial cells were isolated from peripheral lungs of normal 21-day-old rats (22). Strips
of peripheral lungs were removed, minced, and incubated in gelatin-coated tissue culture flasks in DMEM containing 10% FBS and endothelial cell growth factor. After 65 h, tissues were removed. Cultures consisted primarily of endothelial cell. Cells were maintained in culture and grown to 80–90% confluence at passage 2 by 10 days. For all experiments, cells were used at 80–90% confluence at passage 2 and plated in DMEM-FBS at 5 × 10⁵ cells per well in 24-well tissue culture plates and incubated at 37°C in 5% CO₂. Cells were stimulated with appropriate agonists suspended in DMEM-FBS and allowed to grow for 24 h.

Binding of rat IL-18 to solid phase human IL-18bp

Fifty microliters of human IL-18bp (10 µg/ml in borate-coating buffer was used to coat each well in a 96-well ELISA plate overnight at 4°C. A 1% BSA solution in Dulbecco’s PBS was used to block nonspecific binding (1 h at 37°C). Various concentrations of rat IL-18 and BSA (100 µl) were added per well and incubated for 1 h at 37°C. After the washing step, goat anti-rat IL-18 (R&D Systems) was added and incubated for 1 h at 37°C. After a 1-h incubation with donkey anti-goat IgG-HRP conjugate (Santa Cruz Biotechnology, Santa Cruz, CA), the assay was developed by addition of o-phenylenediamine substrate. The developing reaction was stopped by adding 50 µl of 3 M H₂SO₄ and read at 492 nm.

Statistical analysis

All data are presented as means ± SEM. Sample size was 4–6 animals per group unless otherwise noted. One-way analysis of variance was used to compare treatment groups. Significant differences between groups were determined using Tukey’s test. Statistical significance equal to p < 0.05.

Results

IL-18 mRNA and protein expression in IgG immune complex-induced inflamed rat lungs

IL-18 mRNA content varied in normal rat tissues based on Northern blot analysis. In the adult rat, IL-18 mRNA (~1.35 kb) was most abundant in liver, with moderate expression in spleen and low but measurable constitutive expression in the lungs, kidneys, and heart (Fig. 1A). Little if any mRNA for IL-18 was found in skeletal muscle and testes. Equal loading of RNA was confirmed by stripping the blot and reprobing with radiolabeled actin probe (data not shown). To assess changes in endogenous IL-18 mRNA and protein during the pulmonary inflammatory response, lungs from injured animals were evaluated (0–4 h) for changes in mRNA and protein expression. Northern blot analysis of Ilg immune complex-inflamed lungs (using 1.25 mg anti-BSA intratracheally) revealed increases (~50%, as determined by densitometry) in mRNA for IL-18, 2 and 4 h after initiation of the inflammatory response (Fig. 1B, bottom). Equal loading was confirmed by probing with GAPDH (Fig. 1B, bottom).

As determined by Western blot analysis with anti-rat IL-18 rabbit polyclonal IgG, changes in IL-18 content in BAL fluids (5-fold concentrated) are shown in Fig. 2A as a function of time. In lane 1, (rr)IL-18 gave a single band in the 18-kDa position, consistent with the mature form of IL-18. At time 0 (lane 2), a single band reactive with anti IL-18 Ab was found in BAL fluids in the 26-kDa position, which is consistent with the “pro” form of IL-18. Thereafter, between 1 and 8 h, IL-18 bands were found in both the 26- and 18-kDa positions (lanes 3–6), whereas at 16 and 24 h (lanes 7 and 8) bands in the former position were prominent, but bands in the 18-kDa position were barely visible. When the anti-IL-18 preparation (1.0 µg/ml) was preabsorbed with addition of (rr)IL-18 (1.0 µg/ml final concentration), both the 18-kDa and the 26-kDa bands disappeared (lane 9, using the 4-h BAL sample), indicating that both bands are related to IL-18. As is also shown in Fig. 2A, the double banding pattern found in BAL fluids obtained 4 h after initiation of this inflammatory response (lane 10) disappeared from similarly treated rats that also received 400 µg of anti-IL-18 intratracheally at time 0 (lane 11).

By using a custom-prepared ELISA for rat IL-18, BAL fluids (which were concentrated 5-fold) were evaluated after induction of lung inflammation. As shown in Fig. 2B, IL-18 levels in BAL fluids from normal lungs were <1 ng/ml, whereas there was a progressive increase in BAL IL-18, peaking at 4 h (at ~9 ng/ml), followed by a decline. When lung homogenates were evaluated over the same period of time, normal lungs contained 1025 ± 128 ng/g lung weight (Fig. 2B). In the inflamed lungs, there was very little measurable increase in IL-18 during the first 2 h of inflammation, but by 4 and 6 h, the IL-18 content was 2585 ± 172 and 2441 ± 301 ng/g lung, respectively. These data indicate that IL-18 is induced in lungs of rats during deposition of IgG immune complexes.

Effects of (rr)IL-18 on vascular permeability and BAL content

The effects of the exogenously administered rat IL-18 during IgG-induced inflammation were determined in the lung model. For these studies, the dose of anti-BSA used was 1.25 mg, to detect in a more sensitive manner increases in the parameters of lung inflammation and vascular leak. The vascular permeability (leakage of 125I-labeled albumin) was measured at 4 h, the time at which the permeability index peaks (18). The mean permeability value in the negative control lungs was 0.17 ± 0.01 (Fig. 3A). Instillation of 2.0 µg of IL-18 alone did not significantly augment vascular permeability in otherwise normal lungs (0.19 ± 0.03). Positive control rats not otherwise treated demonstrated a 40% increase in vascular permeability (to a value of 0.29 ± 0.02). In a companion group of rats that also received 2.0 µg of IL-18 in the presence of

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**FIGURE 1.** Northern blot for IL-18 mRNA in various rat tissues. A, Two micrograms of poly(A)⁺ RNA from eight different rat tissues were loaded into 1.2% formaldehyde agarose gel. The Northern blot was probed with a 32P-labeled full-length CDNA for rat IL-18. Equal loading was confirmed by β-actin probing (data not displayed). B, mRNA for IL-18 in lung 0–4 h after initiation of the inflammatory response. GAPDH content for equal loading is shown. The lowest frame is a densitometry of the upper portion of B.
absence of exogenously administered IL-18 (108 ± 14.8 pg/ml). The coadministration intratracheally of anti-BSA and 1.0 or 2.0 μg IL-18 further increased IL-1β production, to 132 ± 5.4 and 177 ± 16.5 pg/ml, respectively, (p < 0.05 for the latter).

Noninflamed lungs contained low levels of CINC (146 ± 36.2 ng/ml). The instillation of IL-18 alone (in the absence of immune complexes) caused a modest (but significant) increase in CINC (to 543 ± 23.5 ng/ml; Fig. 3E). Positive control rats demonstrated a 3-fold increase in BAL levels of CINC (1837 ± 97.5). In the positive control group with coinflation of 2.0 μg of IL-18, CINC levels were increased further, by 20% (to 2211 ± 104 ng/ml; p < 0.05). Thus the copresence of IL-18 with IgG immune complexes in the lung enhanced lung vascular permeability and augmented levels of neutrophils, IL-1β, TNF-α, and CINC.

**Effects of anti-IL-18 on vascular permeability and BAL content**

Because we observed an increase in vascular permeability after the coinstillation of IL-18 with anti-BSA, we evaluated whether an affinity-purified, neutralizing goat polyclonal Ab to mouse IL-18 could alter events in positive control animals. As indicated above, this Ab is reactive with rat IL-18. As shown in Fig. 4A, the extravascular leakage of 125I-labeled albumin was measured 4 h after the initiation of injury in lungs receiving 2.5 mg of anti-BSA together with 400 μg of pemmiog mouse IgG. As compared with the negative control group, the positive control group receiving 400 μg of normal rabbit IgG intratracheally had a more than 4-fold increase in vascular permeability (to a value of 0.60 ± 0.04). The addition of 400 μg of anti-IL-18 IgG resulted in a 30% reduction in vascular permeability (to a value of 0.42 ± 0.04; p < 0.05). BAL fluids collected at 4 h were evaluated for neutrophil content (Fig. 4B). Intrapulmonary deposition of IgG immune complexes caused a 10-fold increase in BAL neutrophil numbers (to 6.8 ± 106). The coinflation of anti-BSA and 400 μg of anti-IL-18 decreased neutrophil counts by 50% (to 3.5 ± 106; p < 0.05).

In parallel, we determined whether BAL content of inflammatory mediators would be altered by airway instillation of 400 μg of neutralizing Ab to IL-18. BAL fluids were obtained 4 h after initiation of lung inflammatory reactions and cytokine levels determined. In rats receiving 400 μg of normal rabbit IgG intratracheally, BAL fluids of all three mediators that are known to be involved in the pathogenesis of these inflammatory responses were significantly increased (Fig. 4, C–E) to 2880 ± 229 pg/ml for TNF-α, to 250 ± 25 pg/ml for TNF-α, and to 3984 ± 210 ng/ml for CINC. The coadministration of 400 μg of anti-IL-18 IgG with anti-BSA decreased levels of TNF-α by 33% (to 1908 ± 330 pg/ml), IL-1β levels by 60% (98 ± 15 pg/ml), and CINC levels by 27% (2892 ± 368 ng/ml). In companion experiments, we also assessed the effects of administration of anti-IL-18 on BAL levels of IFN-γ, because, as described above, IL-18 is well known to induce expression of IFN-γ. In noninflamed (negative control) lungs, BAL levels of IFN-γ were 50 ± 4.0 pg/ml. In the inflamed lungs of all animals treated with pemmiog IgG, the levels rose to 120 ± 8.2 pg/ml (Fig. 4F). In the presence of anti-IL-18, BAL levels of IFN-γ fell to negative control levels, 50 ± 3.9 pg/ml. These data suggest that intrapulmonary blockade of IL-18 prevents the expression of IFN-γ, consistent with the known biological activity of IL-18. Collectively, these data suggest that endogenous IL-18 enhances the lung inflammatory response in this model by enhancing production of mediators.

**Immunostaining of alveolar macrophages for IL-18**

To assess the possible source(s) of endogenous IL-18, immunostaining for rat IL-18 was done by using BAL alveolar macrophages retrieved from normal and lungs with immune complex

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**FIGURE 2.** Rat IL-18 protein expression in BAL fluids (concentrated 5-fold) after IgG immune complex deposition. As determined by Western blot analysis as a function of time (A), lane 1 contains (rr)IL-18, whereas lane 9 contains the same, but the anti-IL-18 was first preabsorbed with (rr)IL-18. Lane 10 is BAL fluid 4 h after initiation of the reaction, whereas lane 11 is similar, but in rats receiving 400 μg of anti-IL-18 intratracheally at time 0. B, Rat IL-18 was measured in BAL fluids as a function of time after initiation of lung inflammation. Whole lung homogenates were evaluated for IL-18 by ELISA, as a function of time after initiation of the lung inflammatory response. anti-BSA, the permeability index was further increased, to 0.44 ± 0.04 (a 35% increase) as compared with the positive control group (p < 0.05). Next, we determined whether these changes were associated with enhanced neutrophil recruitment. BAL fluids were collected at 4 h and evaluated for neutrophil content (Fig. 3B). In the negative control animals, low levels (<5 × 105) neutrophils were found; the intratracheal administration of 2.0 μg of IL-18 caused no significant increase in BAL content of neutrophils. Intratracheal instillation of 1.25 mg of anti-BSA together with 10 mg of BSA (which was intravenously administered) resulted in a 4-fold increase in neutrophil counts (to 1.6 × 106). The coinflation of IL-18 (1.0 μg or 2.0 μg) together with anti-BSA further increased neutrophil counts (to 3.1 × 106 and 5.0 × 106, respectively, p < 0.05).

Because TNF-α, IL-1, and CINC are important cytokines and chemokines involved in the recruitment of inflammatory cells in this lung model (19, 23, 24), we examined the effects of exogenously administered IL-18 on BAL levels of these mediators after IgG immune complex-induced lung inflammation. TNF-α levels in the BAL fluids (obtained at 4 h) were determined. Negative control animals had low BAL levels of TNF-α (5.6 ± 0.8 pg/ml; Fig. 3C). Intrapulmonary instillation of 2.0 μg of IL-18 alone caused a small increase in TNF-α content (to 9.0 ± 1.1 pg/ml). After immune complex deposition, BAL levels of TNF-α substantially increased (to 1279 ± 118 pg/ml). The coadministration of 1.25 mg of anti-BSA and 1.0 or 2.0 μg of IL-18 further increased TNF-α levels, to 1590 ± 109 and 2776 ± 130 pg/ml, respectively (p < 0.05).

IL-1β levels also were determined in BAL fluids by ELISA, using the same experimental conditions (Fig. 3D). Negative control animals had low levels of IL-1β (13.4 ± 0.50 pg/ml). The instillation of IL-18 into otherwise normal lungs caused no significant changes in BAL levels of IL-1β. A nearly 8-fold increase in BAL levels of IL-1β was found in the positive control group in the
deposits (at 4 h). Alveolar macrophages isolated from the noninflamed lungs demonstrated low constitutive expression of rat IL-18 (Fig. 5A). After intrapulmonary deposition of IgG immune complex-induced injury, alveolar macrophages showed strong staining for rat IL-18 (Fig. 5B). Thus, alveolar macrophages represent a source of IL-18 in the inflamed lung.

Lung cell sources of IL-18
As indicated above, several lung cells types were used in vitro: fibroblasts, alveolar macrophages, type II alveolar epithelial cells, and lung microvascular endothelial cells. The cells were cultured in the absence or presence of LPS (10 μg/ml) for 24 h at 37°C, and the supernatant fluids then were evaluated for IL-18 by ELISA. As shown in Table I, stimulated fibroblasts and alveolar macrophages produced 0.38 ± 0.37 and 1.14 ± 0.61 ng/ml, respectively, whereas fluids from the other cell cultures were negative (<200 pg/ml). Thus, the source of IL-18 in the inflamed lung appears to be restricted.

Effects of exogenously administered IL-18bp
As a further strategy to assess functional effects of IL-18 in the lung inflammatory model, we used recombinant human IL-18bp that can intercept IL-18. Human IL-18bp binding to rat IL-18 was determined by the methods described above. Fifty microliters of human IL-18bp (10 μg/ml) were used to coat each well of the microtiter plate. Rat IL-18 bound to human IL-18bp in a dose-dependent manner but not to solid phase BSA (Fig. 6A). The binding was detected at a dose of rat IL-18 as low as 15.6 ng/ml. These data demonstrate that rat IL-18 can bind to human IL-18bp. To investigate the effects of exogenously administered IL-18bp in the IgG immune complex model, recombinant human IL-18bp (0–18 μg) was added to 2.5 mg of anti-BSA IgG before their intratracheal instillation. The permeability index and BAL content of neutrophils and cytokines/chemokines were determined at the 4-h time point. The results are shown in Fig. 6. At a dose of 1.5 or 6 μg of IL-18bp, there was no statistically significantly effect on the permeability index. However, at the 18-μg dose of IL-18bp, there was a 52% decrease in the lung permeability index (Fig. 6B). Under the same conditions, neutrophil counts in BAL fluids dropped (by 43%), from 6.43 × 10⁶ to 3.65 × 10⁶ (p < 0.05; n = 4; Fig. 6C). Reduction in BAL content of TNF-α, IL-1β, and CINC were 38% (p < 0.05; n = 4), 55% (p < 0.01; n = 4), and 34% (p < 0.01; n = 4), respectively (Fig. 6, D–F). These data suggest that IL-18bp attenuates lung inflammation induced by IgG immune complexes and support the concept that endogenous IL-18 functions as a proinflammatory factor in this model of lung injury by enhancing production of proinflammatory mediators.
Discussion
In the current study, we used a model of acute lung inflammation induced by the intrapulmonary deposition of IgG immune complexes. Alveolar deposition of IgG immune complexes was achieved by the intratracheal instillation of rabbit polyclonal IgG against BSA followed by the intravenous injection of BSA. This results in damage of capillary endothelial cells and alveolar epithelial cells, neutrophil influx, and intraalveolar hemorrhage. This attendant activation of lung macrophages leads to induction of the early response cytokines TNF-α and IL-1β. These cytokines not only activate macrophages but lead to the expression of vascular adhesion molecules and chemokines involved in the recruitment of

FIGURE 4. Protective effects of anti-IL-18 on acute lung inflammation. Pulmonary vascular permeability index (A) and BAL content of neutrophils (B), TNF-α (C), IL-1β (D), CINC (E), and IFN-γ (F).

FIGURE 5. Immunohistochemical staining of alveolar macrophages for rat IL-18 after IgG immune complex lung inflammation. A, BAL macrophages from PBS-instilled lung; B, BAL macrophages obtained 4 h after deposition of IgG immune complexes. Counterstained with hematoxylin, ×100.
neutrophils (16). Complement, especially C5a, plays a key role in these events (25). Oxidants and proteases released from neutrophils and activated lung macrophages damage lung cells and tissue matrix. Previously, we have shown that ILs have various regulatory roles during the immune response. For instance, IL-4, IL-6, IL-10, and IL-13 have strong anti-inflammatory effects in this lung injury model (26–29). The role of IL-18 in this model of lung injury has not been explored. The current study provides evidence that endogenous IL-18 acts as a proinflammatory cytokine and contributes to IgG immune complex-induced lung inflammation.

IL-18 mRNA is constitutively expressed in various rat tissues, including lung. Constitutive expression of IL-18 also has been observed in specific cell types, including Kupffer cells, macrophages, keratinocytes, and articular chondrocytes (8, 30). Up-regulation of IL-18 mRNA was found after onset of pulmonary inflammation initiated by intrapulmonary deposition of IgG immune complex (Fig. 1). The early up-regulation of IL-18 has been observed after insults induced by mycobacteria, contact allergens, and cold stress (10, 31, 32). In numerous studies, it has been concluded that the 1.35-kb mRNA encodes the precursor (pro) form, which, once translated, is cleaved by ICE (caspase-1) to a functional, mature protein of ~18.3 kDa. By Western blot analysis of BAL fluids, the IL-18 precursor protein (~26 kDa), but not the mature form (~18.3 kDa), was found in noninflamed lungs (Fig. 2). The mature form of IL-18 was detected in BAL fluids 1–6 h after initiation of lung injury. The absence of the mature form of IL-18 in noninflamed lungs is consistent with the finding of others who have observed the absence of the mature form of IL-18 in unstimulated cells (reviewed in Ref. 12). Preabsorption of anti-IL-18 by (rr)IL-18 revealed that the bands at positions 26 and 18 kDa in BAL fluids from inflamed lungs were both related to IL-18, because both bands essentially disappeared after absorption by IL-18 (Fig. 2, lane 9). It should be noted that IL-18 mRNA was evaluated by using whole-lung RNA. When concentrated BAL fluids were evaluated, mature IL-18 protein was found by 1 h (Fig. 2A), whereas the mRNA increase wasn’t apparent until 2 h (Fig. 1). This discrepancy probably is attributable to the two different sources of mRNA (whole lung) and protein (BAL fluids). The data in Fig. 2, B and C, suggest that BAL fluids are a more sensitive indicator of the presence of IL-18 than are lung homogenates because of earlier detection of IL-18 (1 h as seen in Fig. 2, A and B, vs 4 h as seen in Fig. 2C). Alternatively, it is likely that alveolar macrophages are the chief source of IL-18, resulting in earliest presence in the BAL compartment. Tight regulation in expression of the mature form of IL-18 also has been found in stimulated macrophages and chondrocytes (11, 33). In the current study, it is possible that ICE plays a key role in expression of the mature form of IL-18.

Table I. IL-18 production by cultured lung cells

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Fibroblasts</th>
<th>Macrophages</th>
<th>Type II cells</th>
<th>Endothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>NDa</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LPS (10 μg/ml)</td>
<td>0.38 ± 0.37</td>
<td>1.14 ± 0.61</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a ND, not detectable (<200 pg/ml).

FIGURE 6. Protective effects of IL-18bp on immune complex-induced lung inflammation. A, binding of (rr)IL-18 to solid-phase human IL-18bp. A total of 50 μl of human IL-18bp (10 μg/ml) was used to coat each well. Bound rrIL-18 was detected with polyclonal goat anti-mouse IL-18. B, Effects of airway instillation of IL-18bp on lung vascular permeability index at 4 h. When used, 0–18 μg were coinstilled with 2.5 mg of anti-BSA. BAL content of PMN (C), TNF-α (D), IL-1β (E), and CINC (F).
Experimental studies have suggested that IL-18 protects against fungal, bacterial, and viral agents (34–37). This protection is thought to be attributable in part to the infiltration of inflammatory cells after treatment with IL-18. In the current studies, we show that the in vivo exogenous administration of IL-18 after IgG immune complex deposition increases neutrophil recruitment as well as vascular permeability, a marker of lung injury. Conversely, IL-18 blockade by neutralizing Ab or by IL-18bp reduced evidence of lung inflammation. These results suggest that the protection afforded by IL-18 against infectious agents may be related to its ability to facilitate recruitment of inflammatory cells to sites of infectious agents. Reduction in inflammatory injury by treatment with anti-IL-18 has been observed in endotoxin-induced injury and in experimental autoimmune encephalomyelitis (5, 38, 39). IL-18bp appears as a soluble decoy receptor for IL-18 and has been described as a natural inhibitor for biological activities of IL-18 (40, 41). Our studies suggest that endogenous IL-18 has a role in enhancing the inflammatory response by causing enhanced cyto- kine and chemokine generation, which is consistent with our findings.

In vitro studies suggest that IL-18 acts as a proinflammatory cytokine during injury. Mature IL-18 is induced in human peripheral blood mononuclear cells after exposure to IL-8, MIP-1α, MCP-1, or TNF-α. Inhibition of TNF-α resulted in a 80% reduction in IL-18 production, suggesting that the primary action of IL-18 is via a TNF-α-dependent pathway (42). Previously, we have shown that lung inflammation and related events require the production of TNF-α, MIP-2, and CINC (19). In the current studies, we have shown the airway instillation of IL-18 together with deposition of IgG immune complexes enhanced TNF-α and IL-1β levels in BAL fluids. Conversely, the addition of IL-18bp or neutralizing Ab to IL-18 suppressed the increase in BAL levels of cytokines after injury. This suggests that IL-18 has an endogenous role in enhancing production of early response cytokines during acute lung inflammation. It is unknown whether the increase in these inflammatory cytokines is attributable in part to the induction of IFN-γ (or another cytokine) and the subsequent activation of macrophages by IL-18. The effectiveness of IL-18 blockade in vivo by Ab is suggested by the reduction to baseline levels of IL-18bp and CINC (19). In the current studies, we have shown that IL-18bp is a product of activated macrophages and has the ability to cause autocrine stimulation of macrophages, resulting in enhanced generation of cytokines and chemokines (46). It is clear that IL-18 is a pleiotropic cytokine with numerous functions. Recent studies have shown IL-18 directly induces IFN-γ promoter activity, up-regulates ICAM-1 expression in human myelomonocytic cells, and has synergistic effects when combined with IL-12 (47). Our recent data indicate that some, but not all polyclonal rabbit Abs to rat chemokines, are protective in the IgG immune complex model of acute lung injury. For example, in this model Abs to MIP-2, CINC, MIP-1α, and MIP-1β were protective (46, 48, 49) but not Abs to MCP-1 or RANTES (49). In contrast, the same anti-RANTES Abs significantly delayed rejection of allografted rat hearts (50) and the same anti-MCP-1 enhanced the lethal effects of infused LPS in mice (51). Conversely, infusion of MCP-1 in the same mouse model was protective (52). Thus, there is a certain specificity of these blocking Abs, depending on the inflammatory model under study. The current studies identify an in vivo proinflammatory role for IL-18 in the lung inflammatory model used.

Acknowledgments
We thank Robin Kunkel, Lisa Riggs, and Beverly Schumer for their assistance in the preparation of this manuscript.

References