Published in final edited form as:

Am J Respir Cell Mol Biol. 2009 December ; 41(6): 661–670. doi:10.1165/rcmb.2008-0182OC.

Role of Proinflammatory Cytokines IL-18 and IL-1β in Bleomycin-Induced Lung Injury in Humans and Mice

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Abstract

Administration of several chemotherapeutic drugs, such as bleomycin, busulfan, and gefitinib, often induces lethal lung injury. However, the precise mechanisms responsible for this drug-induced lung injury are still unclear. In the present study, we examined the role of the proinflammatory cytokines IL-18 and IL-1β in the mechanism of bleomycin-induced lung injury. We performed immunohistochemical analysis of IL-18 and IL-18 receptor (R) α chain expression in the lungs of five patients with bleomycin-induced lethal lung injury. Enhanced expression of both IL-18 and IL-18Rα was observed in the lungs of all five patients with bleomycin-induced lung injury. To support the data obtained from patient samples, the levels of IL-1β and IL-18 mRNA and protein, pulmonary inflammation, and lung fibrosis were examined in mouse models of bleomycin-induced lung injury. Intravenous administration of bleomycin induced the expression of IL-1β and IL-18 in the serum and lungs of wild-type C57BL/6 mice. IL-18–producing $F4/80^+$ neutrophils, but not $CD3^+$ T cells, were greatly increased in the lungs of treated mice. Moreover, bleomycin-induced lung injury was significantly attenuated in caspase-$1^{-/-}$, IL-$18^{-/-}$, and IL-18R$\alpha^{-/-}$ mice in comparison with control mice. Thus, our results provide evidence for an important role of IL-1β and IL-18 in chemotherapy-induced lung injury.

Keywords

bleomycin; lung injury; cytokine; mouse model

Bleomycin is a member of the glycopeptide group of antibiotics derived from *Streptomyces verticillus* (1). This agent is a chemotherapeutic drug used clinically for a variety of human

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Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

malignancies, including lymphoma. It has been reported that administration of a high dose of bleomycin often leads to lethal lung injury and pulmonary fibrosis in human patients, as well as in rodent models. Treatment with a high dose of bleomycin results in acute alveolitis and interstitial inflammation, characterized by recruitment of neutrophils, lymphocytes, and macrophages in the acute phase. Subsequently, fibrotic responses occur, characterized by an increase in fibroblast proliferation and extracellular matrix synthesis. Previous studies have suggested that various mediators (i.e., cytokines and chemokines, including TNF-α [2], transforming growth factor-β [3], IL-1β [4], macrophage-inflammatory protein-1α [5], monocyte chemoattractant protein-1 [6], reactive oxygen species [ROS] [7], and Fas/Fas ligand interactions [8]) mediate bleomycin-induced pulmonary inflammation and fibrosis in mice. However, the pathogenesis of bleomycin-induced lung injury is still not well understood.

IL-18 is a proinflammatory cytokine that belongs to the IL-1 family of ligands (9, 10). The mature forms of IL-1p and IL-18 are produced intracellularly from a biologically inactive precursor (pro–IL-1β and pro–IL-18, respectively) after cleavage by caspase-1, originally identified as IL-1β-converting enzyme. The IL-18 receptors, although distinct from IL-1 receptors, also belong to the IL-1 receptor (R)/Toll receptor family. The IL-18R complex consists of at least two receptor chains: a ligand-binding chain, termed the IL-18Rα chain, and a coreceptor, termed the IL-18Rβ chain; both chains are thought to be required for signaling. IL-18 synergistically induces IFN-γ production when IL-12, IL-2, antigens, and IFN-α are added as costimulating signals. We and others have also reported that IL-18 potently induced T helper (Th) type 2 cytokines (e.g., IL-4, IL-5, IL-10, IL-13) from T cells, natural killer (NK) cells, NK-T cells, basophils, and mast cells, and IgE production in the absence of IL-12 (9, 11-15). Thus, IL-18 can act as a cofactor for both Th1 and Th2 cell development. IL-18 also plays an important role in various diseases, such as tuberculosis, tuberculoid leprosy, Sjögren's syndrome, rheumatoid arthritis, bone malformation, skin disorders, and Crohn's disease (9, 16-18). We have previously reported a new murine model of human interstitial lung diseases (ILD) in which daily administration of IL-18 plus IL-2 induced lethal lung injury (19). We also showed that constitutive overproduction of mature IL-18 in the lungs of transgenic mice resulted in severe emphysema lesions. In contrast, when IL-18 production was induced in lung tissues for 4 weeks through the use of a doxycycline-dependent surfactant protein (SP)-C promoter, interstitial inflammation was induced (20). Furthermore, we demonstrated that, in patients with idiopathic pulmonary fibrosis, the majority of pulmonary cells strongly expressed both IL-18 and IL-18Rα (21), and we have recently reported that a significant correlation exists between serum levels of IL-18 and pulmonary function (%FEV_1) in chronic obstructive pulmonary disease (22). Overall, our results suggest that the IL-18/IL-18R complex is involved in the pathogenesis of pulmonary inflammatory diseases. However, the roles of IL-1β and IL-18 in bleomycin-induced lung injury are unclear. The aim of our present study was to evaluate the roles of IL-1β and IL-18 in the pathogenesis of bleomycin-induced lung injury.

MATERIALS AND METHODS

Patients with Bleomycin-Induced Lung Injury

Five patients (four men and one woman, aged 56–69 yr) diagnosed as having cancer (female genitalia, lower jaw, lung, hypopharyngeal, and skin cancer) were monitored at Kurume University Hospital. All five patients had squamous cell carcinoma. The patients were treated with bleomycin, and died of severe lung failure. Lung tissues were obtained at autopsy, fixed with 10% buffered formalin, and paraffin embedded. The details of these patients are shown in Table 1. Patients with congestive heart failure, infectious diseases, collagen diseases, and other ILDs, such as pneumoconiosis, pulmonary sarcoidosis, hypersensitivity pneumonitis, eosinophilic pneumonitis, nonspecific interstitial pneumonia, or cryptogenic organizing pneumonia, were excluded. As control samples, we used 13 lung sections: six from patients who had died in accidents (obtained from the Department of Forensic Medicine, Kurume University) and seven sections of noncancerous lung from patients who had undergone surgery for lung cancer, as previously reported (22). Sample collection was performed in accordance with Kurume University guidelines and approved by the institutional ethics committee. Prior informed consent was obtained from all patients or relatives of deceased patients.

Immunohistochemical Assay

Immunohistochemical analysis was performed as reported previously (21, 22). Briefly, anti–human IL-18 (clone 8 [IgG2a] or clone 81 [IgM]) monoclonal antibodies (mAbs) (kindly provided by Dr. Do-Young Yoon, Laboratory of Cellular Biology, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea [23]) and anti–human-IL-18Rα (H44 [IgG1]) mAb (established by our laboratory [21]) were incubated with samples at 4°C for 18 hours to detect human IL-18 and IL-18Rα, respectively. Mouse purified IgG2a, IgM, and IgG1 Abs (Caltag Laboratories, Burlingame, CA) were used as negative controls. Positive reactivity was identified using biotin-labeled goat anti-mouse IgG, streptavidin–peroxidase, and 3-3′-diaminobenzidine-4HCl employing an LSAB2 kit (Dako, Kyoto, Japan). The H44 mAb is commercially available from PharMingen (San Diego, CA), eBio-science (San Diego, CA), BioLegend, Inc. (San Diego, CA), and Serotec (Oxford, UK).

Mice

Wild-type (WT) C57BL/6N (B6) mice (Charles River Japan Inc., Yokohama, Japan) and B6 × 129 (B6129F1 hybrid) mice (Taconic MandB, Ry, Denmark) were used. B6 × 129 background IL-18Rα (IL-1R–related protein)–deficient (−/−) (24) (B6 IL-$18^{-/-}$) mice (25) (kindly supplied by Dr. Shizuo Akira, Osaka University, Osaka, Japan), B6 × 129 caspase-$1^{-/-}$ mice, and B6 caspase-$1^{-/-}$ mice (kindly supplied by Dr. Kenji Nakanishi, Hyogo Medical College, Nishinomiya, Japan) (26) were also used. In addition, we established B6 IL-18R$\alpha^{-/-}$ mice at our laboratory by backcrossing B6 × 129 IL-18R$\alpha^{-/-}$ mice more than five times with WT C57BL/6N mice. All mice used for experiments in this study are described in Table 2. Mice were bred and maintained in accordance with the procedures outlined in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 86-23, 1985). All mice in this study were maintained under specific pathogen-free conditions and used for experiments at 5 to 9 weeks of age. All

procedures were approved by the Committee on the Ethics of Animal Experiments, Kurume University (approval no. 914, 2003).

Administration of Bleomycin to Mice

Mice were treated with an intravenous tail vein or an intraperitoneal injection of 2 mg bleomycin (Nippon Kayaku, Tokyo, Japan) dissolved in 200 μl sterile PBS once at Day 0, twice at Days 0 and 7, thrice at Days 0, 7, and 14, or four times at Days 0, 7, 14, and 21, respectively. Mice were then killed at several time points, as described in the Results section. Lungs and sera were obtained, and wet lung weight and body weight were measured as previously reported (7).

Isolation of Bronchoalveolar Lavage Fluid from Mice

A tubing adaptor was inserted into the trachea, and the lungs were washed three times with 3 ml of PBS. The recovered bronchoalveolar lavage fluid (BALF) was evaluated with a hemocytometer. Aliquots of cells were centrifuged onto glass slides, dried in air, and stained with Wright-Giemsa. Cell populations were then calculated as reported previously (27). The remaining BALF was centrifuged, and the supernatants were then collected and stored at −80°C until ELISA assay.

ELISA and RNase Protection Assays

The whole lung tissues were homogenized in 2 ml of lysis buffer (1% Triton X-100, 10 mM Tris-HCL, 5 mM EDTA, pH 7.6) containing a protease inhibitor cocktail (Complete Mini, Boehringer Mannheim GmbH, Mannheim, Germany) and centrifuged at 20,000 × *g* for 15 minutes, and the supernatants were collected and stored at − 80°C until ELISA assay. The sandwich ELISA kits were used for mouse IL-1β, mouse IFN-γ, mouse transforming growth factor-β (R&D Systems, Minneapolis, MN), and mouse IL-18 (MBL, Nagoya, Japan). Total RNA was isolated from the lungs with an RNeasy Midi Kit (Qiagen Inc., Valencia, CA). Cytokine and chemokine mRNA expression was analyzed by RNase protection assay using a RiboQuant kit (PharMingen, San Diego, CA). mRNA levels was quantitated by a densitometer (Typhoon 8600; Amersham Biosciences, Piscataway, NJ). Mouse glyceraldehyde 3-phosphate dehydrogenase was used as the control for quantitation in both RNase protection assay, as described previously (7).

Establishment of Anti-Mouse IL-18 mAb (M5)

An anti-mouse IL-18 mAb (M5, rat IgG2a, κ) was established by fusion of the mouse myeloma cell line, P3X63Ag8.U.1, with spleen cells isolated from a Sprague-Dawley rat immunized with the recombinant mouse IL-18 protein (MBL), as we have reported previously (21). We have generated ascites using this cell line, and purified this antibody using a protein G column. Purified mAb was labeled with FITC, as reported previously (28).

Western Blot Analysis

Western blotting using the established rat anti-mouse IL-18 mAbs was performed as reported previously (13).

Surface Antigen Analysis by Flow Cytometry

Three-color analysis was performed using an FC500 flow cytometer (Beckman Coulter, Palo Alto, CA). Anti-mouse CD16/CD32 mAb (2.4G2; PharMingen) was used to block the nonspecific binding. Isolated BALF cells from mice were stained with phycoerythrin (PE)-conjugated anti-mouse F4/80 (Caltag Laboratories) and PE-Cy7–conjugated anti-mouse CD3ε (145-2C11; eBioscience). For intracellular cytokine staining, cells were stained with FITC anti–mIL-18 (M5) and/or FITC rat IgG2a (Caltag Laboratories), as reported previously (11, 13).

Histological Examinations

For the histological analysis, mice were killed with an intraperitoneal injection of sodium pentobarbital (2.5–5 mg per mouse). After the thorax had been opened, the lungs were immediately fixed by intratracheal instillation of 20% buffered formalin for 15 to 20 minutes at a constant pressure of 27 cm H_2O. After gross examination, the extracted tissues were placed into 20% buffered formalin and further fixed for at least 24 hours. Sections (4-(μm thick) were cut from paraffin-embedded tissues, placed on poly-l-lysine–coated slides, and then incubated overnight at 55 to 60°C. Deparaffinized sections were stained with hematoxylin and eosin (H&E) and sequential sections were alternatively stained with the Elastica van Gieson or Azan methods. For the analysis of bleomycin-induced lung fibrosis, the fibrotic responses of the lung were scored as previously reported (29). Briefly, H&E sections were observed at 400× magnification and the lesions were defined as follows: Score 0, no lesions; Score 1, occasional small localized subpleural fibrotic foci; Score 2, thickening of intraalveolar septa and subpleural fibrotic foci; and Score 3, thickened continuous subpleural fibrous foci and intra-alveolar septa. These sections were examined by two independent pathologists with no knowledge of the treatment conditions.

Hydroxyproline Assay

The hydroxyproline assay was performed by capillary electrophoresis, as described previously (30). Briefly, lung tissues were freeze dried for 24 to 48 hours. The dried lung was then hydrolyzed in 1 ml of 6 N HCl at 110°C for 22 hours. An aliquot (500 μl) of the hydrolysate was incubated with 1 ml of 25 mM trisodium phosphate at 60°C for 20 minutes, and then 1 ml of 25 M sodium borate was added. A 280-μl aliquot of the sample was conjugated with 40 μl of fluorescamine solution (3 mg/ml fluorescamine in acetone, containing 20 μl of pyridine) for 2 minutes, and analyzed for hydroxyproline at 190–350 nm by capillary electrophoresis (P/ACE System MDQ Capillary Electrophoresis System; Beckman Coulter), as described previously (30). Hydroxy-L-proline (1 mg/ml) and L-proline (1 mg/ml) were dissolved in 0.1 M sodium tetraborate buffer (pH 9.0) and used as a control.

Statistical Analysis

Results are expressed as the mean (±SD) of the number per group. ANOVA or unpaired Student's *t* test was used to compare differences between groups. SAS 9.1.3 software, Japanese edition (SAS Institute, Cary, NC), was used for statistical analysis.

RESULTS

IL-18 and IL-18Rα Expression in Lung Tissue from Patients with Bleomycin-Induced Lung Injury

Table 1 gives details (age, sex, date of autopsy, original cancers, dose of bleomycin, and treatment) of all five subjects whose tissues were analyzed immunohistochemically. Our hospital does not possess all of the medical records for these five patients, as they died between 1970 and 1976. Therefore, we were unable to obtain details of the treatment given to Patient 3. We analyzed H&E-stained lung sections from the five patients with bleomycin-induced lung injury. Representative histopathologic features of these patients are shown in Figure 1A. A diffuse alveolar damage–like pattern accompanied by random and non-uniform foci of inflammation, exudation of alveolar macrophages, mononuclear cell infiltration, degeneration of the bronchoalveolar epithelium, and fibroblasts were widely recognized in the lungs of all five patients. Alveolitis rather than lung fibrosis was predominant in all cases, although both fibrosis and alveolitis were present to some degree. In contrast, no specific inflammatory responses were observed in the lung tissues of any of the 13 control subjects. Representative histopathologic features observed in a 66-year-old male are shown in Figure 1B. IL-18 was expressed constitutively, but weakly, in the bronchoalveolar epithelium, alveolar macrophages, and endothelium of small vessels in control subjects. Bronchoalveolar epithelium and alveolar macrophages in control lung tissues expressed IL-18Rα weakly, as reported previously (21, 22). We found that the expression of both IL-18 and IL-18Rα was strongly enhanced in inflammatory cells infiltrating the lung, including mononuclear cells, neutrophils, and alveolar macrophages in all five patients with bleomycin-induced lung injury. The lining epithelial cells also strongly expressed both IL-18 and IL-18Rα proteins. One noteworthy observation was that IL-18 was expressed in intra-alveolar exudates, although we were unable to distinguish between the pro- and mature forms of IL-18. IL-18Rα was also enhanced in native pulmonary cells, as well as in inflammatory infiltrating cells. However, it is unclear whether IL-1β was also strongly expressed in pulmonary inflammatory cells in these patients, as commercially available antibodies against human IL-1β were not suitable for immunohistochemistry.

Increased Serum Levels of IL-1β and IL-18 in Mice after Intravenous Administration of Bleomycin

Next, we investigated whether bleomycin can induce IL-1β and IL-18 in mice. WT B6 mice were given an intravenous tail vein injection of 2 mg bleomycin ($n = 4$ per group) and then killed 0, 6, 24, or 72 hours after treatment. Serum and lung samples were obtained as described in Materials and Methods; results are shown in Figure 2A. Serum IL-1β levels were significantly ($P = 0.0122$) higher than those in nontreated mice at 6 hours after bleomycin treatment. Serum IL-18 levels were significantly ($P = 0.0212$) higher than those in nontreated mice at 24 hours after treatment. At 72 hours after the treatment, serum levels of IL-1β and IL-18 were decreased, and were similar to those in nontreated mice. Serum IFN-γ was undetectable (<2 pg/ml) at any of the time points analyzed. Lung IL-1β levels were significantly ($P = 0.0133$) higher than those in nontreated mice 6 hours after the treatment. Lung IL-1β, IL-18, and IFN-γ levels were increased at 24 hours after the treatment. However, statistical analysis using AVOVA showed that IL-1β ($P = 0.0835$), IL-18

($P = 0.1935$), and IFN-γ ($P = 0.0569$) protein levels in the lungs were not significantly increased at 24 hours after the treatment when compared with those in nontreated mice. Intravenous PBS treatment did not increase the levels of IL-1β, IL-18, and IFN-γ in the lungs and sera of normal mice at any of the time points analyzed (data not show). Moreover, IL-1β and IL-18 were undetectable in the lungs and sera of bleomycin-treated caspase-$1^{-/-}$ mice (data not shown), consistent with the fact that pro–IL-1β and pro–IL-18 cannot be cleaved and released from the cell surface in the absence of caspase-1 (26).

RNAse protection analysis revealed that mRNAs corresponding to IL-1 family members (IL-1β, IL-1R antagonist [IL-1Ra], and IL-18), and macrophage migration inhibitory factor were induced in the lungs of bleomycin-treated mice at 6 hours (*lanes 4–7*) and 24 hours (*lanes 8–10*) after intravenous administration of bleomycin. In contrast, IFN-γ mRNA was not detected in the lungs of bleomycin-treated mice at any of the time points analyzed (Figure 2B). Quantitative analysis (Figure 2C) revealed that IL-1β, IL-1Ra, and IL-18 mRNA levels were significantly ($P < 0.05$) increased at 6 hours after intravenous administration of bleomycin in comparison with the controls (24 h after PBS treatment). Migration inhibitory factor mRNA levels were significantly ($P < 0.05$) increased at 24 hours after intravenous administration of bleomycin in comparison with the control animals. Overall, intravenous administration of bleomycin significantly increased the serum and lung levels of IL-1β and IL-18 in normal mice.

IL-18–Producing Inflammatory Cells Are Increased in the Lungs of Bleomycin-Treated WT B6 Mice

Next, we examined whether the increased numbers of inflammatory cells in BALF were able to produce IL-18 protein. We newly established seven clones of rat anti-mouse IL-18 mAb (clones M1–M7). One anti-mouse IL-18 mAb (M5, rat IgG2a) is applicable to intracellular staining for flow cytometry. M5 mAb can recognize recombinant mouse IL-18, but not recombinant rat IL-18 or recombinant human IL-18, and can also be used for Western blotting and immunohistochemistry (data not shown).

We treated WT B6 mice with an intraperitoneal injection of 2 mg bleomycin dissolved in 200 ml sterile PBS, or with 200 μl PBS as a control, on Day 0. BALF was isolated from these mice on Day 4. The total numbers of pulmonary cells in the BALF of PBS- and bleomycin-treated mice were 6.5 (±1.4) × 10^4 and 8.1 (±0.2.2) × 10^4 cells per lung ($n = 5$ per each group), respectively. These results show that inflammatory cells were increased, although not significantly, in the lungs of WT B6 mice after intraperitoneal injection of bleomycin. We then performed experiments to study inflammatory cells in BALF by intracellular staining. A representative staining pattern is shown in Figure 3. Flow cytometry showed that bleomycin markedly induced IL-18–producing F4/80-positive cells, but not $CD3^+$ T cells, in the BALF of WT B6 mice on Day 4. Our results showed that IL-18–producing inflammatory cells, especially F4/80-positive macrophages, were greatly increased in the lungs of bleomycin-treated, but not PBS-treated, WT B6 mice.

Prevention of Bleomycin-Induced Lung Injury in Caspase-1$^{-/-}$, IL-18Rα$^{-/-}$, and IL-18$^{-/-}$ Mice

As mentioned previously here, administration of bleomycin is known to induce lung injury in both mice and humans. Initially, we examined whether IL-18$^{-/-}$ mice on a B6 background were resistant to lung injury in comparison with WT B6 mice used as control animals. Histological analysis showed that bleomycin-induced lung injury did barely occur in IL-18$^{-/-}$ mice (Figure 4A). Next, we performed quantitative analysis for lung injury using the fibrosis score (29). The fibrosis score estimated at Day 28 in bleomycin-treated IL-18$^{-/-}$ mice (0.5 ± 0.5) was significantly ($P = 0.003$) lower than that in control WT B6 mice (2.5 ± 0.6). As shown in Figure 4B, bleomycin-induced lung injury was significantly decreased in IL-18$^{-/-}$ mice, but not in WT B6 mice. Furthermore, the mean wet lung weight in bleomycin-treated IL-18$^{-/-}$ mice was significantly lower than that in WT B6 mice (Figure 4C), and the lung hydroxyproline content in bleomycin-treated IL-18$^{-/-}$ mice was significantly lower than that in control WT B6 mice (Figure 4D). It is of note that WT B6 mice are more susceptible to bleomycin-induced lung injury than WT B6 × 129 mice (Figures 4B and 4C). A previous study also reported similar observations (i.e., B6 and C3H/HeN mice are considered to be susceptible to bleomycin-induced lung fibrosis or injury, and Balb/c and C3H/fKam mice are relatively resistant to bleomycin) (31). Therefore, we next examined whether B6 × 129 caspase-1$^{-/-}$ and B6 × 129 IL-18Rα$^{-/-}$ mice were also resistant to lung injury as compared with WT B6 × 129 mice. Mice were given an intraperitoneal injection of 2 mg bleomycin on Days 0, 7, and 14, and then killed on Day 28. Representative histological results from three independent experiments are shown in Figure 4A. The fibrosis score estimated on Day 28 in bleomycin-treated IL-18Rα$^{-/-}$ mice (0.3 ± 0.2) and caspase-1$^{-/-}$ mice (0.2 ± 0.1) was significantly ($P < 0.01$) lower than that in control WT B6 × 129 mice (1.5 ± 0.6) (Figure 4B). The mean wet lung weight in bleomycin-treated caspase-1$^{-/-}$ and IL-18Rα$^{-/-}$ mice was significant lower than that in bleomycin-treated B6 × 129 mice (Figure 4C), and the lung hydroxyproline content in bleomycin-treated caspase-1$^{-/-}$ and IL-18Rα$^{-/-}$ mice was significantly lower than that in control B6 × 129 mice (Figure 4D). It is noteworthy that histological analysis using the fibrosis score is more sensitive than wet lung weight or hydroxyproline content for assessing bleomycin-induced lung injury. Based on this analysis, we found that bleomycin-induced lung injury was not completely prevented in IL-18$^{-/-}$, IL-18Rα$^{-/-}$, or caspase-1$^{-/-}$ mice. Thus, our results suggest that IL-18– or caspase-1–independent mechanisms may also be involved in the pathogenesis of bleomycin-induced lung injury. The lung fibrosis scores evaluated using sections stained with H&E, Elastica van Gieson, or Azan methods were not significantly different (data not shown).

Prevention of Neutrophil Accumulation in Bleomycin-Treated Caspase-1$^{-/-}$ Mice

As described above, genetic background can influence susceptibility to bleomycin in several mouse strains (31). Therefore, we established B6 background IL-18Rα$^{-/-}$ mice to investigate inflammatory cells in BALF from this strain. B6 background caspase-1$^{-/-}$, B6 IL-18$^{-/-}$, B6 IL-18Rα$^{-/-}$, and control WT B6 mice were intraperitoneally injected twice with bleomycin (2 mg) or PBS (vehicle) on Days 0 and 7. The recovered BALF cells were isolated and examined on Day 28, and the results are shown in Figure 5. Neutrophils were significantly ($P < 0.05$) decreased in BALF of bleomycin-treated B6

caspase-1$^{-/-}$ mice on Day 28 in comparison with bleomycin-treated control WT B6 mice. In contrast, lymphocytes were significantly ($P < 0.05$) increased in BALF of bleomycin-treated B6 caspase-1$^{-/-}$ mice on Day 28 when compared with control B6 mice. Interestingly, neutrophils were decreased, although not significantly, in BALF of bleomycin-treated B6 IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice on Day 28 when compared with control B6 mice.

DISCUSSION

Bleomycin is known to kill neoplastic cells by inducing DNA strand scission and interfering with DNA synthesis (1). These effects are thought to be mediated by O_2 radicals. The O_2-derived species may result from bleomycin's ability to chelate multivalent metals, such as iron. Iron easily facilitates single-electron (e^-) transfers to other molecules, donating and accepting electrons as it undergoes cyclic oxidation and reduction. The metal-bleomycin complex may initiate single-electron transfers within the cells, consistent with this model. It has been reported that lethal pulmonary fibrosis develops in patients treated with bleomycin, especially after radiation. In such patients, there are many potential sources of ROS, including inflammatory cells (neutrophils, monocytes, macrophages), alveolar macrophages, parenchymal cells, circulating oxidant-generating enzymes (xanthine oxidase), and inhaled gases with high concentrations of oxygen that are often used during mechanical ventilation. The antioxidants, superoxide dismutase (32) and N-acetyl-L-cysteine (33), partly inhibit bleomycin-induced lung injury in mice. A strong antioxidant, thioredoxin 1 (34), prevented lethal bleomycin-induced lung injury in mice (7). Taken together, the data are consistent with the concept that bleomycin-induced lung injury in humans and mice can be mediated, at least in part, by the generation of ROS.

Previously, we reported a new mouse model of human ILD. In this model, daily administration of IL-18 with IL-2 induces rapid lung injury, but no other tissue damage, and results in death from interstitial pneumonia (19). Both the pro–IL-1β and pro–IL-18 forms are constitutively expressed in pulmonary cells, such as neutrophils, monocytes, macrophages, alveolar macrophages, and parenchymal cells, in healthy subjects (21). In addition, previous studies have shown that human alveolar macrophages produce IL-1β in response to bleomycin *in vitro* (4). Therefore, we speculated that both IL-1β and IL-18 are involved in the pathogenesis of bleomycin-induced lung injury in humans and in the mouse model. Our present results suggest that caspase-1–dependent IL-1β and IL-18 secretion is induced by bleomycin and causes lung injury in mice. Moreover, enhanced expression of IL-18 and IL-18Rα was observed in lung tissue obtained from patients who had succumbed to bleomycin-induced lung injury. Taken together, activated caspase-1 activity may play a role in the pathogenesis of bleomycin-induced lung injury.

In this study, we established an anti-mouse IL-18 mAb for intracellular staining by flow cytometry, and performed additional experiments to analyze inflammatory cells in BALF. Our results show that bleomycin markedly induced inflammatory cells, including F4/80-positive cells, but not CD3$^+$ T cells, in the BALF of WT B6 mice on Day 4. These inflammatory cells, especially F4/80-positive cells, expressed IL-18 protein. Neutrophils were significantly decreased in BALF of bleomycin-treated B6 background caspase-1$^{-/-}$, IL-18$^{-/-}$, and IL-18Rα$^{-/-}$ mice on Day 28 when compared with PBS-treated control

mice. In addition, bleomycin-treated caspase-1$^{-/-}$, IL-18$^{-/-}$, and IL-18Rα$^{-/-}$ mice showed significant attenuation of lung fibrosis in comparison with control mice. These results suggest that IL-18–producing pulmonary inflammatory cells, including neutrophils, may be involved in lung fibrosis.

A previous study has shown that immobilization stress induces pro–IL-18 via induction of adrenocorticotropic hormone and a superoxide-mediated caspase-1 activation pathway, resulting in conversion of pro–IL-18 to mature IL-18 (35). Moreover, a new cytokine, IL-33, has recently been shown to act as a specific ligand for ST2. The caspase-1 processing of precursor IL-33 activates ST2 to promote Th2-type responses (36, 37). Taken together, these findings suggest that (*1*) bleomycin induces ROS (and/or single-electron transfers), and (*2*) indirectly activates caspase-1, resulting in (*3*) cleavage of pro–IL-1β, pro–IL-18, and pro–IL-33 and (*4*) subsequent secretion of mature IL-1β, IL-18, and IL-33 from macrophages. Overexpression of IL-1 family members, including IL-1Ra, IL-1β, IL-18, and IL-33, in the lungs may then induce pulmonary cytokine and chemokine production, resulting in severe lung injury. We are currently investigating this issue.

More than 100 different drugs have been associated with significant pulmonary toxicity. These drugs include pharmacologic groups, such as cancer-therapeutic agents, antibiotics, anti-inflammatory agents, narcotics, and cardiac medications, such as IFN-α, bleomycin, mitomycin C, busulfan, and gefitinib (2, 38, 39). Drug-induced lung injury is an increasingly frequent and important problem in clinical pulmonary medicine, as more and newer therapies are being used for the treatment of human disease. Here we found that the expression of both IL-18 and IL-18Rα was strongly enhanced in inflammatory cells, including mononuclear cells, neutrophils, and alveolar macrophages, and also pulmonary cells, in patients who succumbed to bleomycin-induced lung injury. In particular, IL-18 was strongly expressed in intra-alveolar exudates. Additionally, we recently reported that IL-18 levels in the lungs, serum, and BALF were increased in patients with idiopathic pulmonary fibrosis, but not in healthy subjects (21). Analysis of IL-18 and its receptor in the lungs, or of serum/BALF IL-18 levels, may be helpful for the diagnosis, analysis of disease activity, and the prognosis of drug-induced lung injury. However, we have not analyzed the serum levels of IL-1β and IL-18 in patients with bleomycin-induced lung injury. Further study will be required to determine if the serum levels of these cytokines can be used to predict subsequent lung injury.

Nakatani-Okuda and colleagues (40) have reported that intratracheal bleomycin instillation (2 mg/kg) induced much worse lung injury in IL-18$^{-/-}$ mice than that in WT B6 mice, as assessed by myeloperoxidase activity and survival rate, histology, and leukocyte infiltration in BALF. In WT B6 mice, administration of IL-18 before intratracheal instillation of bleomycin resulted in suppression of lung injury. Although we used the same B6 IL-18$^{-/-}$ mouse strain, our results clearly show that lung fibrosis was significantly attenuated after intraperitoneal administration of bleomycin. Nakatani-Okuda's article showed that total number of cells and lymphocytes was increased in BALF of intratracheal bleomycin–treated B6 IL-18$^{-/-}$ mice on Days 7 and 14 when compared with control WT B6 mice. However, our experiments show that total number of cells, lymphocytes and neutrophils was not significantly different in BALF of intraperitoneally bleomycin-treated B6 IL-18$^{-/-}$ mice on

Days 4 and 14 when compared with control WT B6 mice (data not shown). Total number of cells, neutrophils, macrophages, and lymphocytes was also not significantly increased in BALF of bleomycin-treated B6 IL-18$^{-/-}$ mice on Day 28 when compared with control WT B6 mice (Figure 5). All of the intraperitoneal bleomycin–treated B6 IL-18$^{-/-}$ mice and control WT mice survived in these experiments. However, some of B6 IL-18$^{-/-}$ mice and control WT mice treated with a high dose (>6 mg) of bleomycin died (data not shown). We consider that the difference in experimental protocols (intratracheal versus intraperitoneal or 2 mg/kg versus 100 mg/kg bleomycin) may have accounted for the difference between the results obtained in the two studies. Further studies will be needed to understand the difference in these results.

Currently, the best therapeutic approach for drug-induced lung injury is withdrawal of the drug responsible. Use of corticosteroids for severe cases of toxicity has demonstrated only marginal efficacy, and cannot halt the progression of the disease (1). We demonstrate here that bleomycin-induced lung injury was prevented in caspase-1$^{-/-}$, IL-18Rα$^{-/-}$, and IL-18$^{-/-}$ mice, but not in control WT mice. Our results raise the possibility that blocking of IL-1β and IL-18 expression may be feasible *in vivo*. IL-1β and IL-18 inhibitors, including caspase-1 inhibitors (e.g., IDN-6556 [41]), anti–IL-18R antibodies, anti–IL-1R antibodies, and/or IL-18 binding protein (BP) (42), may be clinically beneficial for the treatment of patients with serious drug-induced lung injury and a consequential poor prognosis.

Acknowledgments:

The authors thank Keiko Nomiyama and Emiko Kuma for technical assistance, Dr. Kitasato (Kurume University) and Dr. Yoshiro Koda (Forensic Medicine, Kurume University) for providing the control lung sections, Dr. Shizuo Akira (Osaka University) for providing IL-18-deficient mice and IL-18Rα-deficient mice, Dr. Kenji Nakanishi and Dr. Hiroko Tsutsui (Hyogo Medical College) for providing caspase-1 deficient mice, and Dr. Do-Young Yoon (Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea) for providing anti-human IL-18 monoclonal antibodies.

This work was supported by a Grant-in-Aid for Scientific Research (B) (no. 17390244) and a Grant-in-Aid for Exploratory Research (no. 18659244) from the Ministry of Education, Science, Sports, and Culture of Japan, a grant from the Kakihara Foundation (Fukuoka, Japan), a grant from the Takeda Science Foundation (Osaka, Japan) (T.H.), and a Grant-in-Aid for Scientific Research (B) (no. 18390244) to H.A.

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CLINICAL RELEVANCE

Enhanced expression of both IL-18 and IL-18 receptor (R) α was observed in the lungs of all five patients with bleomycin-induced lung injury. IL-18–producing $F4/80^+$ macrophages, but not $CD3^+$ T cells, were greatly increased in the lungs of bleomycin-treated mice. Moreover, bleomycin-induced lung injury was significantly attenuated in caspase-$1^{-/-}$, IL-$18^{-/-}$, and IL-18R$\alpha^{-/-}$ mice in comparison with control mice. Our results provide evidence for an important role of IL-1β and IL-18 in chemotherapy-induced lung injury.

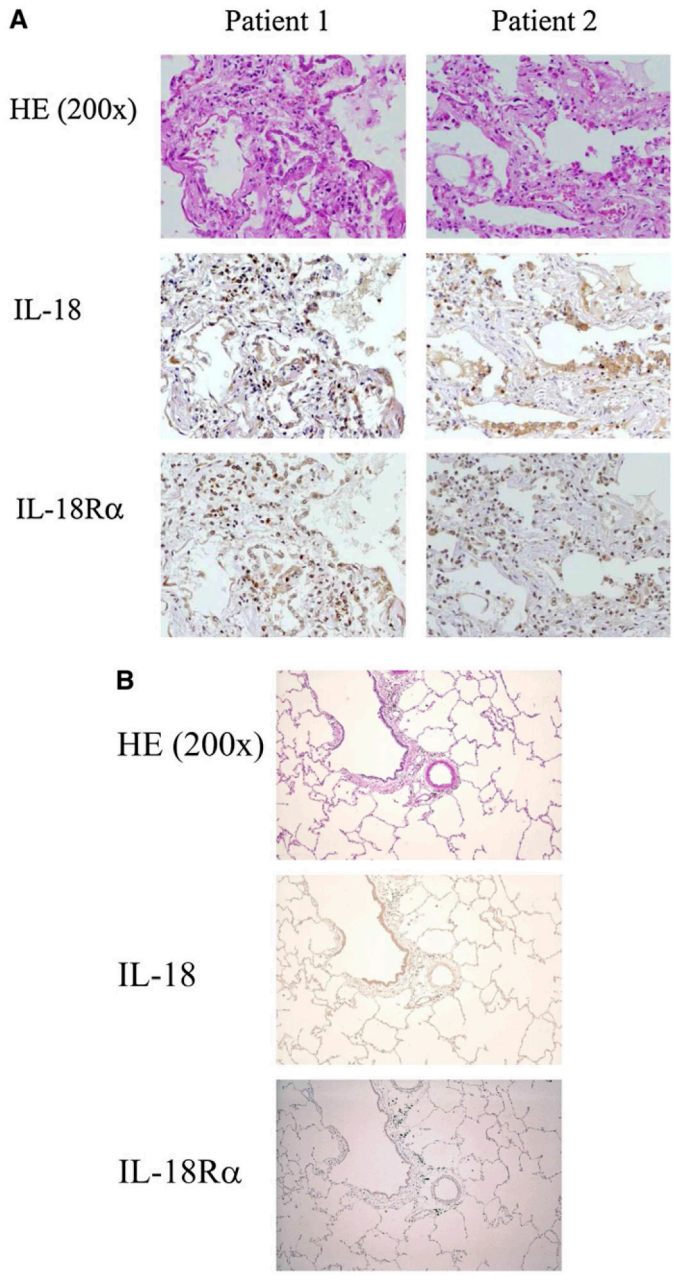

Figure 1.

Enhanced IL-18 and IL-18 receptor (R) α expression in the lungs of patients with bleomycin-induced lethal lung injury. Lung tissues were obtained from two patients with bleomycin-induced lethal lung injury at autopsy. Hematoxylin and eosin (H&E) staining, immunostaining with anti–IL-18 monoclonal antibodies (mAbs), and immunostaining with anti–IL-18Rα mAb were performed as reported previously (21). (*A*) Patient 1 (67-yr-old female treated with 300 mg of bleomycin) demonstrated alveolar interstitial edema and diffused fibrinous exudates with hyaline membrane formation. Patient 2 was a 66-year-old male treated with 190 mg bleomycin. Original magnification, ×200. (*B*) Lung tissues obtained from a 66-year-old male control subject. Original magnification, ×200.

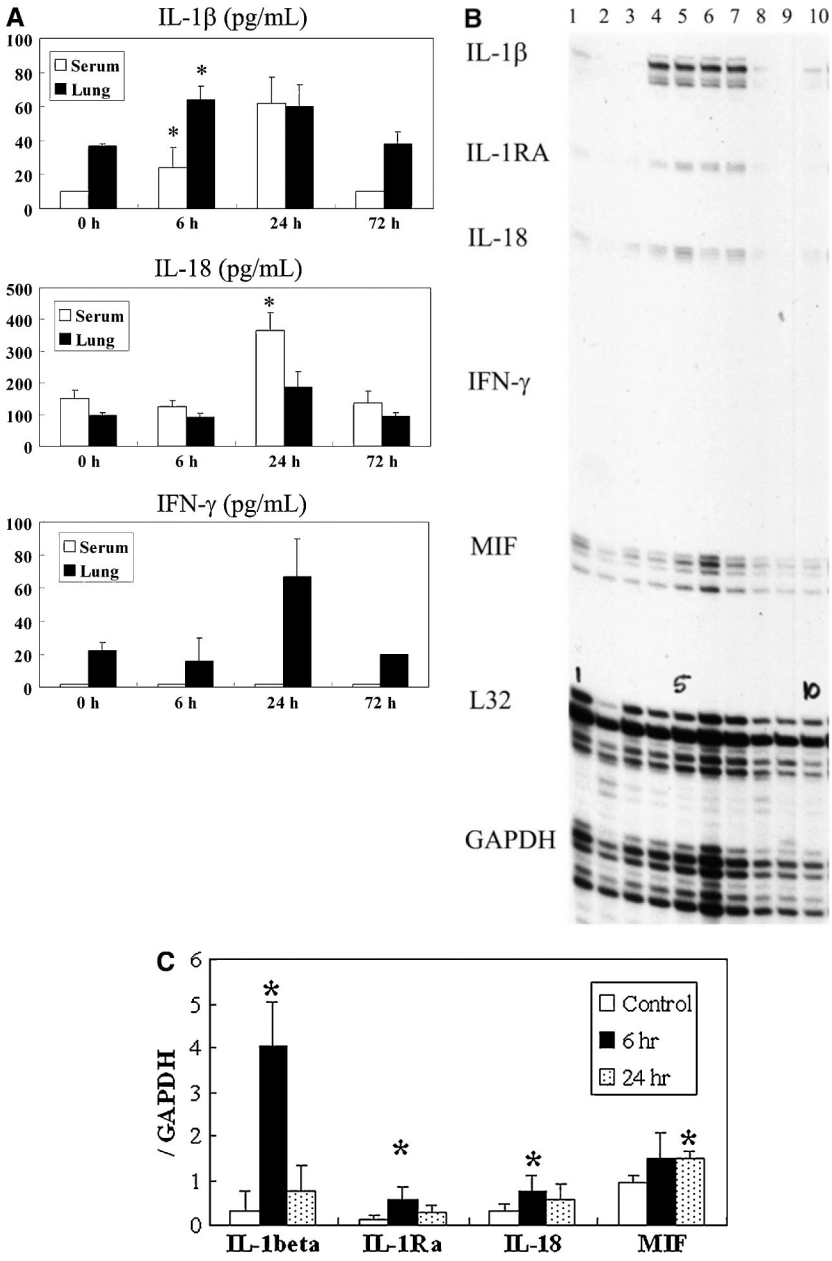

Figure 2.

Induction of expression of IL-1β and IL-18 by intravenous administration of bleomycin in mice. (*A*) Wild-type (WT) C57BL/6 (B6) mice received an intravenous tail vein injection of 2 mg bleomycin suspended in 200 μl of sterile PBS. Mice ($n = 4$ per group) were killed 0, 6, 24, and 72 hours after the treatment, and serum samples were obtained and subjected to ELISA assay. The whole lung tissues were homogenized in 2 ml of lysis buffer (1% Triton X-100, 10 mM Tris-HCL, 5 mM EDTA, pH 7.6) containing a protease inhibitor cocktail (Complete Mini; Boehringer Mannheim GmbH) and centrifuged at 20,000 × *g* for 15 minutes, and the supernatants were collected and stored at −80°C until ELISA assay. The sandwich ELISA kits were used for mouse IL-1β, mouse IFN-γ (R&D Systems) and mouse IL-18 (MBL). *$P < 0.05$ versus 0 hours. (*B*) B6 mice received an intravenous

tail vein injection of 2 mg bleomycin suspended in 200 μl of sterile PBS, and were then killed 6 and 24 hours after the treatment. As a control, the lung tissue was immediately harvested, and total RNA (1 μg) was used for mRNA analysis using a multiprobe RNAse protection assay. *Lanes 1–3*, 24 hours after PBS treatment (control); *lanes 4–7*, 6 hours after bleomycin treatment; *lanes 8–10*, 24 hours after bleomycin treatment. (*C*) Quantitative RNase protection analysis ($n = 3$ or 4 per group) was performed, and mRNA levels were quantitated using a Typhoon 8600 densitometer. Mouse glyceraldehyde 3-phosphate dehydrogenase was used as the control. $*P < 0.05$ versus 0 hours.

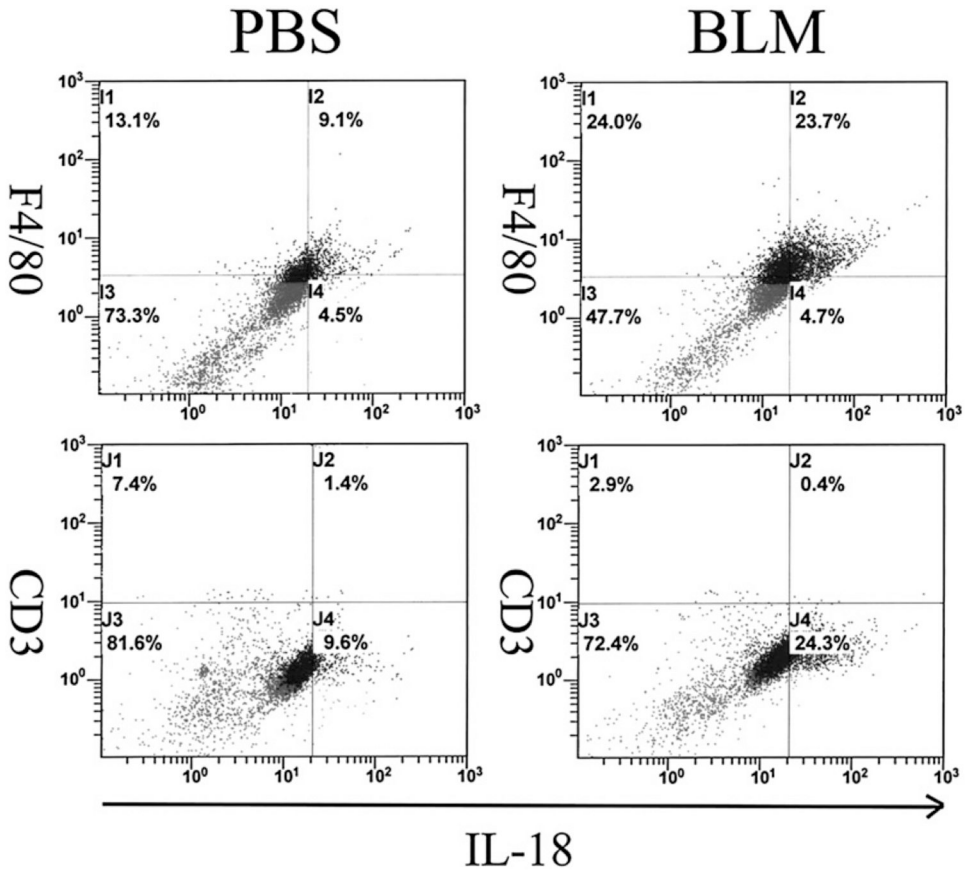

Figure 3.

Increase of IL-18–producing F4/80-positive macrophages, but not $CD3^+$ T cells, in bronchoalveolar lavage fluid (BALF) from bleomycin-treated, WT B6 mice. Recovered BALF cells were isolated from WT B6 mice treated intraperitoneally with bleomycin or control PBS on Day 4. Isolated BALF cells were stained with PE-Cy7–conjugated anti-mCD3 and PE-F4/80 mAb in the presence of anti-mCD16/CD32 mAb. The cells were then fixed, permeabilized, and stained with FITC anti–mIL-18 (M5). Three-color analysis was performed for analysis of cytoplasmic IL-18 expression in $F4/80^+$ neutrophils and $CD3^+$ T cells.

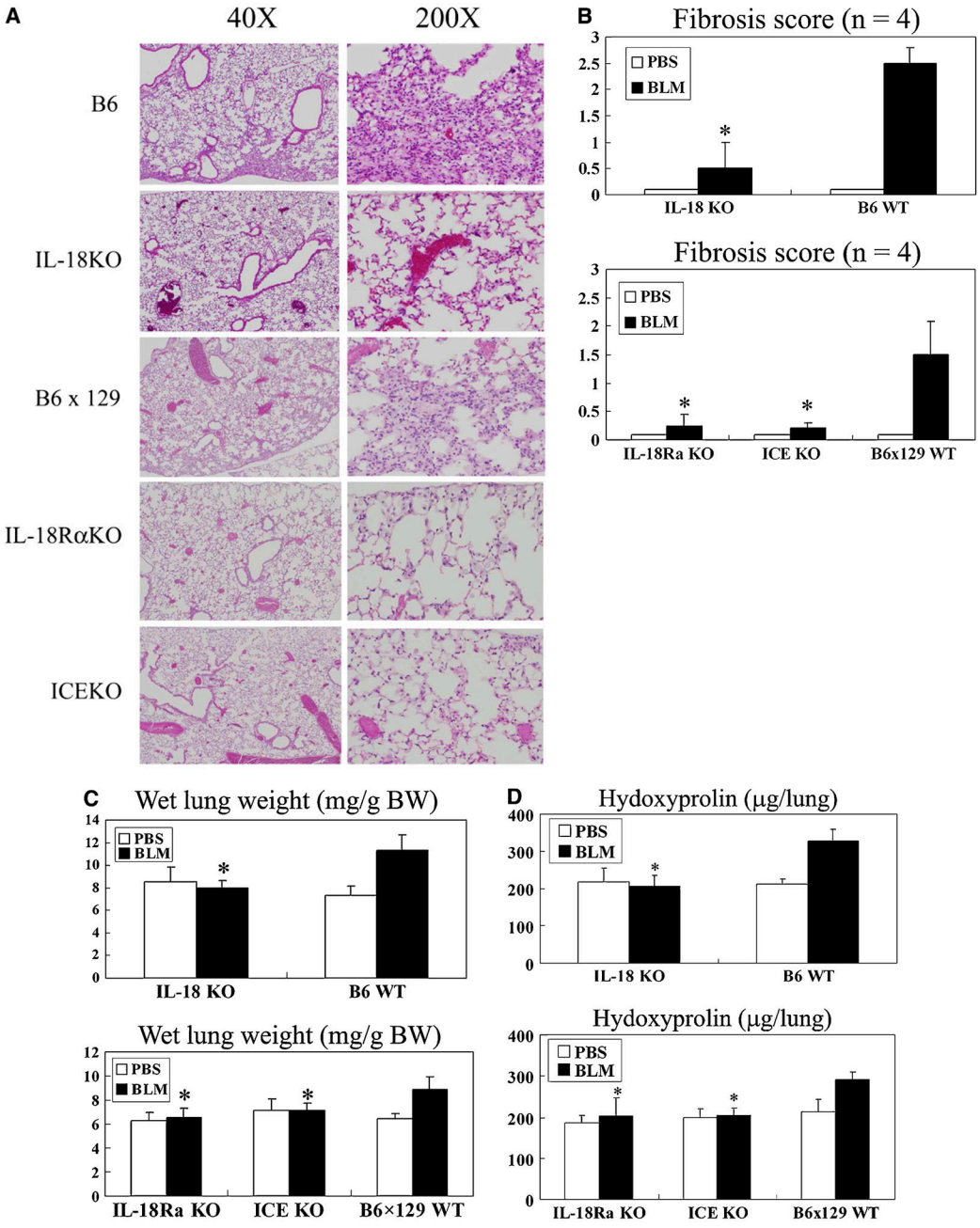

Figure 4.

Prevention of bleomycin-induced lung injury in IL-18–deficient (−/−), IL-18Rα$^{-/-}$, and caspase-1$^{-/-}$ mice. Juvenile (<10 wk old) female IL-18$^{-/-}$ mice and control WT B6 mice were intraperitoneally injected twice with bleomycin (2 mg) on Days 0 and 7, then killed on Day 28. Juvenile female caspase-1$^{-/-}$, IL-18Rα$^{-/-}$, and control WT B6 × 129 mice were intraperitoneally injected three times with bleomycin (2 mg) on Days 0, 7, and 14, then killed on Day 28. (*A*) The lung tissue was examined microscopically after H&E staining. Original magnification, ×40 and ×200. (*B*) Semiquantitative histopathological analysis was performed as previously reported (29). Briefly, H&E sections were observed at 400× and the lesions were defined as follows: Score 0, no lesions;Score 1, occasional small localized

subpleural fibrotic foci; Score 2, thickening of intra-alveolar septa and subpleural fibrotic foci; and Score 3, thickened continuous subpleural fibrous foci and intraalveolar septa. IL-18$^{-/-}$ mice and control WT B6 mice were intraperitoneally injected three times with bleomycin (2 mg) on Days 0, 7, and 14, then killed on Day 28. Caspase-1$^{-/-}$, IL-18Rα$^{-/-}$, and control WT B6 × 129 mice were intraperitoneally injected four times with bleomycin (2 mg) on Days 0, 7, 14, and 21, then killed on Day 28. (*C*) Wet lung weights and (*D*) lung hydroxyproline content were measured at Day 28 ($n = 5$ in each group) as described in Materials and Methods. Results are expressed as the mean (±SD) for five mice per group. *$P < 0.05$ versus bleomycin-treated control WT mice.

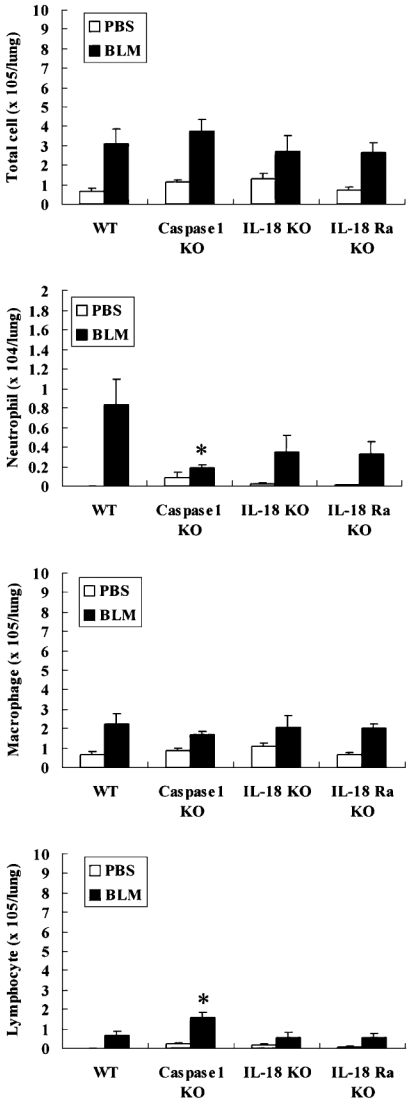

Figure 5.

Prevention of neutrophil accumulation in bleomycin-treated caspase-1$^{-/-}$, IL-18Rα$^{-/-}$, and IL-18$^{-/-}$-mice. Juvenile (< 10 wk old) female B6 background caspase-1$^{-/-}$, B6 IL-18$^{-/-}$, B6 IL-18Rα$^{-/-}$, and control WT B6 mice were injected intraperitoneally twice with bleomycin (2 mg) or PBS (vehicle) on Days 0 and 7. Recovered BALF cells were isolated on Day 28. BALF cells were centrifuged onto glass slides at 800 rpm for 10 minutes, air dried, and stained with Wright-Giemsa. Cell populations were calculated as described in Materials and Methods. *$P < 0.05$ versus bleomycin-treated control WT B6 mice.

TABLE 1.

CHARACTERISTICS OF THE FIVE PATIENTS WITH LETHAL BLEOMYCIN-INDUCED LUNG INJURY

Patient No.	Age (*Yr*)	Sex	Year of Autopsy	Original Cancer*	Dose of BLM (*mg*)	Treatment
1	67	Female	1976	Genitalia	300	Radiation (4,000 rad)
2	66	Male	1973	Lower jaw	190	Radiation (6,000 rad × 2)
3	56	Male	1970	Lung	Unknown	Unknown
4	56	Male	1976	Hypopharynx	195	Radiation (3,600 rad)
5	69	Male	1976	Skin	300	Radiation (6,000 rad) + 5FU (5,500 mg)

Definition of abbreviations: 5FU, 5-fluorouracil; BLM, bleomycin.

*Squamous cell carcinoma.

TABLE 2.

MICE USED IN THIS STUDY

Genetic Background	Genotype	Experiments (Figure No.)
C57BL6/N (B6)	Wild type	ELISA assay (Figure 2A)
		RPA assay (Figures 2B and 2C)
		Flow cytometry (Figure 3)
		Histology (Figures 4A and 4B)
		Wet lung weight (Figure 4C)
		Hydroxyproline (Figure 4D)
		BALF (Figure 5)
B6 × 129 (B6129 F1 hybrid)	Wild type	Histology (Figures 4A and 4B)
		Wet lung weight (Figure 4C)
		Hydroxyproline (Figure 4D)
B6 × 129	IL-18Rα$^{-/-}$	Histology (Figures 4A and 4B)
		Wet lung weight (Figure 4C)
		Hydroxyproline (Figure 4D)
B6	IL-18Rα$^{-/-}$	BALF (Figure 5)
B6 × 129	Caspase-1$^{-/-}$	Histology (Figures 4A and 4B)
		Wet lung weight (Figure 4C)
		Hydroxyproline (Figure 4D)
B6	Caspase-1$^{-/-}$	BALF (Figure 5)
B6	IL-18$^{-/-}$	Histology (Figures 4A and 4B)
		Wet lung weight (Figure 4C)
		Hydroxyproline (Figure 4D)
		BALF (Figure 5)

Definition of abbreviations: BALF, bronchoalveolar lavage fluid; RPA, RNase protection assay.