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Dual Roles of IL-4 in Lung Injury and Fibrosis¹

François Huaux,*[†] Tianju Liu,* Bridget McGarry,* Matt Ullenbruch,* and Sem H. Phan²*

Increased lung IL-4 expression in pulmonary fibrosis suggests a potential pathogenetic role for this cytokine. To dissect this role, bleomycin-induced pulmonary inflammation and fibrosis were analyzed and compared in wild type (IL-4^{+/+}) vs IL-4-deficient (IL-4^{-/-}) mice. Lethal pulmonary injury after bleomycin treatment was higher in IL-4^{-/-} vs IL-4^{+/+} mice. By administration of anti-CD3 Abs, we demonstrated that this early response was linked to the marked T lymphocyte lung infiltration and to the overproduction of the proinflammatory mediators such as TNF- α , IFN- γ , and NO in IL-4^{-/-} mice. In contrast to this early anti-inflammatory/immunosuppressive role, during later stages of fibrosis, IL-4 played a profibrotic role since IL-4^{-/-} mice developed significantly less pulmonary fibrosis relative to IL-4^{+/+} mice. However, IL-4 failed to directly stimulate proliferation, α -smooth muscle actin, and type I collagen expression in lung fibroblasts isolated from the wild-type mice. Upon appropriate stimulation with other known fibrogenic cytokines, fibroblasts from IL-4^{-/-} mice were relatively deficient in the studied parameters in comparison to fibroblasts isolated from IL-4^{+/+} mice. Taken together, these data suggest dual effects of IL-4 in this model of lung fibrosis: 1) limiting early recruitment of T lymphocytes, and 2) stimulation of fibrosis chronically. *The Journal of Immunology*, 2003, 170: 2083–2092.

he pulmonary fibrotic reaction is characterized by an exaggerated accumulation of collagen and other extracellular matrix components leading to an irreversible distortion of normal tissue architecture and loss of function. The cellular mechanism associated with the fibrotic process is currently thought to be due to repeated episodes of acute injury and inflammation leading to an excessive and uncontrolled wound healing characterized by a vigorous replication of mesenchymal cells and exuberant deposition of extracellular matrix proteins (1). Abundant literature has documented that in lung fibrosis, in particular these two sequential stages, can be orchestrated and amplified by cytokines (2). By detecting, neutralizing, or increasing cytokine expression in different experimental models of lung fibrosis, it has been demonstrated that proinflammatory cytokines such as TNF- α as well as fibroblast growth factors such as TGF- β are probably the key mediators in the development of pulmonary fibrosis (3-5). In addition, the elegant mouse models using transgenic overexpression of targeted cytokines specifically in lung using the CC-10 promoter (6, 7), as well as several in vitro studies (8, 9), have highlighted the potential profibrotic roles of novel cytokines including IL-4, IL-10, IL-11, and IL-13. However, their exact functional roles and fibrotic implications still need to be better characterized.

IL-4 has a wide range of biological activities on different cell types. For instance, it has been well demonstrated that IL-4 is a key cytokine in the stimulation of B cell functions (10), in the differ-

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entiation of naive CD4⁺ T cells toward a TH2 phenotype (11), and in the chemotaxis of eosinophils (12) as well as monocytes/macrophages (6). In addition to these immunostimulatory properties, IL-4 paradoxically has important immunosuppressive and antiinflammatory activities. Thus, IL-4 inhibits synthesis of proinflammatory mediators such as TNF- α , IL-1, IL-6, IL-12, PGE₂, and the chemokines IL-8, macrophage inflammatory protein-1 produced by monocytes or macrophages (13). Furthermore, IL-4 enhances the production of the IL-1R antagonist and the release of decoy IL-1RII molecules, which both possess anti-inflammatory properties by antagonizing IL-1 activity (14). These conflicting activities of IL-4 make it impossible to predict a role for this cytokine in pulmonary fibrosis, wherein the role of inflammation remains controversial.

IL-4 is implicated in the pathogenesis of fibrosis by in vitro studies showing that this cytokine can regulate fibroblast function including chemotaxis, proliferation, collagen synthesis, and myo-fibroblast differentiation (15, 16). In accordance with these observations, reports have shown increased IL-4 expression in bleomycin (blm)-,³ silica-, and radiation-induced lung injury and suggested that activated macrophages represent the major source of IL-4 during the establishment of active lung fibrosis (17–19). In human studies, the progression of idiopathic pulmonary fibrosis is also associated with a sustained IL-4 production (20, 21). However, the actual in vivo role of IL-4 remains unknown, and is complicated by its contradictory activities with respect to inflammation and the immune response as cited above.

In the present study, the pulmonary responses to blm of IL-4deficient mice were analyzed and compared with their control wild-type mice to better characterize the potential functions of IL-4 during the development of blm-induced lung inflammation and fibrosis. The results showed that removal of IL-4 from the pulmonary response to blm amplified the early pathological manifestations in treated mice such as T cell influx, proinflammatory mediator production, and early collagen synthesis. This heightened lung inflammation and injury in IL-4-deficient mice was associated

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³ Abbreviations used in this paper: blm, bleomycin; BAL, bronchoalveolar lavage; BALF, BAL fluid; α -SMA, α -smooth muscle actin; PDGF, platelet-derived growth factor.

with significantly higher mortality than that in wild-type mice. These results indicated that IL-4 may play a selective antiinflammatory role during initial lung injury stages by limiting the early influx of pathogenetic T cells. In contrast, the absence of IL-4 expression was associated with a reduction in collagen deposition during the later stages of the experimental pulmonary fibrotic disease, indicating that IL-4 also plays a detrimental role in the process of healing after injury. It may be responsible, at least in part, for promoting lung fibrosis probably due to its activity on lung fibroblast function and phenotype.

Materials and Methods

Mouse fibrosis model

C57BL/6 and IL-4-deficient mice on C57BL/6 background (10) as well as CBA/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Female mice weighing between 18 and 22 g were purchased at 8 wk of age. blm (Blenoxane, Mead Johnson, NJ) was suspended in sterile PBS at 1 U/ml and 0.02 or 0.05 U was administered by intratracheal instillation (1 and 2.5 μ l/g of mouse, respectively). Wild-type and IL-4-deficient mice were anesthetized with a mix of Ketalar (N.V. Warner-Lambert, Zaventem, Belgium) and Rompun (Bayer, Leverkusen, Germany) (1 and 0.2 mg, respectively/mouse, i.p.) and the appropriate blm dose was injected into the lungs via the trachea upon visualization with a surgical incision.

Bronchoalveolar lavage (BAL) and whole-lung homogenates

At selected time points after blm instillation, the animals were sacrificed with sodium pentobarbital (20 mg/animal, i.p.). BAL was performed after cannulating the trachea by flushing the lungs six times with 1 ml sterile 0.9% NaCl. The BAL fluid (BALF) was centrifuged (1200 rpm, 10 min, 4°C), and the cell-free supernatant used for albumin measurements. BAL was then repeated twice with two other milliliters of sterile 0.9% saline. After pooling and centrifugation (1200 rpm, 10 min, 4°C), cell pellets from all the lavage fractions were combined for each animal. Aliquots of the cell suspensions were used to determine total cell numbers and cell differentials. These were done on the cells pelleted onto glass slides by cytocentrifugation and subjected to Diff-Quik staining (Dade, Brussels, Belgium). Polymorphonuclear and mononuclear cells were then counted by light microscopy at $\times 200$ magnification (total of 300 cells counted).

Separately, nonlavaged whole lungs were excised and placed into a Falcon tube chilled on ice, followed by addition of 2 ml of cold 0.9% NaCl. After homogenization for 30 s using a Polytron PT1200 homogenizer (KINEMATICA, Littau, Lu, Switzerland), the tubes were centrifuged at 4° C (10,000 rpm, 15 min) and the supernatants were kept frozen at -80° C until use.

ELISAs and NO assay

Mouse IFN- γ , IL-13, TNF- α , and TGF- β concentrations were measured in lung homogenates, lymphocytes cultures, or serum by ELISA kits obtained from R&D Systems (Minneapolis, MN) following the manufacturer's protocols. Total NO in the same samples was measured using a kit from R&D Systems (Minneapolis, MN) in accordance with the manufacturer's protocol. The detection limits of these assays were 2 pg/ml for cytokine ELISAs and 1.35 μ mol/L for NO assay.

Fibronectin was measured in lung homogenates using a standardized sandwich ELISA. Nunc-immuno ELISA plates (MaxiSorp, Rochester, NY) were coated with rabbit anti-fibronectin capture polyclonal Ab (10 μ g/ml; DAKO, Glostrup, Danemark) in a coating buffer (0.6 M NaCl, 0.26 M H₃BO₄, 0.08 M NaOH, pH 9.6) for 16 h at 4°C. The unbound capture Ab was washed away, and each plate was blocked with 2% BSA-PBS for 2 h at room temperature. Each ELISA plate was then washed with PBS Tween 20 (0.05%; v/v), and samples were added and incubated for 2 h at room temperature. The ELISA plates were then thoroughly washed and polyclonal rabbit anti-fibronectin Ab conjugated with peroxidase (0.22 µg/ml; DAKO) was added and incubated for 2 h at room temperature. After washing the plate, chromogen substrate (Invitrogen, Carlsbad, CA) was added, and optical readings at 492 nm were obtained using an ELISA plate reader. Purified murine fibronectin (from fibroblast culture; Calbiochem, Darmstadt, Germany) was used to generate the standard curves for calculation of fibronectin concentration in each lung homogenate sample. The detection limit of this ELISA was consistently 40 ng/ml.

Type I collagen was measured in lung homogenates and in supernatants of fibroblast culture using a standardized direct ELISA. Samples and standards were diluted in PBS (Invitrogen) and coated directly in Nunc-immuno ELISA plates (MaxiSorp) overnight at 4°C. After blocking with BSA, polyclonal anti-mouse type I collagen Abs (1:200 times; BioDesign, Saco, ME) were then added and incubated 2 h at room temperature. Polyclonal HRP-conjugated goat anti-rabbit Ig Ab (1:1000; BD Biosciences, Mountain View, CA) was used to measure the fixation of primary Abs. Purified mouse type I collagen obtained from Novotec (St. Martin La Garenne, France) was used as standard to calibrate each assay. The detection limit of this ELISA is 40 ng/ml.

The same procedure was used to measure myofibroblast differentiation as defined by expression of α -smooth muscle actin (α -SMA). Sonicated lung fibroblast cultures were coated in ELISA plate and Ab directed against α -SMA were added (1:1000, clone 1A4; Sigma-Aldrich, St. Louis, MO). After washing, specifically bound primary Ab was quantitated using a polyclonal HRP-conjugated goat anti-mouse Ig Ab (1:1000; BD Biosciences).

Collagen assays

Collagen deposition was estimated by measuring the hydroxyproline content of the whole lung and soluble collagen in lung homogenates. For OH-proline, the lung was excised, homogenized in acetic acid (0.5 M), and hydrolyzed in 6 N HCl overnight at 110°C. Hydroxyproline was assessed by colorimetric analysis and data were expressed as micrograms of hydroxyproline per lung. Soluble collagen levels were estimated by Sircol collagen assay following the manufacturer's protocols (Biocolor, Westbury, NY).

In vivo anti-CD3 treatment

Hamster anti-CD3 mAb (clone 145-2C11) and control hamster IgG anti-TNP-PE (clone A19-3) were purchased from BD PharMingen (San Diego, CA). Groups of 5–10 mice were injected i.p. with a total of 150 μ g of anti-CD3 or hamster IgG as control (3 × 50 μ g, every 7 days and starting 1 day before blm instillation). This dosing regimen is known to cause T lymphocyte depletion for up to 2 wk (22).

Isolation of pulmonary T lymphocytes

Lungs from mice were excised, washed in HBSS, cut into small pieces, agitated, and digested enzymatically for 80 min at 37°C. The digestion buffer was composed of collagenase type III (10 mg/lung) and DNase (250 µg/lung) (Worthington Biochemical, Lakewood, NJ) supplemented in HBSS (15 ml/lung) with 2% FBS and antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml fungizone; Invitrogen). The resulting cell suspension was filtered through 100 and 40 μ m filters (BD Biosciences). After centrifugation (1200 rpm, 10 min), cells were washed with PBS and lymphocytes and granulocytes were isolated by density centrifugation in 40% Percoll (23). Cells were washed twice and T lymphocytes were isolated by positive selection with anti-CD90 (Thy-1.2), anti-CD4, and anti-CD8 magnetic beads using the MACS magnet system (Miltenyi Biotec, Auburn, CA). The resulting lymphocyte purity was >90% by microscopic examination of Diff-Quick-stained cytocentrifuge preparations. Purified T cells were resuspended at 2×10^{6} /ml in complete RPMI medium supplemented with 10% FBS and antibiotics then plated at 0.2 ml/ well in 96-well plates precoated or not with anti-CD3 Ab for 2 h at 37°C (BD Biosciences). After 48 h of culture, supernatants were collected and analyzed by ELISA for IL-4 and IFN- γ secretion.

Flow cytometry

Erythrocytes in lung lymphocyte-granulocyte cell suspensions obtained as described above were lysed according to instructions using a blood erythrocyte lysing kit from R&D Systems. Fluorescent labeling of cells was undertaken upon resuspension in HBSS (Invitrogen) with 3% decomplemented FBS (Cocalico Biologicals, Reamstown, PA) and 10 mM NaN₃. The following rat Abs were used: anti-CD4 (Caltag Laboratories, Burlingame, CA), anti-CD8a (BD PharMingen), anti-CD90 (Thy1.2; BD PharMingen), anti-CD19 (Caltag Laboratories), and anti-NK1.1 (BD PharMingen). Cell-fixed Abs were recognized by a secondary R-PE-conjugated goat anti-rat Ig polyclonal Ab (BD PharMingen). After staining, cells were fixed in paraformaldehyde (1.25%) and 10⁴ cells/sample were analyzed on a FACScan apparatus (BD Biosciences). Analysis of the lymphocyte population was undertaken with appropriate gating according to side and forward light scatter to exclude granulocytes, macrophages, and dead cells.

Mouse lung fibroblast culture

Mouse lung fibroblasts were isolated from lung tissue by mincing and enzymatic digestion as described above. After filtration, released cells were centrifuged, washed, and cultured in 6-well in complete medium composed of DMEM (Invitrogen) supplemented with 10% plasma-derived serum (Cocalico Biologicals); human recombinant platlet-derived growth factor



FIGURE 1. Mortality rate of IL-4^{-/-} and IL-4^{+/+} mice after instillation of 0.05 U/mouse or 0.02 U/mouse of blm. A minimum of 10 mice were included in each group and data are representative of two separate experiments. There was no statistical difference in the mortality rate of low-dose blm-treated IL-4^{-/-} and IL-4^{+/+} mice. After high-dose blm treatment, IL-4^{-/-} mice had significantly higher mortalities than IL-4^{+/+}.

(PDGF)-BB (5 ng/ml; R&D Systems); recombinant human epidermal growth factor (10 ng/ml; R&D Systems); insulin, transferrin, and selenium liquid media supplement (1:100; Sigma-Aldrich); and antibiotics. Fibroblasts in this study were used after the first cell passage. Confluent cell monolayers were treated for 24 h with various concentrations of human recombinant TGF- β , mouse rIL-4, and IL-13 (R&D Systems) diluted in medium supplemented with 0.5% plasma-derived serum. Fibroblast proliferation was estimated by [³H]thymidine incorporation in 96-well. Type I collagen and α -SMA were measured by ELISA after sonication of the lung fibroblasts cultivated in 24-well plates.

Histology

Animals were euthanized and perfused via the right ventricle with saline. Lungs were inflated with 1 ml of 10% neutral buffered formalin and fixed overnight. After dehydration in 70% ethanol, the lungs were then processed using standard procedures and embedded in paraffin. Sections were cut, mounted on slides, and stained with H&E or Masson's Trichrome.

Statistics

Treatment-related differences were evaluated using Student t tests or oneway analysis of variance, followed by pairwise comparisons using the Student-Newman-Keuls test, as appropriate. For flow cytometry data, statistical analyses were performed by Mann-Whitney *U* test for unpaired values using Instat software (GraphPad software, San Diego, CA). Statistical significance was considered at p < 0.05.

Results

IL-4 deficiency and early pulmonary response to blm

We first compared the sensitivity of IL-4^{-/-} vs IL-4^{+/+} mice to administration of high-dose blm, which induced lethal lung injury. Following instillation of 0.05 U/mouse of blm, both IL-4^{+/+} and IL-4^{-/-} mice began to die by day 7. However, the mortality rate was higher and occurred sooner in IL-4^{-/-} mice than in IL-4^{+/+}. At day 12 post blm instillation, cumulative mortality in IL-4^{-/-} was 100%, compared with only 60% in IL-4^{+/+} mice (Fig. 1). When the blm dose was reduced to 0.02 U/mouse, there was no significant difference in mortality rate between the two strains (Fig. 1). Thus, deletion of the IL-4 gene had an important effect on the lung response resulting in significantly decreased survival following acute pulmonary injury induced by high-dose blm.

IL-4 deficiency and blm-induced lung fibrosis

The amplitude of the pulmonary fibrosis induced by a standard dose (0.02 U/mouse) of blm was determined at days 7, 14, 21, and 28 by measuring lung hydroxyproline, soluble collagen, fibronectin, and TGF- β contents as well as by histology. No statistically significant differences between the two strains (IL-4^{-/-} vs IL-4^{+/+}) were noted in their pulmonary levels of fibronectin and soluble collagen before day 15. However, IL-4^{-/-} mice exhibited the lowest levels of both these parameters at days 21 and 28 (Fig. 2). OH-proline levels were also significantly decreased in treated IL-4^{-/-} relative to wild-type mice, but only at day 28 (Fig. 2). Finally, the levels of the profibrotic cytokine TGF- β were significantly decreased in treated IL-4^{-/+} mice compared with IL-4^{+/+} animals at all time points examined. In agreement with these quantitative indices of fibrosis, histological examination revealed that on day 28, pulmonary fibrosis in IL-4^{-/-} mice was substantially



FIGURE 2. Time course of OH-proline (*A*), soluble collagen (*B*), fibronectin (*C*), and TGF- β (*D*) levels in lung homogenate obtained from blm-treated IL-4^{+/+} and IL-4^{-/-} mice at days 7, 14, 21, and 28 postinstillation of 0.02 U/mouse of blm. Symbols represent means ± SE of at least six animals. *, p < 0.05; **, p < 0.01; ***, p < 0.001 denote significant differences in values measured in blm-treated IL-4^{-/-} mice compared with corresponding blm-treated IL-4^{+/+} as estimated by Student Newman-Keuls multiple comparison test.

less severe than in IL- $4^{+/+}$ mice. Indeed, lung fibrosis was less intense and less organized in IL-4-deficient mice, affecting smaller areas of the parenchyma than in wild-type mice (Fig. 3).

Collectively, these results showed that lung fibrosis is reduced in $IL-4^{-/-}$ mice relative to wild-type mice, especially during the late phases of blm-induced lung injury.

blm-induced lung injury and inflammation

To appreciate the amplitude of lung injury and inflammation induced by blm (0.02 U/mouse) in the two studied strains, we compared the concentration of albumin and NO, and the number of inflammatory/immune cells harvested by BAL at 1, 3, 7, and 14 days after blm instillation.

At day 7, IL- $4^{-/-}$ mice showed a reduction in BALF alveolar macrophage and eosinophil numbers in comparison to wild-type mice (Fig. 4). In striking contrast, blm-treated IL- $4^{-/-}$ mice displayed an increased number of BALF lymphocytes (day 14) and neutrophils (days 7 and 14) relative to blm-treated IL- $4^{+/+}$ mice (Fig. 4). In addition, the lung injury markers, albumin and NO were present at higher levels in BALF of IL- $4^{-/-}$ mice than that in IL- $4^{+/+}$ animals (Fig. 4).

To complete this cellular analysis, we also determined the number of inflammatory/immune cells present 14 days after blm treatment in the lung tissue of both murine strains. As observed in the BAL cell counts, macrophage/monocyte and eosinophil numbers were lower in IL- $4^{-/-}$ than in IL- $4^{+/+}$ mouse lung tissues (Table I). Analysis of lymphocyte subpopulations by flow cytometry



FIGURE 3. Representative Masson Trichrome-stained lung sections from IL-4^{+/+} (*A*) and IL-4^{-/-} (*B*) mice treated with 0.02 U/mouse of blm at day 28 post blm instillation. Pulmonary fibrosis was substantially less severe in IL-4^{-/-} mice than that observed in IL-4^{+/+} animals (original magnification was ×100).

showed that the number of T cells (CD90.1.2⁺, CD4⁺, and CD8⁺ cells) in lung tissues of blm-treated IL-4-deficient mice was significantly higher than that in the corresponding wild-type mice (Table I). No significant difference in the number of lung tissue NK and B cells was observed between the two strains of mice treated with blm (Table I). After blm administration, inflammato-ry/immune cell influx estimated in BALF and in lung tissue was maximal in both strains at days 7 and 14, but was similar to saline groups after day 21 (data not shown). These results suggested a potential selective immunosuppressive/anti-inflammatory role for IL-4 in the context of blm-induced lung injury and fibrosis.

Effects of anti-CD3 treatment on acute lung injury and pulmonary fibrosis

To further characterize the biological activity of recruited T lymphocytes in blm-induced mortality and lung fibrosis, we investigated the effects of anti-CD3 Ab treatment on $IL-4^{+/+}$ and $IL-4^{-/-}$ mice.

Experiments with high-dose blm (0.05 U/mouse) revealed a similar mortality profile as shown in Fig. 1. Although IL-4^{-/-} mice exhibited significantly higher mortality than in IL-4^{+/+} animals, it was completely abrogated by treatment with anti-CD3 Abs in IL-4^{-/-} (0% survival with control IgG vs 100% survival with anti-CD3 Abs by day 14) and IL-4^{+/+} mice (60 vs 100% survival by day 14). This protection afforded by anti-CD3 Abs enabled analysis of fibrosis at day 21, although results could not be compared with those from mice treated with control IgG since all died before this time point. Lung contents of OH-proline, soluble collagen, and fibronectin were significantly elevated in blm-treated wild-type mice at this time point despite treatment with anti-CD3 Abs (Table II). However, the amplitude of fibrosis as measured by all three criteria was significantly decreased in blm- and anti-CD3treated IL-4^{-/-} mice. When the lower dose of blm (0.02 U/mouse) was used to induce lung injury and fibrosis, a similar but more complete suppression of fibrosis by anti-CD3 Abs was observed (data not shown).

These results demonstrated that the absence of IL-4 was detrimental for animals treated with a lethal dose of blm and that this acute toxic effect was associated with heightened recruitment of T lymphocytes. However, deficiency in IL-4 resulted in decreased lung fibrosis induced by the lower dose of blm (0.02 U/mouse), but this beneficial effect appeared not to be due to alterations in T cell influx.

Characterization of pulmonary T cells

To further characterize the potential roles of recruited T lymphocytes in this animal model, T cells from the lungs of saline or blm-(0.02 U/mouse) treated IL-4^{+/+} and IL-4^{-/-}-treated mice were isolated and purified from lung tissue on day 14. They were then cultivated alone or in coculture with murine lung fibroblasts.

First, purified T lymphocytes were analyzed for proliferative rate as well as their TNF- α , IFN- γ , IL-4, and IL-13 expression in the presence of anti-CD3 Abs or control IgG. Purified T cells from all blm-treated mice, stimulated or not with anti-CD3 Ab, showed an increased proliferative rate and TNF- α expression in comparison to cells obtained from saline-treated control mice (Table III). However, these parameters were significantly higher in cells from IL-4^{-/-} vs IL-4^{+/+} mice. Similarly, anti-CD3-stimulated T cells from blm-treated IL-4^{-/-} mice produced significantly higher levels of IFN- γ than identically stimulated IL-4^{+/+} cells (Table III). However, anti-CD3-induced expression of IL-13 was not significantly different between cells from either strain of mice. IL-4 expression was not detectable in cells isolated from IL-4^{-/-} mice under all experimental conditions examined (Table III).



FIGURE 4. Alveolar macrophage (*A*), eosinophil (*B*), lymphocyte (*C*), and neutrophil (*D*) counts in BAL samples. Levels of albumin (*E*) in BALF and total NO (*F*) in serum obtained from IL- $4^{-/-}$ and IL- $4^{+/+}$ mice at various times after administration of 0.02 U/mouse of blm. Symbols represent means ± SE of four to six animals. *, p < 0.05; ***, p < 0.001 denote significant differences in values measured in IL- $4^{-/-}$ mice compared with corresponding blm-treated IL- $4^{+/+}$ mice as estimated by Student Newman-Keuls multiple comparison test.

In coculture with lung fibroblasts, T cells purified from both blm-treated IL-4^{+/+} and IL-4^{-/-} mice stimulated with similar amplitude the proliferation rate relative to either the medium alone or T cells obtained from saline mice (Fig. 5*A*). Induction of fibroblast proliferation with PDGF was used as a positive control. However, T cells from either murine strain were unable to increase significantly myofibroblast differentiation as estimated by α -SMA expression (Fig. 5*B*) and type I collagen synthesis (Fig. 5*C*). The lung fibroblasts were responsive to TGF- β , which was selected as the positive control for induction of myofibroblast differentiation.

Collectively, these data showed that purified lung T cells from blm-treated IL- $4^{-/-}$ mice were more activated in vitro relative to corresponding T cells from IL- $4^{+/+}$ animals. However, although T cells were also able to stimulate proliferation, they were ineffective in promoting myofibroblast differentiation or collagen production. Furthermore, there were no significant differences between IL-4-deficient and wild-type T cells in their ability or inability to regulate these fibroblast functions.

Effects of IL-4 on pulmonary fibroblasts in vitro

To clarify the basis for the observations in IL-4-deficient mice, we assessed the potential direct activity of IL-4 on pulmonary fibroblasts. We first compared the in vitro activity of various concentrations of recombinant mouse IL-4 to that of TGF- β and IL-13 on lung fibroblasts isolated from IL-4+/+ mice (C57BL/6 background). Although PDGF (10 ng/ml) significantly increased thymidine incorporation in comparison with medium alone, rIL-4 at different concentrations (1-20 ng/ml) was unable to stimulate fibroblast proliferation (Fig. 6A). In addition, no difference in α -SMA and type I collagen expression was observed after addition of IL-4 or IL-13 compared with medium alone, although the cells did respond to TGF- β (10 ng/ml), which served as the positive control (Fig. 6, B and C). In contrast, all three fibroblast parameters were significantly and dose dependently increased by both IL-4 and IL-13 in cultures of lung fibroblasts purified from naive CBA/J mice (Fig. 6, D-F).

To complete the analysis, we also compared the responsiveness of fibroblasts purified from lungs of naive IL- $4^{-/-}$ vs IL- $4^{+/+}$

Table I. Pulmonary cell populations (×10⁶) in saline or blm-treated IL-4^{+/+} and IL-4^{-/-} mice (15 days, 0.02 U of blm/mouse)

IL-4 ^{+/+} + sal ^a	IL-4 ^{+/+} + blm	IL-4 ^{$-/-$} + sal	IL-4 ^{$-/-$} + blm ^b
4.8 ± 0.1	9.1 ± 0.3	4.7 ± 0.02	$8.3 \pm 0.4*$
0.53 ± 0.18	1.78 ± 0.07	0.52 ± 0.16	$0.97 \pm 0.06^{**}$
3.4 ± 0.5	2.1 ± 0.6	3.7 ± 0.9	$7.5 \pm 0.1 **$
2.2 ± 0.3	5.1 ± 0.6	3.0 ± 0.2	$9.1 \pm 1.8^{*}$
1.4 ± 0.2	2.5 ± 0.3	1.5 ± 0.1	$4.2 \pm 0.8^{*}$
0.85 ± 0.01	1.02 ± 0.11	0.90 ± 0.07	$1.51 \pm 0.30*$
0.79 ± 0.09	0.69 ± 0.08	0.78 ± 0.06	0.54 ± 0.10
3.2 ± 0.4	4.6 ± 0.5	3.7 ± 0.3	5.3 ± 1.0
	$\begin{array}{l} \text{IL-4}^{+/+} + \text{sal}^a \\ 4.8 \pm 0.1 \\ 0.53 \pm 0.18 \\ 3.4 \pm 0.5 \\ 2.2 \pm 0.3 \\ 1.4 \pm 0.2 \\ 0.85 \pm 0.01 \\ 0.79 \pm 0.09 \\ 3.2 \pm 0.4 \end{array}$	IL-4 ^{+/+} + sal ^a IL-4 ^{+/+} + blm 4.8 \pm 0.1 9.1 \pm 0.3 0.53 \pm 0.18 1.78 \pm 0.07 3.4 \pm 0.5 2.1 \pm 0.6 2.2 \pm 0.3 5.1 \pm 0.6 1.4 \pm 0.2 2.5 \pm 0.3 0.85 \pm 0.01 1.02 \pm 0.11 0.79 \pm 0.09 0.69 \pm 0.08 3.2 \pm 0.4 4.6 \pm 0.5	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$

^a sal, saline.

^b Asterisks denote significant differences in values measured in blm-treated IL-4^{+/+} mice compared to the corresponding IL-4^{-/-} mice.

*, p < 0.05

**, *p* < 0.01.

mice (Fig. 7). In comparison to wild-type cultures, proliferation rates in IL-4^{-/-} fibroblasts were less responsive to PDGF. In addition, fibroblasts from IL-4-deficient mice were significantly less responsive to TGF- β in terms of α -SMA and type I collagen expression, in comparison to cells from wild-type mice (Fig. 7). Furthermore, constitutive α -SMA expression was lower in cells from the IL-4-deficient mice relative to that in wild-type mice (Fig. 7*B*). These results demonstrated that exogenous IL-4 can in certain circumstances directly activate lung fibroblast functions and that IL-4 may modulate fibroblast activation and myofibroblast differentiation in response to additional factors.

Discussion

In this report, we have shown that IL-4 may play predominantly different roles during the sequential stages of blm-induced lung injury by acting on lung T lymphocytes acutely during early inflammatory stages and on pulmonary fibroblasts chronically during the peak period of active fibrosis. Our observations for the first time indicate that IL-4 overexpressed in blm-injured lungs (17) has the effect of limiting the expansion of detrimental T cells and may be considered at least in part as an anti-inflammatory cytokine. In this capacity it appears to play a beneficial role by limiting the pulmonary inflammation. In contrast, IL-4 could also play a pivotal but undesirable role in the extension of pulmonary fibroblast differentiation, and thus be classified as a profibrotic cytokine.

The inhibitory activity of IL-4 on pulmonary T cell recruitment and activation is demonstrated in this study by the massive T cell infiltrates in lungs of blm-treated IL-4-deficient mice and the higher expression of proinflammatory mediators by these cells when compared with that seen in wild-type mice. IL-4 can mod-

Table II. Levels of OH-proline, soluble collagen, and fibronectin after anti-CD3 Ab administration in saline or blm-treated $IL-4^{+/+}$ and $IL-4^{-/-}$ mice (21 days 0.05 U of blm/mouse)

	OH-Proline (µg/lung)	Soluble Collagen (µg/lung)	Fibronectin (µg/lung)
IL- $4^{+/+}$ + sal	114.7 ± 10.9	111.1 ± 15.6	0.24 ± 0.05
IL- $4^{+/+}$ + blm	214.8 ± 6.5	546.5 ± 42.0	3.35 ± 0.30
IL- $4^{-/-}$ + sal	122.7 ± 14.6	125.4 ± 6.0	0.21 ± 0.04
IL-4 + sal	123.7 ± 14.0	$374.7 \pm 43.0^{**}$	0.21 ± 0.04
$IL-4^{-/-} + blm$	$182.2 \pm 5.2^{a_*}$		$1.71 \pm 0.25^{***}$

 a Asterisks denote significant differences in values measured in blm-treated IL-4 $^{-/-}$ mice compared to the corresponding IL-4 $^{+/+}$ mice.

*, p < 0.05. **, p < 0.01.

***, *p* < 0.001.

ulate T cell subpopulations and function (12, 24-26). Because IFN- γ is increased in cultures of T cells purified from mice deficient in IL-4, we can postulate that the expansion of T cells in this strain correspond to the Th1 subpopulation, which appears to be predominant during the early inflammatory phase of blm-induced pulmonary injury (22, 27). Associated with the increased T cell recruitment, IL- $4^{-/-}$ mice lost weight beginning at the onset of acute blm-induced lung inflammation (7 days), and the majority had succumbed within 2 wk post high-dose blm administration. Given the rapid onset of cachexia in these mice, the overproduction of type 1 and proinflammatory cytokines may be a possible explanation (28). Indeed, T cells from these mice produced abundant IFN- γ and TNF- α , and there was also detectable systemic NO in these animals. These factors plus the marked elevation in lung albumin levels suggest that significant lung damage probably contributed to the higher and more rapid mortality in the IL-4-deficient mice. Because all animals treated with high-dose blm died before the peak of fibrosis on day 21, it is likely that they died from acute lung injury rather than fibrosis.

An important role for recruited T cells in the high-dose blminduced fatal lung injury was demonstrated by administration of anti-CD3 Abs, which completely abrogated the mortality in both blm-treated wild-type and IL-4-deficient mice, confirming a previous report published by Sharma et al. (22). The importance of recruited pulmonary T lymphocytes has been reported also in different animal models of acute and lethal pulmonary inflammatory reactions (29-31). Because of their ability to elaborate oxidants and secrete proteases, activated neutrophils may participate in the acute lung damage and play a deleterious role (32). The increased presence of neutrophils paralleled the influx of T cells and thus could be additionally responsible for the high mortality observed in deficient animals. Although the pathogenic role of neutrophils in blm-induced lung lethal inflammation has been recently rehighlighted (33), the fact that anti-CD3 treatment completely reversed the enhanced mortality in IL- $4^{-/-}$ mice argues for a minimal (if any) role for these granulocytes in the mortality observed in our study. Regardless, our data collected from the IL-4^{-/-} mice document a deleterious role for T cells and polarized type 1 response during the pulmonary inflammatory reaction to blm. In this context, IL-4 produced during this stage appears to play a beneficial role by limiting the expansion of this type of immunological reaction. The anti-CD3 results thus underline the importance of an underlying role for T cells in the early heightened mortality due to acute massive lung injury that appears to be normally (i.e., in wildtype mice) down-regulated by IL-4.

Table III. In vitro proliferation and cytokines production of lung T cells purified from saline or blm-treated $IL-4^{+/+}$ and $IL-4^{-/-}$ mice (day 15, 0.02 U of blm/mouse)

		Proliferation (OD)		TNF-α (pg/ml)		IFN-γ (ng/ml)		IL-4 (pg/ml)		IL-13 (ng/ml)	
		IL-4 ^{+/+}	IL-4 ^{-/-}	IL-4 ^{+/+}	IL-4 ^{-/-}	IL-4 ^{+/+}	IL-4 ^{-/-}	IL-4+/+	IL-4 ^{-/-}	IL-4 ^{+/+}	IL-4 ^{-/-}
Without <cd3< td=""><td>sal blm</td><td>$0.3 \pm 0.02 \\ 0.4 \pm 0.01$</td><td>$\begin{array}{c} 0.3 \pm 0.01 \\ 0.6 \pm 0.03^{b***} \end{array}$</td><td>$nd^a$ 116 ± 28</td><td>nd 240 ± 34**</td><td>nd nd</td><td>nd nd</td><td>nd nd</td><td>nd nd</td><td>nd nd</td><td>nd nd</td></cd3<>	sal blm	$0.3 \pm 0.02 \\ 0.4 \pm 0.01$	$\begin{array}{c} 0.3 \pm 0.01 \\ 0.6 \pm 0.03^{b***} \end{array}$	nd^a 116 ± 28	nd 240 ± 34**	nd nd	nd nd	nd nd	nd nd	nd nd	nd nd
With <cd3< td=""><td>sal blm</td><td>$\begin{array}{c} 0.5 \pm 0.01 \\ 0.8 \pm 0.01 \end{array}$</td><td>$\begin{array}{c} 0.5 \pm 0.01 \\ 1.0 \pm 0.02^{***} \end{array}$</td><td>$\begin{array}{c} 46\pm9\\ 236\pm1 \end{array}$</td><td>$45 \pm 18 \\ 449 \pm 35^{***}$</td><td>$8.7 \pm 0.2$ 12.1 ± 0.1</td><td>$\begin{array}{c} 4.2 \pm 0.2 \\ 13.7 \pm 0.3^{***} \end{array}$</td><td>$159 \pm 5 \\ 457 \pm 13$</td><td>nd nd</td><td>$\begin{array}{c} 1.6 \pm 0.1 \\ 5.4 \pm 0.2 \end{array}$</td><td>$\begin{array}{c} 1.0 \pm 0.1 \\ 5.2 \pm 0.3 \end{array}$</td></cd3<>	sal blm	$\begin{array}{c} 0.5 \pm 0.01 \\ 0.8 \pm 0.01 \end{array}$	$\begin{array}{c} 0.5 \pm 0.01 \\ 1.0 \pm 0.02^{***} \end{array}$	$\begin{array}{c} 46\pm9\\ 236\pm1 \end{array}$	$45 \pm 18 \\ 449 \pm 35^{***}$	8.7 ± 0.2 12.1 ± 0.1	$\begin{array}{c} 4.2 \pm 0.2 \\ 13.7 \pm 0.3^{***} \end{array}$	$159 \pm 5 \\ 457 \pm 13$	nd nd	$\begin{array}{c} 1.6 \pm 0.1 \\ 5.4 \pm 0.2 \end{array}$	$\begin{array}{c} 1.0 \pm 0.1 \\ 5.2 \pm 0.3 \end{array}$

^a nd, Not detected.

^b Asterisks denote significant differences in values measured in saline and blm-treated IL-4^{-/-} mice compared to the corresponding IL-4^{+/+} mice.

**, p < 0.01.

***, p < 0.001.

The exact role of T cells in the chronic fibrotic stages of experimental pulmonary fibrosis remains uncertain. Indeed, studies using nude mice (T cell-deficient mice) and administration of blm (34, 35) or silica particles (36, 37) to induce lung fibrosis have led



FIGURE 5. In vitro [³H]thymidine incorporation (*A*), α -SMA (*B*), and type I collagen expression (*C*) in lung mouse fibroblasts purified from naive IL-4^{+/+} mice in coculture with purified T lymphocytes obtained at day 14 from saline or blm- (0.02 U/mouse) treated IL-4^{+/+} or IL-4^{-/-} animals. Bars represent means ± SE with n = 4-6. ***, p < 0.001 denotes significant differences in values measured in culture with T cells from blm-treated mice compared with corresponding cells from saline-treated mice as estimated by Student Newman-Keuls multiple comparison test. PDGF (*A*, 10 ng/ml) and TGF- β (*B* and *C*, 10 ng/ml) were used as positive controls, respectively, for proliferation and α -SMA as well as type I collagen parameters.

to conflicting conclusions. Although certain reports show that T lymphocyte-mediated processes are not primary determinants of the experimental fibrotic lesions (34, 36), others have shown in contrast that T lymphocytes play a pivotal profibrotic role (35, 37). In support of the latter finding, several studies using Abs neutralizing the lung recruitment and biological functions of CD4⁺ (38, 39), $CD8^+$ (38), or $CD3^+$ (22) T lymphocytes have shown that these different subcellular populations directly contribute to the extension of the lung fibrotic process. On the basis of our coculture experiments, their profibrotic activity may be due, at least in part, to their ability to directly stimulate the proliferation of lung fibroblasts. Because T cells can produce growth factors for fibroblasts such as PDGF, basic fibroblast growth factor (40), and as observed in this study TNF- α , it is probable that the effect observed with blm-stimulated T cells on fibroblast proliferation is mediated by such growth factors. However, purified T cells were unable to stimulate other aspects of fibroblast activation, namely, myofibroblast differentiation and collagen synthesis, at least under the in vitro conditions as described in this study. Thus, T cells appear to play only a limited direct role in terms of promoting fibrosis in this model, although additional indirect roles cannot be excluded.

Aside from the important role of IL-4 on T cells and inflammation, we also demonstrated that this cytokine is an important mediator in the chronic stages of the pulmonary disease. Our findings that IL- $4^{-/-}$ mice showed a reduction in the amplitude of the late-stage fibrotic process to blm are consistent with previous suggestion of a role for IL-4 in hepatic and dermal fibrosis (41-45). Additionally, overexpression of IL-4 using an adenoviral vector in a pulmonary granulomatous inflammation model induces lung type III procollagen expression and increases the granuloma lesion size (46). Indeed, IL-4 has been reported to directly stimulate fibroblast chemotaxis (47) proliferation (16) as well as contraction (48). In addition, IL-4 can stimulate fibroblast production of extracellular matrix components including collagen, fibronectin, and tenascin as well as promote myofibroblast differentiation (8, 15, 16, 49, 50), but apparently not in all fibroblast populations (9). The current study extends these observations by showing that IL-4 failed to directly stimulate proliferation, myofibroblast differentiation, and type I collagen production in C57BL/6 murine lung fibroblasts, but were able to do so in CBA/J murine lung fibroblasts, albeit to a lesser extent than that observed with TGF- β , but with comparable intensity relative to the effects of IL-13. However, lung fibroblasts from both murine strains were equally responsive to TGF- β and PDGF. Thus, these data from the C57BL/6 mice are consistent with a minimal, if any, direct role for IL-4 in the fibrotic response, as previously suggested (9). The basis for this lack of IL-4 responsiveness in C57BL/B6 fibroblasts remains to be determined, but may be due to possible absence of adequate and functional receptor(s) and/or synergistic activation by molecules such as CD40



FIGURE 6. In vitro [³H]thymidine incorporation (*A* and *D*), α -SMA (*B* and *E*), and type I collagen expression (*C* and *F*) in lung fibroblasts purified from naive C57BL/6 mice (*A*-*C*) and from CBA/J mice (*D*-*F*) in the presence of various concentrations of recombinant mouse IL-4 and IL-13. Bars represent means \pm SE with n = 4-6. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 denote significant differences in values measured with medium alone compared with corresponding cells treated with rIL-4 or IL-13 as estimated by Student Newman-Keuls multiple comparison test. PDGF (*A* and *D*, 10 ng/ml) or TGF- β (*B*, *C*, *E*, and *F*, 10 ng/ml) was used as positive control for proliferation or α -SMA as well as type I collagen parameters, respectively.

ligand (51–54). In addition, the fact that transgenic mice overexpressing IL-4 fail to develop significant fibrosis (6, 12) may reflect an intrinsic difference in the responsiveness of murine fibroblasts to IL-4 relative to the mouse background strain used (C57BL/6). This argument is supported by the fact that although IL-4 was unable to directly induce significant profibrogenic responses from C57BL/6 fibroblasts per se, murine lung fibroblasts from IL-4deficient mice with this background were still able to respond to growth factor stimulation, albeit at a lower level than cells from the corresponding wild-type mice (Fig. 7). This would suggest an intrinsic defect in cells from the IL-4-deficient mice in their response to growth factor stimulation, at least relative to cells from wildtype animals.

Several elements support the view that IL-4 is not directly responsible for induction of exaggerated extracellular matrix deposition but acts primarily as an indirect signal, possibly by regulating the expression of other more potent fibrogenic mediators. This concept is supported by recent studies demonstrating that IL-4 can increase TGF- β production by bronchial epithelial cells (9), eosinophils (55), and T cells (56), the latter two cell types having been previously identified as being potentially profibrotic. In addition, IL-4 produced by mast cells up-regulates PDGF and fibroblast growth factor expression by fibroblasts in culture (57). Furthermore, in vivo experiments show that the profibrotic activity of IL-4 in skin fibrosis is due to its ability to increase TGF- β production (44), also supporting an essential but indirect role for IL-4 in fibroblast activation via augmentation of profibrogenic growth factor expression. It is noteworthy that lung recruitment of macrophages and eosinophils is deficient in IL-4^{-/-} animals, confirming that IL-4 is an important regulator of eosinophil and macrophages recruitment (6). Because eosinophils and macrophages are key sources of growth factors such as TGF- β (58, 59), this may represent a potential indirect mechanism by which IL-4 contributes its profibrogenic activity.

IL-13 shares 30% homology with IL-4 and appears to have certain overlapping biological activities in type 2 responses. Both cytokines use the IL-4R α -chain and the STAT6-dependent signal transduction pathway (60). However, despite some overlapping roles, these cytokines do have clearly distinct biological activities in certain areas, such as in T cell proliferation and differentiation for instance (61). Thus, although IL-13 and other cytokines have the capability of regulating the inflammatory/immune response and fibroblast function, the results of this study based on the specific deficiency in IL-4 do suggest some unique and specific roles



FIGURE 7. In vitro [³H]thymidine incorporation (*A*), α -SMA (*B*), and type I collagen expression (*C*) in lung fibroblasts purified from naive IL-4^{+/+} and IL-4^{-/-} mice with or without PDGF or TGF- β stimulation. Bars represent means \pm SE with n = 4-6. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 denote significant differences in values measured in culture from IL-4^{-/-} mice compared with corresponding cells from IL-4^{+/+} mice as estimated by Student Newman-Keuls multiple comparison test.

for IL-4 in controlling acute lung inflammation and promotion of chronic fibrosis. In contrast, the incomplete inhibition of fibrosis in IL-4-deficient mice suggests that one cannot ignore the contribution of other cytokines or mediators with overlapping activities, although the latter cannot compensate completely for the absence of IL-4.

On the basis of the cytokine profile expressed in vitro by purified T cells, it is difficult to discriminate between preferential type 1 and 2 immune response in blm-induced lung fibrosis because T cells express IFN- γ , IL-4, and IL-13 as well. Although fibrosis is thought to be a type 2-related disease, our observations show a concomitant presence of both immune responses. Nevertheless, the type 1 component of this response appears to be important since IFN- γ -deficient mice exhibit attenuated blm-induced pulmonary fibrosis (62). Thus, both type 1 and 2 responses appear to play important roles in fibrosis. To reconcile this apparent conflict, we postulate that while type 1 cytokines such as IFN- γ are important in the promoting T cell inflammation and exacerbation of inflammatory lung injury acutely, IL-4 and perhaps other type 2 cytokines play their profibrotic roles during the later stages of fibrosis when the fibroblast now becomes the main target of the cytokine network and inflammation is diminished. Thus, regulation of fibrosis appears to be complex with different and opposing cytokines playing different roles at different time points, but ultimately with the same effect on the fibrotic endpoint. Furthermore, the same cytokine can be both anti- and profibrotic depending on the stage of the disease process. Thus, IL-4 can suppress T cell inflammation and limit lung injury during early stages of disease, but promote fibrogenesis at later stages when the importance of the fibroblast predominates. However, under conditions that allow the animals to survive and develop extensive fibrosis, IL-4 appear to play predominantly a profibrotic role. Nevertheless, these dual roles of IL-4 make it more complicated to devise therapeutic approaches based on controlling the activity or expression of a single pleiotropic cytokine.

In summary, in this study the use of IL-4-deficient mice has revealed that the roles of IL-4 in lung injury, inflammation, and fibrosis are complex and may be due to its pleiotropic effects on both immune cells as well as fibroblasts. The results show that IL-4 deficiency results in a heightened early inflammatory reaction to a lethal dose of blm, characterized by the generation of a more marked Th1 response and an associated increase in mortality before peak fibrosis occurs. In contrast, if animals were allowed to survive and develop extensive pulmonary fibrosis, IL-4-deficient mice displayed a reduction in pulmonary fibrosis that is associated with a deficiency in lung fibroblast responsiveness to known profibrotic growth factors.

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