

Eosinophils and T Lymphocytes Possess Distinct Roles in Bleomycin-Induced Lung Injury and Fibrosis¹

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Leukocyte infiltration is characteristic of lung injury and fibrosis, and its role during tissue repair and fibrosis is incompletely understood. We found that overexpression of IL-5 in transgenic mice (IL-5^{TG}) or by adenoviral gene transfer increased bleomycin (blm)-induced lung injury, fibrosis, and eosinophilia. Surprisingly, blm-treated IL-5-deficient (IL-5^{-/-}) mice also developed pronounced pulmonary fibrosis but characterized by marked T lymphocyte infiltration and absence of eosinophilia. In both murine strains however, induction of lung TGF- β expression was evident. Purified lung eosinophils from blm-treated IL-5^{TG} mice stimulated α -smooth muscle actin and collagen expression in mouse lung fibroblasts, without affecting proliferation. Furthermore instillation of purified eosinophils into murine lungs resulted in extension of blm-induced lung fibrosis, thus confirming a role for eosinophils. However, lung T lymphocytes from blm-treated IL-5^{-/-} mice were able to stimulate fibroblast proliferation but not α -smooth muscle actin or collagen expression. Blocking T cell influx by anti-CD3 Abs abrogated lung fibrosis, thus also implicating T lymphocytes as a key participant in fibrosis. Pulmonary fibrosis in IL-5^{TG} mice was preferentially associated with type 2 cytokines (IL-4 and IL-13), whereas fibrotic lesions in IL-5^{-/-} animals were accompanied by proinflammatory cytokine (TNF- α , IL-1 β , and IFN- γ) expression. We suggest that eosinophils and T cells contribute distinctly to the development of blm-induced lung fibrosis potentially via their production of different cytokine components, which ultimately induce TGF- β expression that is intimately involved with the fibrosis. *The Journal of Immunology*, 2003, 171: 5470–5481.

The development of fibrotic lesions can be initiated by a variety of factors such as drug and alcohol abuse, radiation, and toxic agents, but fibrotic lesions are also important pathological manifestations of a number of chronic and debilitating illnesses such as several autoimmune, allergic, and infectious diseases. Unfortunately, there is still no effective treatment to directly ameliorate the outcome of such diseases (1, 2).

Lung fibrosis can be defined as uncontrolled or abnormal wound healing characterized by a vigorous replication of mesenchymal cells, accumulation of myofibroblasts, and exuberant deposition of extracellular matrices (3). Leukocytic infiltration is also a characteristic of lung fibrosis and is believed to contribute, at least in part, to the fibrotic response (1). Among these recruited cells, macrophages and their profibrotic activity have been the focus of intense research over the past 20 years. It is now clear that macrophages generate and modulate inflammation and fibrosis extension as well as alterations in the cytokine milieu (4). However, the exact role of other cells, such as lymphocytes, neutrophils, and eosinophils also recruited during the establishment of many forms of fibrosis, is still not fully delineated and remains a controversial topic in the pub-

lished literature (5–8). Interestingly, all of these immune cells are, as the macrophages, potent sources of mediators capable of activating fibroblast functions, such as cytokines; hence, these immune cell populations have the potential of playing comparable roles.

The importance of cytokines produced during fibrosis is well documented, demonstrating that these mediators have the potential of orchestrating and amplifying inflammation and fibrotic responses (9). For instance, several studies have demonstrated the key role of proinflammatory cytokines such as TNF- α and IL-1 β (10, 11) as well as growth factors such as TGF- β and platelet-derived growth factor (PDGF)³ (12, 13). Exaggerated lung responses to these cytokines may represent key events in the development of pulmonary fibrosis (14). Recently, human and experimental studies have highlighted the potential profibrotic roles of additional cytokines, including IL-4, IL-10, and IL-13, all classified as belonging to the class of Th2 type cytokines (15–17).

Recent reports have identified the importance of another Th2 cytokine, IL-5, in eosinophil recruitment to the lung in bleomycin (blm)-induced lung inflammation and fibrosis (7, 18). To clarify the role of IL-5/eosinophils, further in vivo experiments were performed with transgenic mice overexpressing IL-5 (IL-5^{TG}), with IL-5 expressing adenoviral construct (AdIL-5) and with mice deficient in IL-5 (IL-5^{-/-}). Analysis of the amplitude of fibrosis demonstrated the presence of marked blm-induced lung fibrosis in IL-5^{TG} and in AdIL-5 mice but paradoxically also in blm-treated IL-5^{-/-} mice. Pulmonary fibrosis was accompanied in IL-5 overexpression models by a massive influx of eosinophils, whereas the lung response of knockout mice was characterized by specific T cell accumulation. To define the exact role in fibrosis of these two

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³ Abbreviations used in this paper: PDGF, platelet-derived growth factor; blm, bleomycin; BAL, bronchoalveolar lavage; BALF, BAL fluid; MCP, monocyte chemoattractant protein; Ad, adenovirus construct; TG, transgenic; WT, wild-type; SMA, smooth muscle actin.

recruited immune cell populations, we dissected their separate biological activity both in vitro and in vivo as well as their specific cytokine production profiles.

Materials and Methods

Mouse fibrosis model

C57BL/6 (IL-5^{+/+}) and IL-5^{-/-} mice on C57BL/6 background (19) were obtained from The Jackson Laboratory (Bar Harbor, ME). IL-5^{TG} mice were obtained from Glaxo Wellcome (Stevenage, U.K.) (20). Their controls, CBA/Ca (wild-type IL-5) were purchased from Harlan Olac (Bicester, U.K.). Blm (Blenoxane, Mead Johnson, NJ) was suspended in sterile PBS at 1 U/ml. Either 0.02 or 0.05 U was administered by transoral instillation (1 or 2.5 μ g of mouse, respectively).

Bronchoalveolar lavage (BAL) and whole lung homogenates

BAL was performed with 1 ml of 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 2 ml of sterile 0.9% saline. Cells from all the lavage fractions were combined and cell differential counts determined (Diff-Quik staining; Dade Behring, Deerfield, IL). Separately, nonlabeled whole lungs were excised and homogenized on ice in 2 ml of cold 0.9% NaCl. After centrifugation at 4°C (10,000 rpm, 15 min), the supernatants were kept frozen at -80°C until use.

Collagen assays

Collagen deposition was estimated by measuring the hydroxyproline and soluble collagen contents of whole lung homogenates. Hydroxyproline was assessed by colorimetric analysis as previously described (15). Soluble collagen levels were estimated by Sircol collagen assay following the manufacturer's protocols (Biocolor, Westbury, NY).

Cytokine assays and ELISA

TNF- α , IL-1 β , IFN- γ , IL-4, and IL-13 concentrations were measured by ELISA kits (R&D Systems, Minneapolis, MN) following the manufacturer's protocols. TGF- β was measured by an assay using mink lung epithelial cells stably transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct (generous gift of Dr. D. B. Rifkin, New York University, New York, NY) as previously described (21). The relative mRNA expression of 20 cytokines was analyzed in freshly purified eosinophils and T cells (see below) with chemiluminescent GEArray gene arrays (SuperArray, Bethesda, MD) according to the manufacturer's protocol. The gene expression of the following cytokines or cytokine receptors were analyzed: IFN- γ , IL-12p40, IL-12p35, IL-18, TNF- α , IL-1 β , IL-4, IL-5, IL-9, IL-10, IL-13, IL-16, IL-3, eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein (MCP)-1, MCP-3, TGF- β 1, - β 2, and - β 3, CCR2, CCR3.

Albumin levels were measured in BALF by ELISA quantification kit provided by Bethyl Laboratories (Montgomery, TX). Fibronectin, type I collagen and α -smooth muscle actin (α -SMA) were measured using a standardized ELISA already detailed in a previous study (15).

Isolation and culture conditions of pulmonary eosinophils and T lymphocytes

Lungs from mice were digested enzymatically to obtain cell suspension as already detailed elsewhere (15). For eosinophil purification, the digestion solution also contained 5 ng/ml IL-5 and GM-CSF (R&D Systems). Lymphocytes and granulocytes were isolated by density centrifugation in 40% Percoll. Eosinophils and T cells were further isolated using immunomagnetic beads and the magnetic cell separation system (MACS; Miltenyi Biotec, Auburn, CA). Eosinophils were purified by negative selection using rat IgG Abs directed against cells such as: erythrocytes (anti-Ter 119; PharMingen, BD Biosciences, Bedford, MA), lymphocytes (anti-CD3, anti-CD4, anti-CD19; Caltag Laboratories, Burlingame, CA; anti-CD8a, anti-CD90 Thy1.2, NK1.1; PharMingen, BD Biosciences), and neutrophils (anti-GR-1; PharMingen, BD Biosciences). Goat anti-rat IgG microbeads were used to recognize primary rat IgG Ab. The purity of eosinophil preparations was >95% (Diff-Quik staining, Dade Behring). T lymphocytes were isolated by positive selection with anti-CD90 (Thy1.2) magnetic beads. The resulting lymphocyte purity was >90%. Purified eosinophils and 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 with calcium ionophore (A23187, Sigma-Aldrich, St. Louis, MO) and anti-CD3 Ab (BD Biosciences), respectively, as stimulators. After 24 (eosinophil cultures) and 48 h (lymphocyte cultures), supernatants of cell cultures were collected and analyzed by ELISA for cytokine secretion.

Adenoviral constructs

AdIL-5-expressing and control adenoviral (AdCTL) constructs were prepared as previously described (22). A dose of 0.5×10^8 PFU of AdIL-5 or AdCTL vector was diluted in 50 μ l of PBS and delivered in mice by transoral instillation 4 days after blm treatment (0.02 U/mouse). This approach allowed us to maintain high lung expression of IL-5 and eosinophilia, until at least day 14 after blm treatment. For example, on day 14, the lung IL-5 levels in mice transfected with AdIL-5 were 648 ± 69 pg/lung compared with 7 ± 3 pg IL-5/lung in mice transfected with AdCTL. The corresponding lung eosinophil counts at the same time point were $2.5 \pm 0.3 \times 10^6$ /lung and $0.1 \pm 0.04 \times 10^6$ /lung, respectively, for AdIL-5 and AdCTL transfected mice.

In vivo injection of eosinophils and anti-CD3 treatment

Eosinophils from blm-treated IL-5^{TG} mice were purified as previously described. Naive and blm-treated (0.01 U/mouse) wild-type mice were administered by transoral instillation with 5×10^6 eosinophils at days 7 and 14 after blm treatment. Intensity of pulmonary fibrotic lesions was analyzed at day 21.

To deplete T cells, hamster anti-CD3 mAb (clone 145-2C11) and control hamster IgG anti-TNP-PE (clone A19-3) were purchased from Pharmingen (BD Biosciences). 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 (5). Lung fibrosis was assessed 21 days after blm administration.

Mouse lung fibroblast culture

Mouse lung fibroblasts were isolated from lung tissue by mincing and enzymatic digestion as previously described (15). Cells were cultured in complete medium composed of DMEM (Life Technologies, Grand Island, NY) supplemented with 10% plasma-derived serum (Cocalico Biologicals, Reamstown, PA), human recombinant PDGF-BB (5 ng/ml, R&D Systems), insulin, transferring and selenium liquid media supplement (1:100, Sigma-Aldrich), and antibiotics. Fibroblasts used after the first passage were seeded into 24-well or 96-well plates at 40 or 10×10^3 cells/wells, respectively. Subconfluent cell monolayers were treated for 24 h with various concentrations of recombinant cytokines (R&D Systems) or with eosinophils and T cells suspended in medium supplemented with 0.5% plasma-derived serum. Cocultures were performed with 2×10^6 /ml of purified lung eosinophils or T cells. Fibroblast proliferation was estimated by [³H]thymidine incorporation in 96 wells. Type I collagen and α -SMA were measured by ELISA after sonication of the lung fibroblasts cultivated in 24-well plates.

Flow cytometry

The following rat anti-mouse mAbs were used: anti-CD3, anti-CD4, anti-CD19 (Caltag Laboratories), anti-CD8a, anti-CD90 (Thy1.2), anti-NK1.1, and anti-CD62L (PharMingen, BD Biosciences). Cell-fixed Abs were recognized by a secondary R-PE-conjugated goat anti-rat Ig polyclonal Ab (PharMingen, BD Biosciences). R-PE-conjugated hamster anti-mouse $\gamma\delta$ and β -chain T cell receptor (PharMingen, BD Biosciences) were also used. After staining, cells were fixed in paraformaldehyde (1.25%) and 10^4 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.

Histology

Animals were euthanized and perfused via the right ventricle with saline. Lungs were inflated with 1 ml 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 Trichrome stain.

Statistics

Treatment-related differences were evaluated using Student's *t* test or one-way ANOVA, 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

Pulmonary expression of IL-5 after blm treatment

We first determined by ELISA the level of IL-5 in lung homogenates of mice administered with standard doses of blm. Seven days following instillation of 0.02 U/mouse of blm, a significant increase in pulmonary IL-5 content was noted in CBA/Ca wild-type (IL-5^{WT}) mice in comparison to saline-treated control mice (Fig. 1A). At all other time points studied, IL-5 levels were comparable to the saline values. In blm-treated IL-5^{TG} animals, IL-5 expression was dramatically increased (>10-fold increase over wild type) at each time point studied in comparison to the transgenic mice injected with saline (Fig. 1A). In contrast and as expected, IL-5 was undetectable in lungs of blm-treated IL-5 knockout mice (IL-5^{-/-}), whereas their matching wild-type C57BL/6 controls (IL-5^{+/+}) showed significant elevations at days 3 and 7 after blm treatment (Fig. 1B).

IL-5 overexpression or deficiency and blm-induced lung injury and inflammation

To assess the amplitude of lung injury and inflammation induced by blm (0.02 U/mouse), we compared the lung wet weight, the lung concentration of albumin, and the number of inflammatory/immune cells present in the lung at 7 days after blm instillation. The lung weight and BALF albumin levels were increased by blm treatment, but the increase was significantly higher in IL-5^{TG} mice than in the treated IL-5^{WT} animals (Table I). Analysis of lung immune cell subset distribution revealed an abnormally high eosinophil accumulation in the BALF of blm-treated IL-5^{TG} mice in comparison to their corresponding treated wild-type mice (Table I). BALF neutrophils were absent in IL-5^{TG} mice and macrophages as well as lymphocyte numbers were slightly increased compared with that observed in IL-5^{WT} (Table I). We also compared the response of IL-5^{TG} mice vs IL-5^{WT} mice to administration of a higher dose of blm, which induced lethal lung injury. Following instillation of 0.05 U/mouse blm, mice of both strains began to die by day 10. However by day 21 post-blmm instillation, cumulative mortality in IL-5^{TG} mice was greater than that noted in IL-5^{WT} animals (100% in IL-5^{TG} compared with 80% in IL-5^{WT}). All together, these data indicate that in response to blm treatment, IL-5 overexpressing transgenic mice exhibited more acute pulmonary lesions associated with a specific/selective and massive eosinophil accumulation.

To confirm the importance of IL-5 and eosinophils, the same analysis was undertaken in IL-5-deficient mice. Indeed, after blm

treatment, the lung injury parameters studied (lung weight and albumin) were reduced in IL-5^{-/-} mice compared with those in IL-5^{+/+} animals (Table I). Blm-treated IL-5^{-/-} mice displayed a complete absence of BALF eosinophils relative to blm-treated IL-5^{+/+} mice (Table I). The numbers of BALF neutrophils and macrophages were also significantly decreased in IL-5-deficient animals. Lymphocyte numbers were however comparable in both strains. As observed in the BAL cell counts, eosinophils were relatively absent in IL-5^{-/-} minced lung preparations but not in IL-5^{+/+} at day 7 post-blmm instillation (data not shown). No change in the counts of pulmonary tissue macrophages and neutrophils was noted between these two last strains (data not shown). However, an unexpected finding was a massive increase in the recovery of lymphocytes from lung parenchyma in blm-exposed IL-5^{-/-} mice. Indeed after instillation of blm, lung tissue lymphocyte accumulation was markedly higher in IL-5-deficient mice than in wild-type mice ($17.0 \pm 1.1 \times 10^6$ in IL-5^{-/-} vs 10.9 ± 0.5 in IL-5^{+/+}). Similar disparity in lung tissue lymphocyte numbers between the two groups of mice was also seen at days 14 and 21 (data not shown). Because lymphocyte influx estimated in pulmonary tissue was maximal in both strains at 14 days after blm treatment, detailed analysis of lymphocyte subpopulations by flow cytometry in lung parenchyma was performed at this time point. The results revealed an increase in the percentage of lymphocytes expressing CD90.1.2 in blm-treated IL-5^{-/-} mice compared with the corresponding IL-5^{+/+} mice (52.6% vs 39.9%). We noted in contrast an important reduction in lymphocytes expressing CD62L in blm-treated IL-5^{-/-} mice in comparison to IL-5^{+/+} mice (35.5% vs 48.3%). Thus, the major lymphocyte population present in blm-treated IL-5-deficient animals was differentiated/activated T cells. The other lymphocytes subsets (CD3, CD4, CD8, NK, CD19 and TCR- $\alpha\beta$ and - $\gamma\delta$ epitopes) did not differ significantly between the two strains treated with blm (data not shown). However, when absolute counts for the different cell subsets were analyzed, the number of T cells (CD90.1.2⁺, CD3⁺, CD4⁺, and CD8⁺, TCR- $\alpha\beta$ and - $\gamma\delta$ T cells) in lung interstitium of blm-treated IL-5-deficient mice was significantly higher than those in the corresponding wild-type mice (Table II). 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 II). IL-5^{-/-} mice were relatively resistant to lethal pulmonary injury induced by high-dose blm (0.05 U/mice) compared with treated IL-5^{+/+} mice. Thus the mortality rate in the deficient animals at day 21 was only 50%, whereas

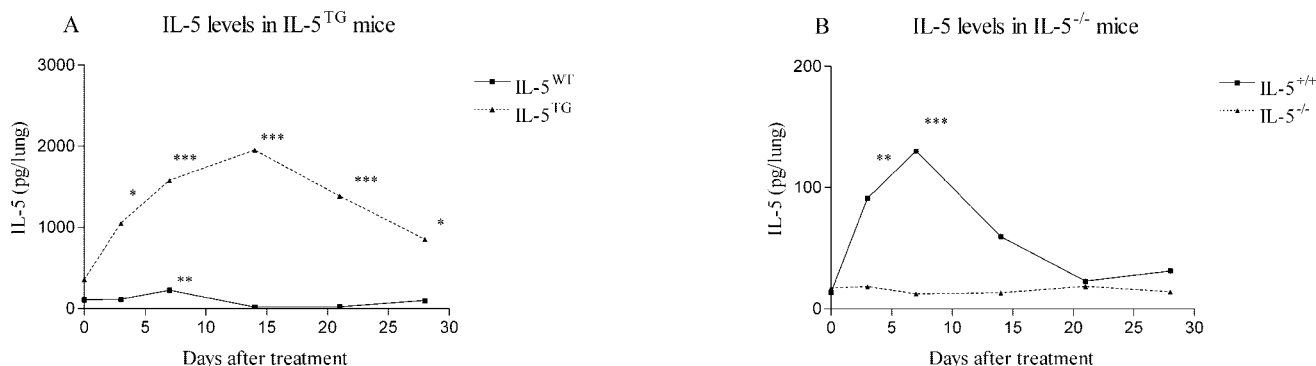


FIGURE 1. Kinetics of lung IL-5 expression. At days 0, 7, 14, 21, and 28 post-blmm instillation (0.02 U/mouse), IL-5 protein content was determined by ELISA in lung homogenates from saline- or blm-treated IL-5 transgenic (IL-5^{TG}) and corresponding wild-type (IL-5^{WT}) mice (A), as well as from IL-5-deficient (IL-5^{-/-}) and corresponding wild-type (IL-5^{+/+}) mice (B). Results are shown as mean $\pm$ SEM of at least six animals. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ denote the level of significant differences in mean values measured in blm-treated IL-5^{TG} or IL-5^{-/-} mice compared with the corresponding blm-treated wild-type mice (IL-5^{WT} and IL-5^{+/+}, respectively) as analyzed by the Student-Newman-Keuls multiple comparison test.

Table I. Lung wet weight and biochemical as well as cellular parameters in BALF^a

		Lung Weight (mg)	Albumin ($\mu\text{g/ml}$)	Macrophages ($\times 10^3$)	Lymphocytes ($\times 10^3$) ^b	Neutrophils ($\times 10^3$)	Eosinophils ($\times 10^3$)
IL-5 ^{WT}	saline	45.7 $\pm$ 4.5	30.1 $\pm$ 10.0	0.03 $\pm$ 0.002	ND	ND	ND
	blm	109.7 $\pm$ 11.5	2187.2 $\pm$ 269.4	0.21 $\pm$ 0.03	0.11 $\pm$ 0.01	0.12 $\pm$ 0.01	0.04 $\pm$ 0.01
IL-5 ^{TG}	saline	56.2 $\pm$ 4.0	52.0 $\pm$ 4.0	0.07 $\pm$ 0.01	ND	ND	0.01 $\pm$ 0.002
	blm	189.5 $\pm$ 16.3***	3159.2 $\pm$ 285.9*	0.42 $\pm$ 0.12	0.56 $\pm$ 0.21	ND	4.1 $\pm$ 0.3***
IL-5 ^{+/+}	saline	40.5 $\pm$ 8.5	149.2 $\pm$ 18.5	0.06 $\pm$ 0.01	ND	ND	ND
	blm	128.1 $\pm$ 8.6	3257.7 $\pm$ 238.8	1.3 $\pm$ 0.2	0.93 $\pm$ 0.07	0.19 $\pm$ 0.02	0.1 $\pm$ 0.01
IL-5 ^{-/-}	saline	58.1 $\pm$ 4.4	202.5 $\pm$ 11.6	0.1 $\pm$ 0.02	ND	ND	ND
	blm	85.1 $\pm$ 6.7**	2735.4 $\pm$ 229.5	0.25 $\pm$ 0.03***	0.91 $\pm$ 0.05	0.12 $\pm$ 0.02*	ND

^a Parameters for 7 days after transoral instillation of bleomycin (0.02 U/mouse) in transgenic (TG) and knockout (-/-) mice for IL-5 and their respective counterpart mice (IL-5^{WT} and IL-5^{+/+}).

^b ND, Not detected.

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; denote significant differences in values measured in blm-treated transgenic or knockout mice (IL-5^{TG} and IL-5^{-/-}) compared to their corresponding blm-treated wild type mice (IL-5^{WT} and IL-5^{+/+}).

it reached 100% in IL-5^{+/+} mice. Collectively these results suggest that deletion of the IL-5 gene had an important effect on the lung response resulting in significantly reduced blm-induced lung injury and mortality. The lung responses in IL-5-deficient animals were also characterized by a relative absence of eosinophilia but a more pronounced T cell influx.

IL-5 overexpression or 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. In comparison to blm-treated IL-5^{WT}, treated IL-5^{TG} mice showed more severe lung fibrosis. Indeed at each time point examined, levels of lung hydroxyproline, soluble collagen, fibronectin, and TGF- β were higher in blm-treated IL-5^{TG} relative to IL-5^{WT} mice (Fig. 2, A–D). Histological examination also showed more extensive blm-induced fibrotic lesions and collagen deposition (Masson Trichrome stain) in transgenic mice in comparison to wild type (data not shown). Thus increased IL-5 was associated with an enhanced fibrotic response, suggesting a role for this cytokine in pulmonary fibrosis.

To confirm such a role, the studies were repeated in IL-5-deficient mice. Although there was less lung inflammation and injury during the early stage of blm toxicity in the lungs of IL-5^{-/-} mice, this strain unexpectedly exhibited more severe chronic disease when compared with the blm-treated IL-5^{+/+} mice. At days 21 and 28, levels of all parameters used to estimate the intensity of lung fibrosis were increased in deficient mice relative to wild-type mice (Fig. 2, E–H). In agreement with these quantitative indices of fi-

brosis, histological examination revealed that on day 21, pulmonary fibrosis in IL-5^{-/-} mice was substantially more severe than in IL-5^{+/+} mice. Indeed, lung fibrosis was more intense and more organized in IL-5-deficient mice (data not shown).

Collectively, these results showed that blm-induced lung fibrosis was increased paradoxically in both IL-5^{TG} and IL-5^{-/-} mice in comparison to their respective blm-treated wild-type mice.

Blm lung response after adenovirus construct for IL-5 administration

To clarify the role of IL-5 and to support the observations in IL-5^{TG} mice, we studied the effects of enhanced lung IL-5 expression (as a result of treatment with an AdIL-5) on blm-responses in IL-5^{WT} as well as in low responder (to blm-induced pulmonary fibrosis) BALB/c mice. The effects of AdIL-5 treatment on blm-induced pulmonary fibrosis were evaluated by measuring lung content of hydroxyproline, soluble collagen, type I collagen, and fibronectin at day 21. The results showed that blm-induced fibrosis, as assessed using all criteria, was significantly increased in IL-5^{WT} mice treated with AdIL-5 relative to those treated with AdCTL (Table III). In addition increasing the dose of adenovirus (10^8 PFU) caused a greater increase in the mortality rate of IL-5^{WT} mice treated with AdIL-5 relative to AdCTL (50% vs 80% in AdIL-5 and AdCTL, respectively). Treatment of BALB/c mice with blm did not cause significant fibrosis (data not shown), but additional treatment with AdIL-5 caused significant fibrosis, although the level of fibrosis was still less than that seen in IL-5^{+/+} mice (Table III). No significant differences were noted between the saline-treated control group of IL-5^{WT} and BALB/c mice receiving either construct (data not shown). Collectively, these data support

Table II. Pulmonary lymphocyte populations in saline or bleomycin-treated IL-5^a

	Populations ($\times 10^6$)			
	IL-5 ^{+/+} + sal	IL-5 ^{-/-} + blm	IL-5 ^{-/-} + sal	IL-5 ^{-/-} + blm
CD90.1.2 ⁺ T cells	1.4 $\pm$ 0.2	7.0 $\pm$ 0.4	1.2 $\pm$ 0.1	12.9 $\pm$ 0.7***
CD3 ⁺ T cells	2.4 $\pm$ 0.2	6.6 $\pm$ 0.2	3.5 $\pm$ 0.2	9.6 $\pm$ 0.7**
CD4 ⁺ T cells	0.8 $\pm$ 0.05	4.1 $\pm$ 0.2	1.0 $\pm$ 0.1	5.9 $\pm$ 0.3**
CD8 ⁺ T cells	0.5 $\pm$ 0.03	1.1 $\pm$ 0.1	0.5 $\pm$ 0.02	1.6 $\pm$ 0.1**
TCR- β T cells	2.1 $\pm$ 0.1	6.4 $\pm$ 0.2	3.5 $\pm$ 0.2*	9.56 $\pm$ 0.7**
TCR- δ T cells	0.22 $\pm$ 0.02	0.18 $\pm$ 0.01	0.15 $\pm$ 0.01	0.48 $\pm$ 0.04**
NK cells	0.53 $\pm$ 0.04	1.3 $\pm$ 0.1	0.69 $\pm$ 0.12	1.3 $\pm$ 0.2
B cells	2.4 $\pm$ 0.2	6.9 $\pm$ 0.3	4.0 $\pm$ 0.2	8.6 $\pm$ 0.8

^a Lymphocyte population in saline (sal) or bleomycin (blm)-treated IL-5^{+/+} and IL-5^{-/-} mice after 15 days (0.02 U of blm/mouse).

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, denote significant differences in values measured in bleomycin-treated IL-5^{+/+} mice compared to the corresponding IL-5^{-/-} mice.

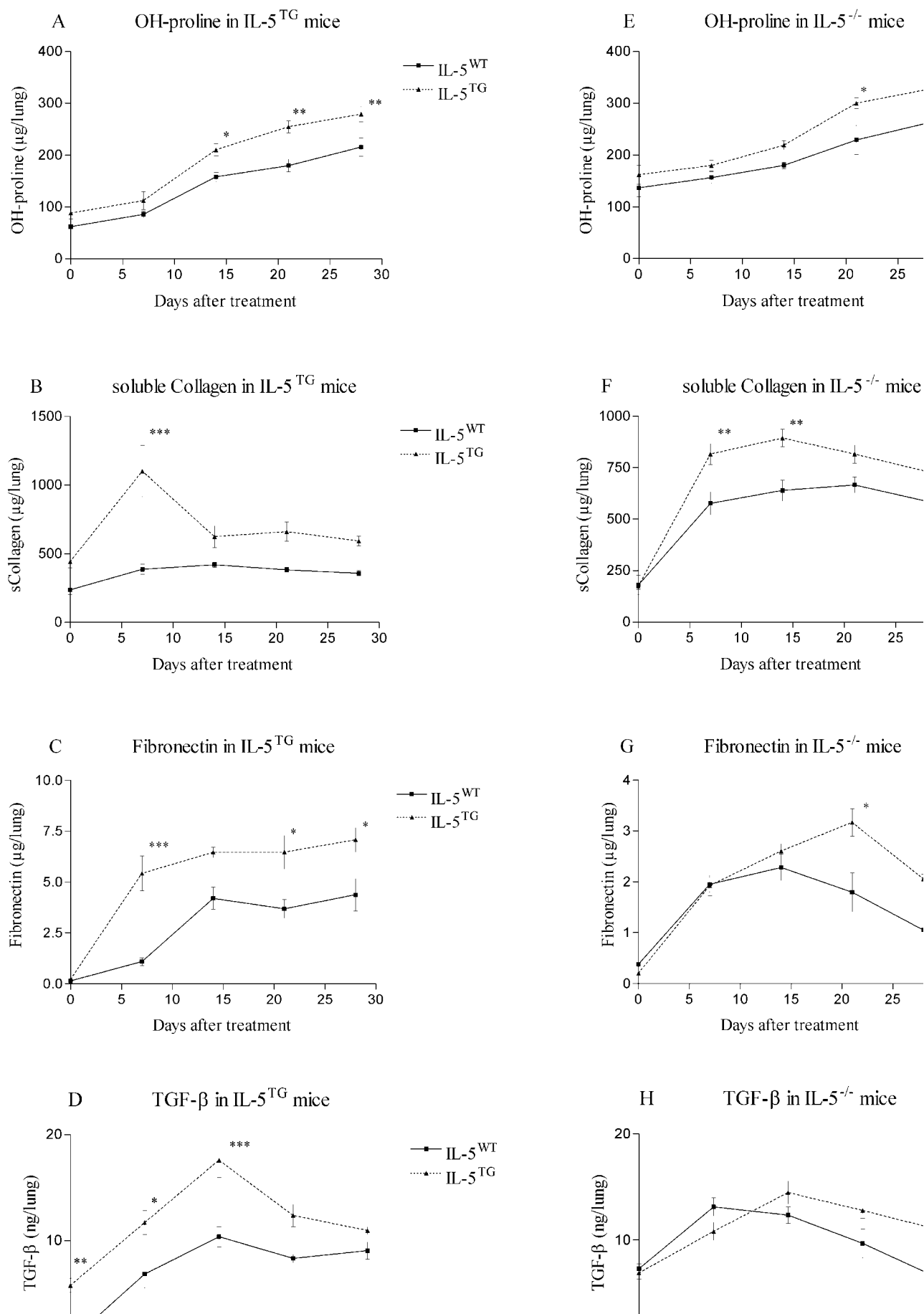


FIGURE 2.

(Legend continues)

Table III. Levels of hydroxyproline, soluble collagen, collagen type I, and fibronectin in bleomycin-treated IL-5^{WT} and Balb/c mice^a

	Hydroxyproline ($\mu\text{g}/\text{lung}$)	Soluble Collagen ($\mu\text{g}/\text{lung}$)	Collagen Type I ($\mu\text{g}/\text{lung}$)	Fibronectin ($\mu\text{g}/\text{lung}$)
IL-5 ^{+/+} blm + adCTL	204.0 $\pm$ 22.5	641.6 $\pm$ 49.1	146.1 $\pm$ 10.4	4.5 $\pm$ 0.2
IL-5 ^{+/+} blm + adIL-5	260.0 $\pm$ 17.6*	844.3 $\pm$ 27.4*	167.5 $\pm$ 10.5	5.2 $\pm$ 0.2*
Balb/c blm + adCTL	128.8 $\pm$ 6.4	120.0 $\pm$ 13.2	70.0 $\pm$ 17.2	1.6 $\pm$ 0.1
Balb/c blm + adIL-5	160.4 $\pm$ 12.1*	220.3 $\pm$ 33.2*	111.0 $\pm$ 12.1*	2.7 $\pm$ 0.3*

^a Evaluation at 21 days (0.02 U of blm/mouse) after adenoviral construct administrations.*, $p < 0.05$; denote significant differences in values measured in AdCTL and blm-treated mice compared to the corresponding AdIL-5 and blm-treated mice.

our observations obtained in blm-treated IL-5^{TG} mice and suggest a key role for IL-5 in the extension of blm-induced pulmonary fibrosis. However these results appear to contradict the findings using the IL-5-deficient mice, wherein reduced eosinophils due to IL-5 deficiency but enhanced lung T lymphocyte influx are associated with enhanced pulmonary fibrosis.

Characterization of pulmonary eosinophils and T cells

To clarify this apparent paradox, the potential roles of selective influx of eosinophils or T lymphocytes in blm-induced fibrosis in IL-5 transgenic and deficient animal models were examined in vitro using coculture studies. These pulmonary immune cells were isolated and purified from lung tissue at day 7 (eosinophils) or day 14 (T cells) after instillation of blm to donor animals. They were then cultivated in coculture with murine lung fibroblasts obtained from respective naive (not treated with blm) wild-type mice (IL-5^{WT} and IL-5^{+/+}). The capacity of these immune cells to regulate fibroblast function was then examined in terms of cell proliferation (³H]thymidine incorporation), collagen production, and myofibroblast differentiation (expression of α -SMA) in culture. Unfortunately, the cell purification techniques used in this study did not permit isolation of sufficient viable eosinophils from the parenchyma tissue of IL-5^{WT} mice treated with saline or blm. In contrast, numerous eosinophils were obtained from saline and blm-treated IL-5^{TG} mice. Thus, we compared only the effect of eosinophils from the transgenic mice purified at days 7 and 14 after blm or saline treatment. Eosinophils from both saline- and blm-treated mice and at either time point, did not significantly affect fibroblast proliferation in coculture (Fig. 3A). However, eosinophils purified from blm-treated IL-5^{TG} at day 7 and to a lesser extent at day 14, significantly induced myofibroblast differentiation as estimated by measuring α -SMA and increased type I collagen expression (Fig. 3, B and C). Although eosinophils from saline-treated IL-5^{TG} weakly stimulated both parameters as compared with the medium alone, this effect was not statistically significant. PDGF and TGF- β were used as positive controls for fibroblast proliferation and α -SMA (as well as type I collagen synthesis), respectively. These data showed that in vitro purified blm-IL-5^{TG} eosinophils could directly activate certain fibroblast functions.

In coculture with pulmonary fibroblasts, T cells purified from blm-treated mice stimulated the proliferation rate with similar amplitude relative to either the medium alone or T cells obtained from saline mice (Fig. 3D). Because the fibroblast monolayer was

washed twice with medium before addition of [³H]thymidine, the contribution from nonadherent T cells was minimized. Induction of fibroblast proliferation with PDGF was used as a positive control. Although T cells were also able to stimulate proliferation, they were ineffective in promoting myofibroblast differentiation (α -SMA expression, Fig. 3E) or collagen production (Fig. 3F). The lung fibroblasts however were responsive to TGF- β , which was selected as the positive control for induction of myofibroblast differentiation. Thus eosinophils have the potential to directly affect fibrosis by stimulating myofibroblast differentiation, whereas T cells do not have this role.

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

Because enhanced T lymphocyte influx in the lung in the absence of eosinophilia is associated with enhanced blm-induced pulmonary fibrosis in IL-5-deficient mice, the role of eosinophils and T cells in vivo was investigated. To further characterize the biological activity of eosinophils and T lymphocytes in blm-induced lung fibrosis, we investigated the effects on blm-induced lung fibrosis of 1) instillation of purified eosinophils from blm-treated IL-5^{TG} mice into the lungs of wild-type mice and 2) T cell depletion by anti-CD3 Ab treatment in IL-5^{+/+} and IL-5^{-/-} mice. Wild-type mice were administered at day 0 with saline or blm at low dose (0.01 U/mouse) to induce submaximal levels of lung injury and fibrosis. At days 7 and 14, these mice received by oral instillation 5×10^6 purified lung eosinophils from blm-treated IL-5 transgenic mice. Eosinophil injection into saline-treated recipient mice slightly increased the level of lung hydroxyproline at day 21 (Fig. 4). This effect of eosinophil injection was much more marked and significant in blm-treated recipient mice. Thus lung hydroxyproline was significantly higher in blm-treated mice receiving eosinophils than in mice receiving blm only (Fig. 4).

With respect to the role of T cells, the effects of T cell depletion on blm-induced fibrosis were analyzed. Pulmonary fibrosis induced by 0.02 U of blm in both IL-5^{-/-} and IL-5^{+/+} mice was completely abrogated by treatment with anti-CD3 Abs in comparison with corresponding blm mice injected with control IgG (Fig. 4B). However increasing the dose of blm to 0.05 U abrogated the inhibitory effect of treatment with anti-CD3 Abs in IL-5^{+/+} mice (Fig. 4B). In contrast, IL-5^{-/-} mice treated with 0.05 U/mouse of blm remained completely protected by anti-CD3 Ab treatment, showing a comparable level of lung hydroxyproline as the saline

FIGURE 2. Kinetics of parameters of lung fibrosis. At days 0, 7, 14, 21, and 28 post-blom instillation (0.02 U/mouse), hydroxy(OH)-proline (A and E), soluble collagen (B and F), fibronectin (C and G), and TGF- β (D and H) levels were measured in lung homogenates obtained from saline- or blm-treated IL-5^{TG}, IL-5^{WT}, IL-5^{-/-}, and IL-5^{+/+} mice. The results are shown as mean $\pm$ SEM of at least six animals. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ denote the level of significant difference in mean values measured in blm-treated IL-5^{TG} or IL-5^{-/-} mice compared with their corresponding blm-treated wild-type mice (IL-5^{WT} and IL-5^{+/+}, respectively) as analyzed by the Student-Newman-Keuls multiple comparison test.

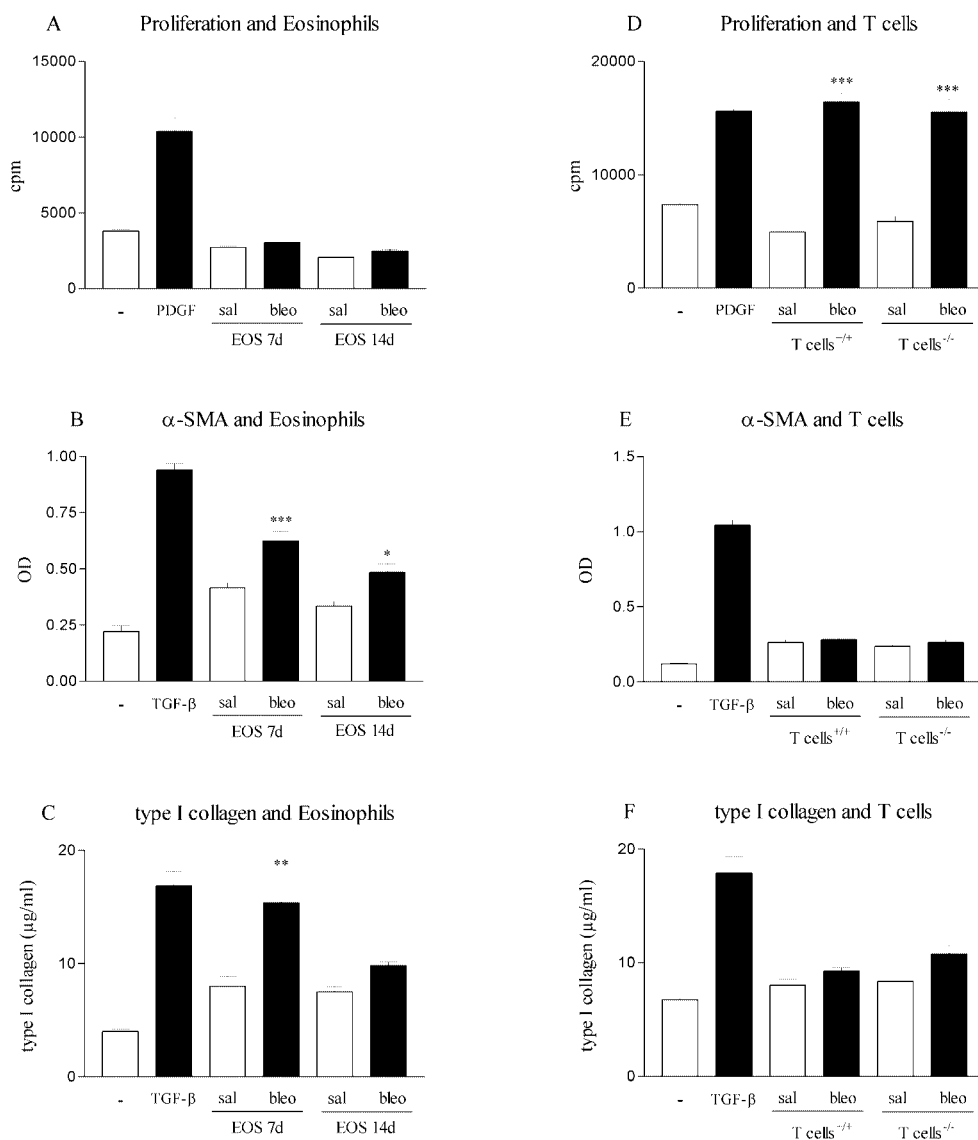


FIGURE 3. Effects of lung eosinophil or T cell coculture with lung fibroblasts. Lung fibroblasts isolated and purified from naive (untreated) IL-5^{WT} or IL-5^{+/+} mice were cocultured with either purified lung eosinophils or T lymphocytes obtained from IL-5^{TG} and IL-5^{-/-} animals on day 7 or day 14 after saline or blm (0.02 U/mouse) instillation. Cell proliferation was measured by [³H]thymidine incorporation (A and D), and myofibroblast differentiation was determined as α-SMA (B and E) protein expression. Fibrogenic effect was assessed by type I collagen expression (C and F). Bars represent means ± SEM with $n = 4-6$. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ denote level of significant difference in mean values measured in culture with purified leukocytes compared with medium alone as analyzed by the Student-Newman-Keuls multiple comparison test. PDGF (10 ng/ml) (A and D) and TGF-β (10 ng/ml) (B, C, E, and F) were used as positive controls for proliferation and α-SMA, respectively, as well as type I collagen parameters.

control group (Fig. 4B). These results demonstrated that the pulmonary fibrosis observed in IL-5 transgenic and deficient animals were at least in part associated with heightened recruitment of eosinophils and T lymphocytes, respectively. They also show that the fibrosis in IL-5-deficient mice is totally dependent on T cells, even at a high dose of blm, but independent of T cells in IL-5-sufficient mice treated with high-dose blm.

Cytokine profile of purified eosinophils and T cells

To determine the mechanism by which eosinophils and T cells participate to the extension of fibrosis, we then explored their expression of cytokines implicated in the pathogenesis of fibrosis. GEArrays gene arrays allowed us to profile the expression of 20 preselected genes known to activate fibroblast functions and to be produced specifically by both these immune cell types. Transcript levels of type 2 cytokines such as IL-4, IL-13, and IL-10 were

significantly increased in eosinophils purified from IL-5^{TG} mice administrated with blm in comparison to eosinophils from saline-treated IL-5^{TG} animals. Similarly higher expression of TGF-β1 and -β3 was found in eosinophils from blm- vs saline-treated mice (data not shown). Analysis of T cells using a similar array revealed that blm-treated IL-5^{-/-} and IL-5^{+/+} mice significantly expressed TNF-α, IL-1β, and IFN-γ, although expression was higher in deficient cells. Similarly, TGF-β1 was more highly induced in T cells from IL-5^{-/-} vs IL-5^{+/+} mice (data not shown).

On the basis of these array analyses, we further focused our work on selected cytokines. Isolated and purified eosinophils and T lymphocytes were analyzed for TNF-α, IL-1β, IFN-γ, IL-4, IL-13, and TGF-β production in the presence of calcium ionophore (eosinophils) or anti-CD3 Abs (T cells). Consistent with the array data, purified and activated eosinophils from saline and blm-treated IL-5^{TG} mice expressed IL-4, IL-13, and TGF-β (Table IV).

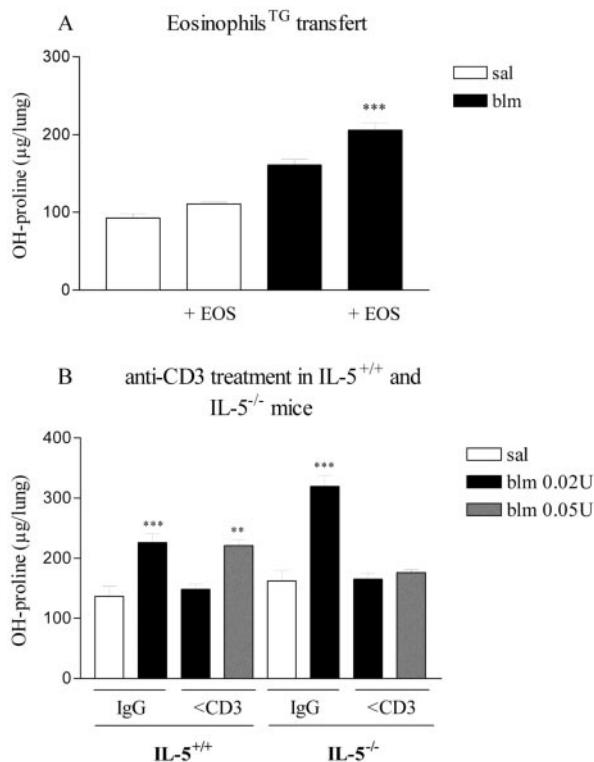


FIGURE 4. Effects of eosinophil instillation or T cell depletion on lung fibrosis. Purified eosinophils from day 7 blm-treated IL-5 transgenic donor mice were instilled into wild-type recipient mice treated with saline (sal) or blm (0.01 U/mouse). At day 21 after sal or blm treatment, lung hydroxy(OH)-proline levels were determined in recipient mice (A). In another experiment mice were treated with saline (sal), 0.02 or 0.05 U blm/mouse, plus either control IgG or anti-CD3 Abs. At day 21 after sal or blm treatment, lung hydroxyproline levels were determined as a measure of fibrosis (B). Only the IgG controls at the 0.02 U blm dose were shown because the majority of IgG-treated animals died from the high-dose blm before the end of the experiment. The bars represent mean $\pm$ SEM of at least six animals in each group. **, $p < 0.01$ and ***, $p < 0.001$ denote level of significant difference in mean values of groups treated with or without eosinophil instillation (A) or anti-CD3 Abs (B) as analyzed by the Student-Newman-Keuls multiple comparison test.

Eosinophils from blm-treated transgenic mice however produced significantly higher levels of these cytokines than those from saline-treated transgenic mice. Very low or undetectable levels of TNF- α , IL-1 β , and IFN- γ were expressed by all eosinophil cultures, and no difference was noted between cells from blm- vs saline-treated mice (Table IV). Without in vitro stimulation, eosinophils from both blm- and saline-treated mice did not produce detectable levels of cytokines albeit freshly purified cells expressed the transcripts. This observation may reflect a lack of sen-

sitivity of our ELISA or denote the relative incapacity of eosinophils to produce spontaneously cytokines in vitro without strong stimulating factors such as calcium ionophore.

T cells from blm-treated mice (both IL-5^{-/-} and IL-5^{+/+}) produced significant levels of all studied cytokine (Table IV). TNF- α , IL-1 β , and IFN- γ expression were slightly decreased in cultures from blm-treated IL-5^{-/-} mice relative to cultures from blm-treated IL-5^{+/+} mice (Table IV), whereas TGF- β levels were significantly increased. Anti-CD3-induced expression of IL-4 and IL-13 were dramatically diminished in IL-5^{-/-} T cells in comparison to IL-5^{+/+} T cells.

In summary, these data showed that activated saline and blm transgenic eosinophils produced preferentially type 2 cytokines (IL-4 and IL-13) and TGF- β , and the expression level was higher in cells from blm-treated animals. T cells from blm-treated IL-5^{-/-} and IL-5^{+/+} mice expressed both proinflammatory/type 1 and type 2 cytokines as well as TGF- β . However T cells from IL-5-deficient mice expressed significantly less type 2 cytokines relative to T cells from wild-type mice.

To ensure that the cytokine profile observed in vitro was also apparent in vivo, we measured the levels of the studied cytokines in whole lung homogenates. TNF- α or IFN- γ proteins were not detectable in all samples examined. However IL-4 (data not shown) and IL-13 (Fig. 5A) proteins were markedly induced in lungs of blm-treated IL-5^{TG} mice in comparison to similarly treated IL-5^{WT} mice. This increase was maximal in IL-5^{TG} at day 7 after blm treatment. In contrast, IL-1 β levels were significantly lower at days 14 and 21 post-blom treatment in IL-5^{TG} relative to IL-5^{WT} animals (Fig. 5B). The opposite effects on cytokine levels were noted in IL-5^{-/-} mice in comparison to IL-5^{+/+} mice. Thus IL-13 and IL-4 (data not shown) levels were decreased in deficient animals 7 days after blm treatment, whereas IL-1 β lung contents were higher in IL-5^{-/-} vs IL-5^{+/+} mice at days 14 and 21 (Fig. 5, C and D). These data indicate that the more severe fibrotic lesions observed in IL-5^{TG} and IL-5^{-/-} relative to their respective wild-type controls, were associated with different patterns of cytokine expression. Transgenic mice produced preferentially type 2 cytokines such as IL-4 and IL-13 in response to blm, whereas deficient animals developed pulmonary responses characterized by a pronounced presence of proinflammatory/type 1 cytokines. However, the lung response to blm in both strains was associated with an increased level of TGF- β expression (Fig. 2, D and H).

Effects of proinflammatory and type 2 cytokines on pulmonary fibroblasts in vitro

To determine whether cytokine production by purified eosinophils and T cells can be related to their known biological activity in coculture with fibroblasts (Fig. 3), we assessed the potential direct activity of these two groups of cytokines on pulmonary fibroblasts. We compared the in vitro activity of various concentrations of recombinant mouse IL-4, IL-13, and TGF- β on lung fibroblasts

Table IV. In vitro cytokine production by lung eosinophils and T cells^a

			TNF- α (ng/ml)	IL-1 β (pg/ml)	IFN- γ (ng/ml)	IL-4 (ng/ml)	IL-13 (ng/ml)	TGF- β (ng/ml)
EOS	IL-5 ^{TG}	sal	0.04 $\pm$ 0.003	ND	0.008 $\pm$ 0.001	4.0 $\pm$ 0.03	0.8 $\pm$ 0.04	3.5 $\pm$ 0.2
EOS	IL-5 ^{TG}	blm	0.03 $\pm$ 0.002	ND	0.012 $\pm$ 0.002	6.1 $\pm$ 0.1**	1.1 $\pm$ 0.08**	4.2 $\pm$ 0.5*
T cells	IL-5 ^{+/+}	blm	1.2 $\pm$ 0.03	50.4 $\pm$ 1.6	129.4 $\pm$ 4.9	462.4 $\pm$ 15.8	79.8 $\pm$ 2.1	1.6 $\pm$ 0.3
T cells	IL-5 ^{-/-}	blm	1.0 $\pm$ 0.02	32.3 $\pm$ 2.9**	110.6 $\pm$ 4.8*	77.2 $\pm$ 4.5**	25.1 $\pm$ 2.1**	2.0 $\pm$ 0.04*

^a Lung eosinophils (EOS) and T cells purified from respectively saline- or blm-treated IL-5^{TG} (day 7, 0.02 U of blm/mouse) and IL-5^{+/+} and IL-5^{-/-} mice (day 15, 0.02 U of blm/mouse).

^b ND, Not detected.

*, $p < 0.05$; **, $p < 0.001$; denote significant differences in values measured in blm-treated transgenic or knockout (IL-5^{TG} or IL-5^{-/-}) mice compared, respectively, to saline-treated (sal) transgenic mice and blm-treated wild-type (IL-5^{+/+}) mice.

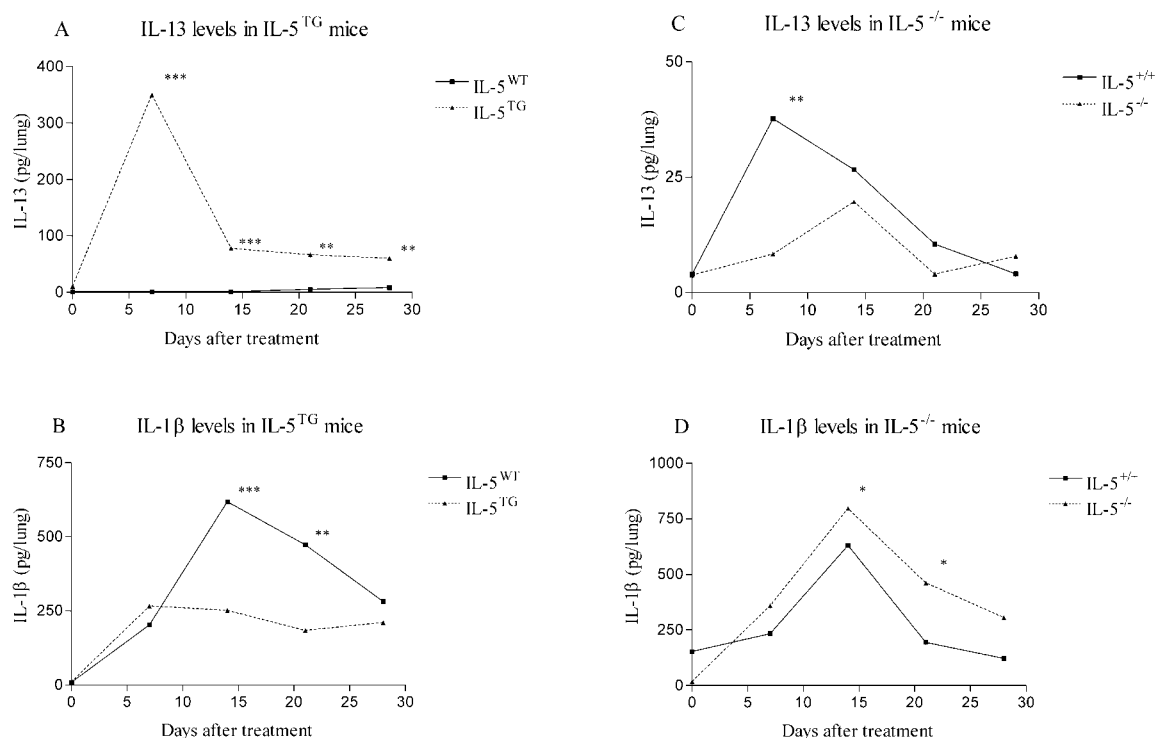


FIGURE 5. At days 0, 7, 14, 21, and 28 post-bltn instillation (0.02 U/mouse) IL-13 (A and C) and IL-1 β (B and D) protein levels were measured in lung homogenates obtained from IL-5^{TG} and IL-5^{WT} mice (A and B) as well as IL-5^{-/-} and IL-5^{+/+} mice (C and D). Values represent means $\pm$ SEM of at least six animals. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ denote the level of significant difference in mean values measured in bltn-treated IL-5^{TG} or IL-5^{-/-} mice compared with the corresponding bltn-treated wild-type mice (respectively, IL-5^{WT} and IL-5^{+/+}) as analyzed by the Student-Newman-Keuls multiple comparison test.

isolated from IL-5^{WT} mice, and the effects of TNF- α , IL-1 β , and IFN- γ on lung fibroblasts isolated from IL-5^{+/+} mice. Recombinant IL-4, IL-13, and TGF- β at different concentrations (1 to 20 ng/ml) stimulated fibroblast proliferation, which was significant at the 20 ng/ml dose for all three cytokines (Fig. 6A). In addition, α -SMA and type I collagen expression were also increased after addition of these three cytokines compared with medium alone, with the greatest response noted with TGF- β (Fig. 6, B and C). Addition of recombinant TNF- α , IL-1 β , and IFN- γ also caused increased proliferation in IL-5^{+/+} fibroblasts (Fig. 6D). In contrast, no major differences in α -SMA or type I collagen expression were observed after addition of these proinflammatory type 1 cytokines compared with medium alone (Fig. 6, E and F), whereas these cells did respond to TGF- β used as a positive control. Recombinant mouse IL-5 was also tested in culture of fibroblasts obtained from all murine strains. At all concentrations used (1 to 20 ng/ml), IL-5 was unable to significantly affect proliferation rate, α -SMA expression and collagen synthesis (data not shown). These results demonstrated that both types of cytokines, type 2 vs proinflammatory type 1, expressed in IL-5 transgenic and deficient mice, respectively, mimicked relatively well the capacity of purified eosinophils and T cells to activate certain functions of fibroblasts in culture.

Discussion

Human lung fibrosis as well as experimental animal models of pulmonary fibrosis are often associated with pulmonary inflammation characterized by accumulation of macrophages, lymphocytes, and granulocytes (23). These immune cells are considered to be the major source of mediators such as cytokines, which possess the ability to directly stimulate certain fibroblast functions critical to fibrogenesis (9). However, it remains difficult to predict a priori the

exact and specific contribution of each lung leukocyte population as well as their unique capacity to produce profibrotic mediators in the pathogenesis of lung fibrosis *in vivo*. Because of its relative ease of isolation, the role of alveolar macrophages in the pathogenesis of pulmonary fibrosis has been clarified by several studies (4). In contrast, the precise roles of other leukocytes, such as eosinophils and lymphocytes mainly present in the lung parenchyma and interstitium and thus less accessible for purification, are not as clear with recent studies showing conflicting data (5–8). By studying the role of IL-5 in bltn-induced inflammation and fibrosis, we have observed that fibrotic lesions can be preferentially accompanied by either eosinophil or T cells accumulation. These pulmonary immune cells were purified and their unique *in vitro* and *in vivo* roles were studied in detail. We demonstrated that both cell populations were able to amplify fibrosis by different mechanisms and by producing different classes of cytokines.

The first significant finding from this study was that the intensity of the pulmonary response to bltn depended, at least in part, on an IL-5-dependent eosinophil infiltrate in animals that are IL-5 sufficient. Lung accumulation of eosinophils accentuated not only alveolitis but also the fibrosis induced by administration of bltn in these mice. In mouse models of IL-5 overexpression or IL-5 deficiency, we demonstrated that eosinophils contributed to the early bltn-induced lung injury and mortality (due to high-dose bltn). Although the absence of IL-5 was associated with a relative limitation of early lung injury and a relative protection from lethal dose of bltn, exaggerated lung IL-5 expression was accompanied by the opposite effects, namely more severe bltn-induced lung injury and mortality. The amplitude of early lesions in our models were specifically linked with the intensity of pulmonary eosinophilia, emphasizing the important role of activated eosinophils in bltn-induced acute injury. We can further postulate that these deleterious effects are due to the activated eosinophil ability to

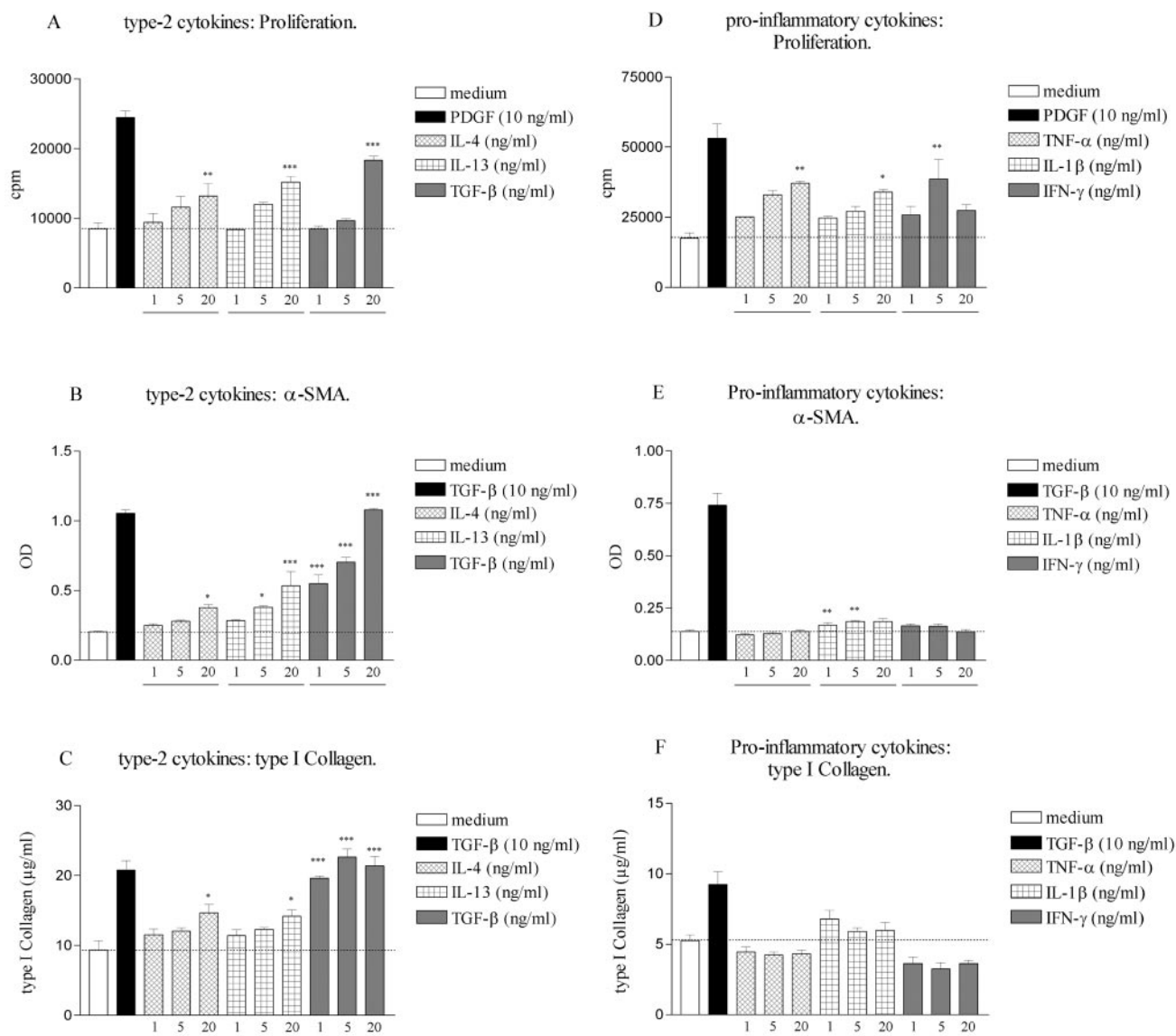


FIGURE 6. Effects of cytokines on lung fibroblasts in vitro. Effects of the indicated recombinant mouse cytokine and its dose on fibroblast [3 H]thymidine incorporation (A and D), α -SMA (B and E), and type I collagen expression (C and F) were determined in cells isolated from naive IL-5^{WT} mice (A–C) and naive IL-5^{+/+} animals (D–F). Bars represent mean $\pm$ SEM with $n = 4$ –6. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ denote level of significant difference in mean values measured with medium alone compared with corresponding cells treated with the indicated recombinant cytokine and dose, as analyzed by the Student-Newman-Keuls multiple comparison test. PDGF (10 ng/ml) (A and D) and TGF- β (10 ng/ml) (B, C, E, and F) were used as positive controls for proliferation and α -SMA as well as type I collagen parameters, respectively.

release a spectrum of cytotoxic mediators such as eosinophil cationic proteins and oxygen radicals (24), and as demonstrated in other models of lung injury (25, 26).

By studying more chronic effects of blm, we also found that recruited and activated eosinophils can specifically and directly interact with mesenchymal cells by stimulating myofibroblast differentiation and collagen synthesis and thus amplify lung fibrosis. This was observed from in vitro studies where eosinophils were cultivated with lung fibroblasts. Our in vitro observations using lung cells are consistent with the data obtained with eosinophils purified from blood and cultivated with lung or dermal fibroblasts (27–29), except pulmonary eosinophils from blm-treated IL-5 transgenic mice were unable to affect fibroblast proliferation in contrast to a previous study (28). This notable difference emphasizes the need to consider the functional heterogeneity of eosinophils from different anatomical compartments, or that eosinophils may adopt dif-

ferent phenotypes depending on anatomic localization and/or the pathology of the particular lesion causing the eosinophilia. The in vivo importance of eosinophils was confirmed specifically by the ability of donor eosinophils instilled into recipient mice to enhance the chronic response to blm-induced pulmonary fibrosis. This was additionally confirmed by the ability of AdIL-5 transfection to enhance blm-induced fibrosis in both responder (IL-5^{+/+}) as well as low-responder (BALB/c) mice. By increasing IL-5 and thus eosinophil recruitment in BALB/c mice, we were able to significantly induce pulmonary fibrosis in this strain known to develop minimal (if any) eosinophilia and lung fibrosis in response to blm. Beside its capacity to elaborate toxic and injurious products, the eosinophil has the potential of being an immunomodulatory cell with a remarkable capacity to produce a whole range of potent mediators such as pro-inflammatory (TNF- α), Th1 (IL-12), and Th2 (IL-4 and IL-13) cytokines, and thus may dramatically and directly

change the phenotype of the immune response (24). In our study, lung eosinophils were identified as a major source of TGF- β , IL-4 and IL-13 but not proinflammatory cytokines, consistent with a preferentially Th2-like phenotype in the course of blm-induced inflammation and fibrosis. It is noteworthy that these cytokines are strongly involved in tissue remodeling and fibrosis (30). Thus, we can further postulate that their profibrotic functions are mediated by type 2 cytokines. In addition, these observations well support data obtained by immunohistochemistry and published by our group showing that eosinophils are key cellular sources of the profibrotic mediators TGF- β and MCP-1 in the blm model (31–33). Finally because the lung eosinophil is also an important source of the chemokines RANTES and IL-16 (data not shown), it is probable that it also participates in the initiation of pulmonary inflammation by promoting the recruitment and the activation of macrophages and lymphocytes as demonstrated in another lung injury model (34). This evidence for a role for eosinophils in IL-5-sufficient mice is consistent with a previous study showing suppression of blm-induced fibrosis by eosinophil depletion using anti-IL-5 Abs (7), but appears to contradict another study showing failure of such Abs to suppress blm-induced fibrosis despite suppression of eosinophilia (8). The basis for this contradiction is unclear, but may be related to differences in dosage of blm used, timing of analysis, method for quantitating lung eosinophilia, and the use of the SCID mouse in the latter study.

A second chief finding was that blm-induced pulmonary fibrosis is also intimately dependent on T lymphocytes and can proceed in the absence of IL-5 and lung eosinophil recruitment. The importance of T cells in the development of lung fibrosis was demonstrated by administration of anti-CD3 Abs, which highly reduced the amplitude of fibrotic lesions in both blm-treated wild-type and IL-5-deficient mice. Our observations are in accordance with previous reports showing that blocking the recruitment of CD4⁺, CD8⁺, or CD3⁺ cells limit the extension of fibrotic lesions in different lung experimental models (5, 35, 36). On the basis of our coculture experiments, we can further propose that profibrotic activity of T lymphocytes 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 factor, (37) and as observed in this study, TNF- α , it is probable that the effect observed with 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 in vitro conditions as described in this study. Thus, T cells appear to play a limited but specific direct role in terms of promoting fibrosis in this model, although indirect roles certainly could play additional and potentially more important roles. Furthermore, a more intense lung T cell recruitment in IL-5-deficient mice could compensate for the loss of lung eosinophil recruitment in the lung fibrotic response to blm. Thus although IL-5-dependent lung eosinophilia plays an important role in blm-induced lung fibrosis, it is not indispensable, especially in transgenically IL-5-deficient mice where compensatory mechanisms have time to develop. This conclusion is consistent with a previous study showing no protection from fibrosis in IL-5-deficient mice (8). Interestingly such compensatory mechanisms are inadequate to fully compensate for the lack of IL-5/eosinophils in the acute injury response to blm. It is noteworthy that induction of lung TGF- β expression remained intact in these IL-5-deficient mice. Thus fibrosis in both IL-5^{TG} and IL-5-deficient mice is associated with induction of TGF- β , which could account for the observed fibrosis despite differences in Th1 vs Th2 profiles.

Evidence has emerged that lung fibrosis is the result of disordered fibroblast regulation. In fibrotic lesions, numerous histological studies have shown the simultaneous presence of scattered foci of fibroblast proliferation as well as the emergence of myofibroblast phenotype (38). Bearing this in mind, we can postulate that the appearance of the myofibroblast phenotype during fibrosis may be dependent at least in part on recruited eosinophils, whereas fibroblast proliferation could be induced at least in part by recruited T cells. However the potential role of additional cell types, such as mast cells, cannot be excluded from the current study.

Recent observations in humans and animals supported the view that lung fibrosis is a type 2-related disease (30). Nevertheless the type 1 component of the lung response to injury also appears to be important because IFN- γ -deficient mice exhibit attenuated blm-induced pulmonary fibrosis (39). The data presented in this study indicate a possible concomitant presence of both types of immune responses in lung injury and fibrosis. On the basis of the cytokine profile expressed in vitro and in vivo, it is difficult to discriminate between preferential type 1 or type 2 response in blm-induced lung disease because we detected in wild-type mice the expression of both types 1 and 2 cytokines, namely IFN- γ , TNF- α , IL-1 β , IL-4, and IL-13. In addition, our observations on transgenic and deficient animals support the notion that either exaggerated type 2 or proinflammatory/type 1 response can promote lung fibrosis, as observed respectively in blm-treated IL-5^{TG} and IL-5^{-/-} mice. This clearly argues for a more complicated mechanism, wherein both types of responses can promote fibrosis either individually or in concert. If this could be confirmed in human lung fibrosis, development of new therapeutic approaches based on controlling the activity of each type of immune responses should take this possibility into consideration. This may imply that selective suppression of either one of these responses is unlikely to adequately control disease progression.

In summary, we describe in this study that recruited eosinophils and T cells both can contribute to the genesis of lung fibrosis induced by blm, partly via their ability to directly affect certain fibroblast functions mediated by elaboration of both types 1 and 2 cytokines. These findings may have similar implications for other models or diseases where tissue remodeling/fibrosis is characterized by eosinophil and T cell infiltration.

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