Bleomycin-induced lung fibrosis in IL-4-overexpressing and knockout mice

GABRIEL IZBICKI,1 REUVEN OR,2 THOMAS G. CHRISTENSEN,3 MICHAEL J. SEGEL,1 ALAN FINE,4 RONALD H. GOLDFSTEIN,4 AND RAPHAEL BREUER1,3,4
1Lung Cellular and Molecular Biology Laboratory, Institute of Pulmonology, 2Bone Marrow Transplantation Department, Hadassah University Hospital and The Hebrew University-Hadassah Medical School, 91120 Jerusalem, Israel; and 3Mallory Institute of Pathology, Department of Pathology and 4Pulmonary Center, Boston University School of Medicine and Boston Veterans Administration Medical Center, Boston, Massachusetts 02118

Received 10 April 2002; accepted in final form 11 June 2002

Interleukin-4 (IL-4) is produced by macrophages and T helper (Th) 2 lymphocytes (47, 48), which infiltrate sites of lung fibrosis (23, 47). Data on IL-4 expression in rodent models of Bleo-induced lung fibrosis are conflicting (10, 14, 23, 43), and the precise role of IL-4 in lung fibrosis is as yet unclear. IL-4 has been shown to stimulate collagen gene expression (11), to increase collagen synthesis (8, 26, 34, 41), and to cause the chemotaxis of fibroblasts (35). In vivo data also point to a potential profibrotic role for IL-4. Neutralizing anti-IL-4 antibodies have been shown to reduce murine Schistosoma mansoni-induced hepatic fibrosis (5) and scleroderma (29). In contrast, IL-4 modulates the expression of proinflammatory cytokines such as IL-1 and TNF-α, which are involved in Bleo-induced lung fibrosis (16).

Because in vivo and in vitro data suggest that IL-4 may play a profibrotic role in the development of lung fibrosis, we aimed to test this hypothesis. Bleo-induced lung fibrosis was studied in transgenic IL-4-overexpressing (IL-4 TG), IL-4 knockout (IL-4 KO), and wild-type (WT) mice of the C57BL/6J strain. Our results indicate that IL-4 is not a key profibrotic cytokine in this model.

MATERIALS AND METHODS

Animals

Three groups of C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME), 11–12 wk old, weighing 25–30 g, were used: IL-4 TG (C57BL/6J-TgN), IL-4 KO (C57BL/6J-IL4tm1Cgn), and control WT mice. The IL-4 gene mutations were confirmed at the protein and molecular levels.

All procedures involving animals were approved by the institutional committee of animal care. Mice were housed in plastic cages on hardwood shavings. A 12-h light/dark cycle was maintained, and mice had free access to water and rodent laboratory chow ad libitum. Mice were acclimated to these conditions at least 1 wk before receiving intratracheal instillation treatment.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

L1110

http://www.ajplung.org
Experimental Design

Bleo (Bristol Laboratories, Syracuse, NY) was dissolved in sterile 0.9% saline (Sal) and administered as a single dose of 0.06 mg in 0.1 ml Sal per animal. All animals received intratracheal instillations with either Bleo or Sal on day 0 as previously described (2, 3, 4, 14, 21, 22, 24, 40).

Animals were studied 14 days after intratracheal instillation, on the basis of a previous time course study of Bleo-induced injury in C57BL/6 mice (18) demonstrating that this time point is optimal. Animals were killed by transection of the abdominal aorta while under an overdose of pentobarbital.

Animals were killed by transsection of the abdominal aorta while under an overdose of pentobarbital.

ELISA Measurements

Splenocytes from IL-4 KO and WT mice were isolated and cultured (5 × 10⁶ cells/ml) in RPMI 1640 containing 10% FCS, 1% l-glutamine, and 1% penicillin-streptomycin. Cells were incubated for 48 h with 1 μg/ml concanavalin A (con-A) and separated by centrifugation (4,000 rpm, 4 min), and supernatants were assayed for IL-4 by a solid-phase ELISA. This assay employs the quantitative “sandwich” enzyme immunoassay technique. ELISA kits were purchased from Quantikine HS R & D Systems (Minneapolis, MN).

RT-PCR

Total RNA was extracted from whole lung tissues of WT and IL-4 TG mice. Equal quantities of RNA were reverse transcribed, and aliquots of cDNA were amplified by PCR using primers specific for IL-4 and the ribosomal protein S12. The number of cycles used in each PCR was predetermined to be within the exponential range (IL-4 and S12, 40 cycles). Aliquots of RNA were used as negative controls for each PCR.

Statistical Analysis

BAL cell counts were analyzed using ANOVA and Bonferroni analyses (44). Hydroxyproline levels and QIA parameters were analyzed by nonparametric Kruskal-Wallis analysis (44). To compare the severity of Bleo-induced lung fibrosis among the different mice groups, we standardized values by z score = (Xi - mean Sal)/SD Sal and compared them using Kruskal-Wallis analysis (44). Probability values of <0.05 were considered statistically significant.

Morphological Examinations

The left lung was fixed by intratracheal infusion through the cannula with 4% formalin and 1% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4, maintained at 25-cm hydrostatic pressure for 5 min and then immersed in fixative for an additional 24 h. Only lungs that were well inflated by the fixative were analyzed. Three 0.3-cm-thick transverse sections were embedded in paraffin, and sequential 4- to 6-μm sections were stained with hematoxylin-eosin (H&E) and modified Masson's trichrome. Fibrotic lung injury was assessed morphologically by quantitative image analysis (QIA) as follows.

Fibrosis fraction. We quantified the degree of fibrosis with the Optimas image analysis computer program (Optimas, Bothell, WA) by analyzing slides that were stained with a modified trichrome stain, as previously described (4, 21, 22), to enhance the blue-stained collagen. By adjusting image contrast, brightness, and color threshold settings, we configured the image analysis program to detect areas of blue-stained collagen within each of 20 randomly selected fields per slide using a ×40 objective lens. The fraction of blue-stained collagen areas for each field, a constant 155 × 95 μm, was summed and averaged for each animal. The area fraction of fibrosis is presented as a percentage.

Alveolar wall area fraction. Alveolar wall thickness may increase due to either fibrosis or interstitial edema. Alveolar wall area fraction was quantified by the Optimas image analysis computer program configured to determine, in sections of H&E-stained slides, the area fraction of alveolar wall tissue. Using a ×10 objective lens and a video camera, we randomly selected fields lacking visible blood vessels or Airways and displayed them on a Sony monitor. Each field of interest measured 455 × 345 μm. The image analysis software was programmed to measure the area of all stained cells × and divide it by the constant field of interest area, thereby calculating an alveolar wall area fraction value for 10 fields that were averaged for each animal. Data are presented as a percentage.

Statistical Analysis

BAL cell counts were analyzed using ANOVA and Bonferroni analyses (44). Hydroxyproline levels and QIA parameters were analyzed by nonparametric Kruskal-Wallis analysis (44). To compare the severity of Bleo-induced lung fibrosis among the different mice groups, we standardized values by z score = (Xi - mean Sal)/SD Sal and compared them using Kruskal-Wallis analysis (44). Probability values of <0.05 were considered statistically significant.

Fig. 1. IL-4 mRNA expression in mouse lungs. Expression of mRNA was measured by semiquantitative RT-PCR. Expression of the ribosomal protein S12 was used as control for the quantity and quality of RNA. Genotype is shown above each lane: IL-4 transgenic (TG) mice (IL-4+/+), control wild-type (WT), and negative control reaction with RNA instead of cDNA (0).
RESULTS

We examined the effect of Bleo on IL-4 KO, IL-4 TG, and WT mice. The absence of IL-4 in IL-4 KO mice was confirmed by RT-PCR, which showed, in contrast to WT, no signal for IL-4 in cDNA preparations from con-A-stimulated splenocytes, and by ELISA of conditioned media of the same cells, in which IL-4 levels were below the limit of detection. IL-4 overexpression in the lungs of Bleo- and Sal-treated IL-4 TG mice was demonstrated using semiquantitative RT-PCR, which clearly showed a stronger signal for IL-4 mRNA in lungs from IL-4 TG mice compared with WT controls (Fig. 1).

To confirm the presence of Bleo-induced lung injury, we compared Bleo-treated IL-4 KO, IL-4 TG, and WT mice with Sal controls for each group of mice. Total and differential BAL cell counts are presented in Fig. 2. Total cell count increased in Bleo- compared with Sal-treated mice in all three groups. The rise of total cell count due to Bleo treatment was not significantly different in IL-4 TG or IL-4 KO compared with WT controls.

Differential cell count: in all three mouse groups, the percentage of lymphocytes was significantly higher and the percentage of macrophages correspondingly lower in Bleo- compared with Sal-treated animals (Fig. 2), confirming that Bleo did cause injury in all mice groups. Despite the decreased percentage in macrophages, the absolute number of macrophages was increased due to the Bleo-induced increases in total cell number. A significantly higher percentage of neutro-

Fig. 2. Total and differential cell count in bronchoalveolar lavage (BAL) fluid. Wild-type (WILD) and mutant mice with knockout (IL-4 KO) or overexpressed IL-4 gene (IL-4 TG) treated by intratracheal instillation of bleomycin (solid bars) or saline (Sal, open bars) are presented. A: total cells; B: macrophages; C: lymphocytes; D: neutrophils. Number of animals in each group is noted in parentheses. Values are means ± SE. *P < 0.05 compared with Sal.

Fig. 3. Hydroxyproline lung content (A) and quantitative image analysis of the fibrosis fraction (B) and alveolar wall area fraction (C) in bleomycin- and Sal-treated wild-type, IL-4 KO, and IL-4 TG (IL-4 OVER) mice groups. Values are presented as means ± SE. Number of animals in each group is noted in parentheses. Group symbols are detailed in Fig. 2. *P < 0.05 compared with Sal.
was observed in the IL-4 TG group.

To compare the severity of Bleo-induced lung fibrosis among the different mouse groups, we compared fibrosis parameters in Bleo-treated mice. Hydroxyproline levels (means ± SE) in the Sal-treated groups were not significantly different among the groups: 308 ± 35 and 208 ± 28 in IL-4 TG and IL-4 KO, respectively (Fig. 3A). IL-4 KO and WT Bleo-treated mice had significantly higher hydroxyproline values when compared with Sal-treated controls, whereas no significant difference was detected between Bleo- and Sal-treated mice in the IL-4 TG group (Fig. 3A).

Fibrosis fraction and alveolar wall area fraction as assessed by QIA are presented in Fig. 3, B and C. A significant increase in fibrosis fraction was observed in Bleo- compared with Sal-treated mice in the IL-4 KO and WT but not in the IL-4 TG group (Fig. 3B). Alveolar wall area fraction (Fig. 3C) was significantly increased in Bleo- compared with Sal-treated mice in the IL-4 KO and WT but not in the IL-4 TG group. Together, the data in Fig. 3 show that Bleo did cause a significant degree of fibrosis in IL-4 KO and WT but not in IL-4 TG mice.

The severity of Bleo-induced fibrosis was compared in the three groups of mice using standardized z score values for hydroxyproline, fibrosis fraction, and alveolar wall area fraction (Fig. 4). Mean z score values were significantly lower for all three fibrosis parameters in IL-4 TG compared with IL-4 KO mice but were not significantly different in either IL-4 KO or IL-4 TG animals compared with WT. However, for all parameters tested, a consistent trend was observed, demonstrating that Bleo-induced fibrosis is inversely related to IL-4 expression; z score values in IL-4 KO compared with IL-4 TG mice were 1.2-, two-, and fourfold higher for alveolar wall area fraction, fibrosis fraction, and hydroxyproline levels, respectively. These differences were highly significant (P < 0.01) for two of the three parameters, namely fibrosis fraction and hydroxyproline content (Fig. 4).

In a Bleo time course study, we showed that BAL neutrophils increase early at 3 or 6 days and not at 14 or 21 days after Bleo instillation (18). Because in IL-4 TG mice BAL neutrophils increased at 14 days after Bleo treatment (Fig. 2), an additional 21-day time point was studied to test whether Bleo-induced lung fibrosis is delayed in these animals. Hydroxyproline and QIA parameters of fibrosis fraction and alveolar wall area fraction of 14- and 21-day mice are presented in Table 1. There were no significant differences between these two time points.

**DISCUSSION**

The role of IL-4 in lung fibrosis is presently unclear. In murine and human fibroblast lines, IL-4 was shown to enhance collagen gene expression (11), collagen synthesis (8, 34, 41), fibroblast proliferation (26), and chemotaxis (35). The administration of neutralizing anti-IL-4 antibodies reduced dermal collagen content in an experimental model of scleroderma (29) and decreased hepatic collagen deposition in mice infected with *S. mansoni* (5). In vivo, Bleo caused lung IL-4 mRNA levels to increase in CBA/J mice (10), and in our laboratory, there was increased IL-4-induced proliferation of peribronchial T lymphocytes in Bleo-treated hamsters (23). Together, these data provide support for IL-4 being a profibrotic agent. In contrast, a possible protective role for IL-4 is suggested by a study from our laboratory, in which Bleo caused diminished IL-4 production in lung interstitial cells of “Bleo-sensitive” C57BL/6 mice and increased IL-4 production in “Bleo-resistant” BALB/c mice (14). Diminished IL-4 production by lung lymphocytes in Bleo-treated C57BL/6 mice was also confirmed by Sharma et al. (43). To further

**Table 1. Fibrotic parameters in bleomycin-treated IL-4 transgenic mice at 14 and 21 days after intratracheal instillation**

<table>
<thead>
<tr>
<th></th>
<th>Hydroxyproline, nmol/lung</th>
<th>Fibrosis Fraction, %</th>
<th>Alveolar Wall Area Fraction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 days (n = 5)</td>
<td>905 ± 33</td>
<td>9.2 ± 1.3</td>
<td>34.5 ± 1.0</td>
</tr>
<tr>
<td>21 days (n = 3)</td>
<td>1,240 ± 68.4</td>
<td>10.4 ± 5.6</td>
<td>39.1 ± 1.9</td>
</tr>
</tbody>
</table>

Values are means ± SE.
clarify the role of IL-4 in lung fibrosis, we evaluated Bleo-induced lung fibrosis in IL-4 KO mice and TG IL-4-overexpressing mice.

The increased number of BAL lymphocytes in all three groups of Bleo-treated mice on day 14 (Fig. 2) confirmed the presence of Bleo-induced injury. Data on parameters of fibrosis, as assessed biochemically by hydroxyproline lung content and histopathologically by the QIA parameters of fibrosis fraction and alveolar wall area fraction, show subtle differences between the groups. By comparing the severity of the Bleo-induced lung fibrosis among the groups (Fig. 4), we show that a clear trend in the severity of Bleo-induced fibrosis was inversely related to IL-4 expression. These findings negate a key profibrotic role for IL-4 in this model. Moreover, in IL-4 TG mice, in contrast to IL-4 KO and WT mice, there was no Bleo-induced lung fibrosis (Figs. 3, 4), suggesting a possible antifibrotic effect of IL-4 in this model. The magnitude of this antifibrotic effect is probably only moderate, since the differences between groups were statistically significant only at the extremes of IL-4 expression, when IL-4 TG were compared with IL-4 KO mice.

The discrepancy between the in vitro effect on collagen production and in vivo effects on fibrosis is as for TNF. In vitro, TNF-α downregulates collagen production (6), whereas no fibrosis is detected in TNF receptor KO mice (30).

The stimulatory effects of IL-4 on fibroblasts may be counteracted by the effects of IL-4 on other cells. IL-4 may ameliorate lung fibrosis since it regulates and may modulate the expression of inflammatory cytokines such as TNF-α, IL-1α, IL-1β, IL-1 receptor antagonist (RA), and IFN-γ, which are involved in Bleo-induced lung fibrosis (18). TNF-α expression is enhanced in lungs of Bleo-treated mice (30), with almost no fibrosis detectable in Bleo-treated double TNF receptor KO mice (30). Administration of anti-TNF-α antibodies suppressed Bleo-induced TGF-β mRNA expression and subsequent lung fibrosis (50). Thus IL-4 may act as a protective cytokine since it has been shown to downregulate TNF-gene expression (7), production (16), and TNF receptors (15).

Another protective role for IL-4 could be through IL-1. Bleo treatment enhances IL-1 synthesis in vitro (38) and in rodents (25), thereby increasing both the growth of fibroblasts and their collagen secretion rate (1, 39). Furthermore, administration of IL-1RA, which completely blocks the activity of IL-1, suppresses collagen deposition in Bleo-treated mice (33). IL-4 may act protectively since it has been shown to downregulate IL-1 and upregulate IL-1RA expression (49).

IL-4 has been shown to downregulate IFN-γ production by activated T cells in vitro (31) but, in contrast, to enhance IFN-γ secretion by stimulated rat splenocytes (27). IFN-γ inhibits fibroblast proliferation and collagen synthesis in vitro (19, 37, 45), and exogenous administration of recombinant IFN-γ to Bleo-treated rodents causes significant reduction in lung collagen accumulation (12, 17, 28).

IL-4 may also enhance IFN-γ production through its effect on dendritic cells (DC). DC are antigen-presenting cells that specialize in the initiation of T-cell responses and are classified in two subsets, DC1 and DC2, that induce Th1 and Th2 differentiation, respectively. IL-4 kills DC2 cells but enhances DC1 maturation (36). Thus IL-4 plays a role in a negative feedback loop by which DC regulate the Th1/Th2 balance. DC are recruited to the lung of Bleo-injured mice (46). Hence, IL-4-induced upregulation of DC1 maturation might promote Th1 development and, subsequently, enhance IFN-γ production, which in turn protects against Bleo-induced lung fibrosis.

Two major populations of functionally distinct murine lung fibroblasts (Thy 1+ and Thy 1−) have been described (32). Stimulation with recombinant IL-4 induced a twofold increase in total collagen only in Thy 1+ fibroblasts (41), whereas type I and III collagen mRNA levels were increased only in Thy 1+ fibroblasts but not in the Thy 1− subset. These results clearly identify IL-4 stimulation of collagen production as a distinct point of heterogeneity between these two fibroblast subsets. This also supports the hypothesis that selective expansion of a functionally distinct fibroblast subset occurs in vivo and may be important in the process of chronic fibrosis. Expansion of the "IL-4-resistant" Thy 1− subset could theoretically explain the lack of Bleo-induced lung fibrosis observed in IL-4 TG mice. Further investigations are needed to determine whether the Thy 1− subset is preferentially activated in this model.

In summary, our data demonstrate that in vivo overexpression or deficiency of IL-4 is not critical and negates a key profibrotic role for IL-4 in the murine model of Bleo-induced lung fibrosis.

This study was supported in part by the Nathan Shainberg Fund, the Cha and, and Louise Katz Boston University School of Medicine, The Hebrew University-Hadassah Medical School Program, the Israel Lung Association Tel Aviv, and by National Heart, Lung, and Blood Institute Grants P50-HL-56386 and R01-HL-66547. G. Izbicki is a recipient of Swiss National Science Foundation grants (fellowship no. 81GE-050068) and Swiss Foundation for Medical and Biological grants.

REFERENCES


33. Piguet PF, Vesin C, Grau GE, and Thompson RC. Interleukin 1 receptor antagonist (IL-1 Ra) prevents or cures pulmonary fibrosis elicited in mice by bleomycin or silica. *Cytokeine* 5: 57–61, 1993.


44. Sharma SK, MacLean JA, Pinto C, and Kradin RL. The effect of an anti-CD3 monoclonal antibody on bleomycin-induced L1115


