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Therapeutic Attenuation of Pulmonary Fibrosis Via Targeting of IL-4- and IL-13-Responsive Cells

Claudia Jakubzick,* Esther S. Choi,* Bharat H. Joshi,‡ Michael P. Keane,* Steven L. Kunkel,* Raj K. Puri,‡ and Cory M. Hogaboam²*

Severe forms of idiopathic interstitial pneumonia (IIP), such as usual interstitial pneumonia, can be impervious to modern steroid and immunosuppressive treatment regimens, thereby emphasizing the need for novel effective therapies. Consequently, research attention has been directed toward understanding the cytokine networks that may affect fibroblast activation and, hence, the progression of certain IIPs. This led us to investigate whether the specific targeting of resident lung cells responsive to IL-4 and IL-13 exerted a therapeutic effect in an experimental model of IIP, namely the bleomycin-induced model of pulmonary fibrosis. IL-4, IL-13, and their corresponding receptor subunits, IL-4Rα, IL-13Rα1, and IL-13Rα2, were maximally expressed at the mRNA and protein levels in whole lung samples on day 21 or 28 after an intratracheal bleomycin challenge. The intranasal administration of an IL-13 immunotoxin chimeric molecule (IL13-PE) from days 21–28, but not for 1-wk periods at earlier times, after bleomycin challenge had a significant therapeutic effect on histological and biochemical parameters of bleomycin-induced pulmonary fibrosis compared with the control group. The intranasal IL13-PE therapy significantly reduced the numbers of IL-4 and IL-13 receptor-positive mononuclear cells and macrophages and the levels of profibrotic cytokine and chemokine in the lungs of bleomycin-challenged mice on day 28. Thus, this study demonstrates that IL-4- and/or IL-13-binding cells are required for the maintenance of pulmonary fibrosis induced by bleomycin and highlights the importance of further investigation of antifibrotic therapeutics that target these cells during pulmonary fibrosis. The Journal of Immunology, 2003, 171: 2684–2693.

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ince the overall cytokine pattern in biopsies and alveolar macrophages from patients with usual interstitial pneumon
ia (UIP) appears to be more Th2-type (i.e., IL-4 and IL-13) than Th1-type (i.e., IL-12 and IFN-γ) (1–4), a highly anticipated antiﬁbrotic strategy within the lung entails the targeted inhibition of both IL-4 and IL-13. Although transgenic overexpression of IL-13 alone in the lung leads to the development of pulmonary fibrosis (5, 6), both IL-4 and IL-13 appear to contribute to the development of pulmonary fibrosis (7, 8), presumably due to their ability to act directly on pulmonary fibroblasts (9) and mononuclear cells/macrophages (10). IL-4 has been shown to be a potent inducer of TGF-B1 production by pulmonary fibroblasts, leading to increased extracellular matrix synthesis (11, 12), and it has also been shown to be a potent chemotactic factor for the directed movement of pulmonary fibroblasts (13). IL-13 has recently joined the ranks of profibrotic cytokines in the lung (5, 14–16) and liver (17, 18). The profibrotic effect of IL-13 in the lung is postulated to involve irreversible fibroblast activation, triggered either directly (19) or indirectly through TGF-β (6, 21). Direct effects of IL-4 and IL-13 on monocytes and macrophages include enhanced generation of chemokines such as CCL6, CCL17, and CCL22, and augmented responsiveness by CXCR1 and CXCR2 to chemokine ligands such as CXCL8 (reviewed in Ref. 22).

IL-4 activates its cellular target via the membrane-associated version of IL-4Rα, whereas IL-13 specifically binds the IL-13Rα1-chain (23). IL-4Rα and IL-13Rα1 form a functional receptor complex that binds both ligands (24, 25). IL-13, but not IL-4 (26), also binds with 100-fold higher affinity for IL-13Rα2 than IL-13Rα1, but the IL-13Rα2-chain does not appear to signal and may actually function as an in vivo inhibitor of IL-13 function (27). Functional IL-4R and IL-13R are expressed on a variety of immune and nonimmune cells, including B cells, NK cells, monocytes, mast cells, endothelial cells, and fibroblasts (9, 19, 23, 28–30).

A therapeutic strategy for specifically targeting the profibrotic activities of both IL-4 and IL-13 in the lung involves a fusion protein comprised of human IL-13, which binds to mouse receptors and a mutated form of Pseudomonas exotoxin (IL-13-PE38QQR or IL-13-PE) (31). IL-13-PE was initially developed to selectively target and kill tumor cells with abnormal responses to IL-13 due to markedly up-regulated expression of IL-4R and IL-13R (31, 32). More recently, we demonstrated that the intranasal delivery of IL13-PE significantly reduced Aspergillus fumigatus-induced peribronchial (16, 33) and Schistosoma mansoni-induced granulomatous (34) fibrosis in vivo through its reduction in the number of IL-4- and IL-13-responsive cells in the lung.

The intratracheal (i.t.) administration of bleomycin causes alveolar interstitial inflammation that precedes an exuberant and inappropriate tissue repair response in the lung (35, 36). Previous

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3 Abbreviations used in this paper: UIP, usual interstitial pneumonia; BAL, bronchoalveolar lavage; HSA, human serum albumin; IPI, idiopathic interstitial pneumon
ia; IL13-PE, IL-13 immunotoxin chimeric molecule; i.t., intratracheal; KC, kerati
ocyte-derived chemokine; MIP-1α, macrophage inflammatory protein-1α; SPP, specific pathogen free.
studies have documented that IL-4 and IL-13 are elevated during the pulmonary response to an intrapulmonary bleomycin sulfate challenge (10, 37), and IL-4 contributes to the chronic fibrotic response to this chemotherapeutic agent (38), but the time course of the expression of receptor subunits that bind these cytokines has not been previously reported. Hence, we examined the effects of intranasal IL-13-PE administration in a bleomycin-induced interstitial pulmonary fibrosis model (35) in which several experimental therapies for pulmonary fibrosis have been tested (39).

Materials and Methods

Bleomycin model

Interstitial pulmonary fibrosis was induced in specific pathogen-free (SPF) female, C57Bl/6 mice (6–8 wk old, The Jackson Laboratory, Bar Harbor, ME) by the i.t. injection of 0.003 U of bleomycin (Biowest, sterile bleomycin sulfate; Bristol-Meyers Pharmaceuticals, Evansville, IN; 0.15 U/kg of mouse body weight) dissolved in 60 μl of PBS as previously described in detail (35, 36). Controls received 60 μl of PBS by the same route. All procedures were conducted in a sterile environment and were approved by the institutional animal care and use committee.

Experimental protocols

Characterization of IL-4, IL-13, IL-4R, and IL-13R expression following bleomycin challenge in mice. The first experiment undertaken in this study addressed the temporal expression of IL-4, IL-13, and their corresponding receptor subunits, IL-4Rα, IL-13Rα1, and IL-13Rα2, during the development of bleomycin-induced pulmonary fibrosis. Mice were i.t. challenged with saline or bleomycin, and 10 mice in each group were euthanized by anesthetic overdose on days 7, 14, and 21 after the i.t. challenge. Unchallenged lungs were also collected from a group of 10 mice, and these samples were referred to as the day 0 point. Lung samples were processed as described below.

Intranasal IL-13-PE therapy after bleomycin challenge in mice. Groups of 10–15 mice i.t. challenged with saline or bleomycin received 1000 ng/dose of IL-13-PE dissolved in 10 μl of PBS containing 0.25% human serum albumin (PBS-HSA) via intranasal instillation starting on day 7, 14, or 21 and continuing every other day until day 14, 21, or 28, respectively, after the i.t. bleomycin challenge. Other groups of saline- or bleomycin-challenged mice received PBS-HSA alone over the same time period. All groups of mice were euthanized on days 14, 21, and 28 by anesthetic overdose, and whole lung samples from each mouse were processed for molecular, biochemical, and histological analysis (see below).

Preparation of RNA and cDNA, and RT-PCR amplification

Total RNA was prepared from dissected whole lung samples, and RNA was isolated using TRIzol reagent according to the manufacturer’s directions (Life Technologies). The purified RNA was subsequently reverse transcribed into cDNA using a BRL reverse transcription kit (Life Technologies) and oligo(dT)12–18 primers. The amplification buffer contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2.5 mM MgCl2. Specific oligonucleotide primers were added (200 ng/sample) to the buffer along with 1 μl of reverse transcribed cDNA sample. The following murine oligonucleotide primers (5′–3′ sequences) were used for RT-PCR analysis: IL-4Rα sense, GAGTGGAGTGGGAGCTCTAGCATC; IL-4Rα antisense, GCTGAAGTAAGTTCCTCCAGG; IL-13Rα1 sense, GAAATTGTGGCCTCTCTGTCGAA; IL-13Rα1 antisense, GGTATGCGCAAATGCACTTGAG; IL-13Rα2 sense, ATGGCTCTTGTGCTCATAAGT; IL-13Rα2 antisense, GAGGTTGTGCTCATTACCTT; procollagen I sense, TCGTGACCGTGACCTTGCG; procollagen I antisense, GGTTATGCCAAATGCACTTGAG; IL-13Rα sense, GAATTTGAGCGTCTCTGTCGAA; and procollagen III antisense, TTCGCGCGCTGCCTTGCCTTCT.

These mixtures were then first incubated for 4 min at 94°C and amplified using the following cycling parameters: IL-4Rα, procollagen I, and procollagen III: cycled 38 times at 94°C for 30 s, 55°C for 45 s, and 66°C for 60 s, and elongated at 72°C for 60 s. IL-13Rα1 and IL-13Rα2: cycled 38 times at 94°C for 30 s and 66°C for 60 s, and elongated at 72°C for 60 s. After amplification the samples were separated on a 2% agarose gel containing 0.3 μg/ml of ethidium bromide, and bands were visualized and photographed using a transilluminant UV source.

Real-time TaqMan PCR analysis

IL-4 and IL-13 gene expression was analyzed by a real-time quantitative RT-PCR procedure using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The cDNAs from whole lung samples before and at various times after bleomycin challenge were analyzed for IL-4, IL-13, and GAPDH (an internal control). All primers and probes used were purchased from PE Applied Biosystems. Cytokine gene expression was normalized to GAPDH, and the fold increases in IL-4 and IL-13 gene expression were calculated via the comparison of gene expression in all bleomycin-challenged lungs with that in unchallenged lungs. Cytokine gene expression in the latter group of lungs was given an arbitrary value of 1.

Whole lung histology

Following anesthesia-induced euthanasia, whole lungs from bleomycin-challenged mice were fully inflated with 10% formalin, dissected, and placed in fresh formalin for 24 h. Routine histological techniques were used to paraffin-embed the entire lung, and 5-μm sections of whole lung were stained with H&E. Each whole lung from the PBS-HSA and IL-13-PE treatment groups was examined for the presence of inflammatory infiltrates and interstitial pulmonary fibrosis on day 28 after bleomycin challenge and graded using a previously described scale (40).

Immunohistochemistry

Paraffin-embedded whole lung samples were analyzed for immunohistochemical localization of IL-4, IL-13, IL-4Rα, and IL-13Rα2. All Abs were obtained from R&D Systems (Minneapolis, MN). We used an immunohistochemical approach for the detection of IL-4 and IL-13 in whole lung samples because of a growing concern that the ELISAs used for measuring these ligands underestimate the actual levels of both in whole lung samples due to the presence of soluble IL-4Rα (30) and/or IL-13Rα2 (41, 42). Five-micron histological sections were dewaxed with xylene, rehydrated in graded concentrations of ethanol, and blocked with normal rabbit serum (Vectorstain ABC-AP kit; Vector Laboratories, Burlingame, CA). A solution containing a 1/1 ratio of water to methanol with 3% hydrogen peroxide was added to each slide to be stained for the presence of IL-4 and IL-13. Goat anti-mouse IL-4, IL-13, IL-4Rα, and IL-13Rα2 Abs and control normal goat IgG were diluted in PBS to a final concentration of 2 μg/ml. Specific Abs or IgG were added to histological sections for 60 min, after which each tissue section was washed thoroughly three times with PBS. A secondary rabbit anti-goat biotinylated Ab (Vector Laboratories) was added to each section for 60 min, then each slide was thoroughly washed and to each was added alkaline phosphatase (Vector Laboratories) conjugated to avidin. Receptor localization was revealed with an alkaline substrate, 5-bromo-4-chloro-3-indolyphosphate/nitro blue tetrazolium. Coverslips were applied to each slide using an aqueous mounting solution.

Hydroxyproline assay

Total lung hydroxyproline levels were determined in groups of five PBS-HSA- or IL13-PE-treated mice on day 28 after i.t. bleomycin or saline challenge using a previously described assay (43). Briefly, a 500-μl sample of lung homogenate prepared for ELISA analysis (see above) was added to 1 ml of 6 N HCl for 8 h at 120°C. To a 5-μl sample of the digested lung, 5 μl of citrate/acetate buffer (5% citric acid, 7.2% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid, pH 6.0) and 100 μl of chloramine-T solution (282 mg of chloramine-T, 2 ml of distilled water, and 16 ml of citrate/acetate buffer) were added. The resulting samples was then incubated at room temperature for 20 min before 100 μl of Ehrlich’s solution (Aldrich, Milwaukee, WI), 9.3 ml of n-propanol, and 3.9 ml of 70% perchloric acid were added. These samples were incubated for 15 min at 65°C, and cooled samples were read at 550 nm in a DU 640 spectrophotometer (Beckman, Fullerton, CA). Hydroxyproline concentrations were calculated from a hydroxyproline standard curve (0–100 μg of hydroxyproline/ml).

ELISA analysis

Murine IFN-γ, IL-12, TNF-α, IL-5, keratinocyte-derived chemokine (KC), macrophage inflammatory protein-1α (MIP-1α/CCL3), and RANTES/CCL5 levels in 50-μl whole lung samples from PBS-HSA- or IL13-PE-treated mice on day 28 after bleomycin challenge were measured using a standardized sandwich ELISA technique as previously described in detail (44). These samples were prepared from whole lung samples homogenized in 2.0 ml of PBS immediately before ELISA. Bronchoalveolar lavage (BAL) fluids from the PBS-HSA and IL-13-PE groups that received i.t. bleomycin 28 days previously were also screened for murine CCL6 and MIP-2. Each ELISA was screened to ensure Ab specificity, and recombinant murine cytokines and chemokines were used to generate the standard curves from which the concentrations present in the samples were derived. The limit of ELISA detection for each cytokine was consistently >50.
pg/ml. The cytokine levels in each sample were normalized to total protein levels measured using the Bradford assay.

**Lung cell cytometric analysis**

Whole lungs of control mice were aseptically removed from PBS-HSA and IL13-PE treatment groups on day 28 after bleomycin challenge and processed as previously described in detail (45). Briefly, lung tissues were pooled, mechanically macerated, constantly agitated in 0.2% (w/v) collagenase (type IV) in RPMI with 10% FBS for 60 min at 37°C, and finally washed in a balanced salt solution to remove aggregates. Lung cell suspensions were treated with ammonium chloride for RBC lysis. The cells were counted with a hemocytometer, and ~5 × 10^6 cells were transferred to 5-ml polypropylene tubes (Sarstedt, Newton, NC) and fixed in 2% paraformaldehyde for 30 min. Subsequently, Fc binding was blocked via a 10-min incubation with anti-murine CD16/CD32 (FcγR III/II, BD PharMingen, San Diego, CA), and separate aliquots of cells were stained with anti-murine Abs directed against CD4, CD8a, CD19, F4/80, pan-NK, or the appropriate IgG isotype control (all purchased from BD PharMingen) for 30 min at 4°C in PBS supplemented with 5% FCS. Each cell population was analyzed by flow cytometry using an EPICS XL (Beckman Coulter, Miami, FL). The isotype control was used to gate the population of interest and as the negative control. The total population of each cell type was corrected against the control, and total cell counts were calculated.

**Statistical analysis**

All results are expressed as the mean ± SEM. A one-way ANOVA and Dunnett’s multiple comparisons test or Student’s t test were used to detect statistical differences between the saline and bleomycin groups and between the control and treatment groups; p < 0.05 was considered statistically significant.

**Results**

**Temporal changes in IL-4 and IL-13 transcript and protein expression during bleomycin-induced fibrosis**

Quantitative TaqMan PCR analysis of whole lung samples showed that IL-4 and IL-13 gene expression was increased at all times after bleomycin challenge compared with transcript levels of both cytokines in whole lung samples from unchallenged mice (Fig. 1). The greatest increase in transcript expression for both ligands was observed on day 28 after bleomycin treatment. Temporal-dependent changes in protein levels of IL-4 and IL-13 were observed in formalin-fixed whole lung samples from bleomycin-challenged mice (Fig. 2). Although neither ligand was detected in whole lung samples immediately before bleomycin challenge (i.e., day 0), immunohistochemical analysis of IL-4 and IL-13 expression in whole lung samples revealed that the level of expression for both Th2-type cytokines was present at all times examined after the bleomycin challenge. Importantly, the expression of both ligands appeared to be greatest on day 28 after the bleomycin challenge (Fig. 2, K and L). On day 28 after bleomycin, most of the expression of both cytokines was associated with mononuclear cells and macrophages present in the lung samples. Coincident with previous studies (36, 46), ELISA revealed that immunoreactive levels of IFN-γ and IL-12 in whole lung samples peaked on day 7 after bleomycin challenge, and both were significantly elevated above the whole lung Th1-type cytokine levels measured before bleomycin challenge (day 0; Table I).

**Gene expression for IL-4R and IL-13R subunits and procollagen 1 and 3 was increased in a temporally dependent manner in the lungs of bleomycin-challenged mice**

RT-PCR analysis of whole lung samples showed that IL-4Rα gene expression was present on days 21 and 28, but not at other times after bleomycin challenge (Fig. 3). IL-13Rα1 gene expression was present before (day 0) and at all times after bleomycin challenge. The greatest expression of IL-13Rα1 was observed on day 28 after bleomycin treatment (Fig. 3). IL-13Rα2 gene expression was only observed on day 28 after bleomycin challenge (see below). RT-PCR analysis also revealed a temporally dependent increase in procollagen 1 (47) and procollagen 3 (48) gene expression in this model of bleomycin challenge.

**Temporal changes in IL-4Rα and IL-13Rα2 protein expression during bleomycin-induced pulmonary fibrosis**

Immunohistochemical analysis of formalin-fixed whole lung samples from bleomycin-challenged mice showed a marked temporally dependent increase in IL-4Rα and IL-13Rα2 protein expression (Fig. 4, A–L). While an Ab for immunohistochemical analysis of murine IL-13Rα1 expression is not presently available, IL-4Rα protein expression was observed at all times examined after bleomycin challenge (Fig. 4, B, E, H, and K), and peak expression of IL-4Rα in activated mononuclear cells and in interstitial areas of the lung was observed on day 21 after bleomycin challenge (Fig. 4H). No IL-4Rα protein expression was detected in whole lung samples from CBA/J mice immediately before i.t. challenge (i.e., day 0). Although no IL-13Rα2 protein expression was observed on day 0 or 7, mononuclear cells weakly expressed IL-13Rα2 on day 14 after bleomycin challenge. Intense IL-13Rα2 protein expression was observed in activated mononuclear cells, airway epithelial cells, and fibrotic interstitial areas on days 21 (Fig. 4I) and 28 (Fig. 4L) in this bleomycin model. Peak IL-13Rα2 expression was present on day 28 after bleomycin treatment (Fig. 4L).

**Intranasal administration of IL13-PE significantly attenuated bleomycin-induced pulmonary fibrosis**

The objective of this experiment was to determine whether intranasal IL13-PE therapy could prevent and/or attenuate the interstitial fibrotic response elicited by an i.t. bleomycin challenge. The timing of IL13-PE therapy in this experiment corresponded to the appearance of IL-4, IL-13, IL-4R, and IL-13R levels in whole lung samples from bleomycin-challenged mice. Accordingly, 1 μg of IL13-PE (34) suspended in PBS-HSA was administered by intranasal bolus to bleomycin-challenged mice beginning on day 7, 14, or 21 after bleomycin treatment. Control groups of mice received PBS-HSA in the same manner at the same times after bleomycin treatment. A total of four IL13-PE or PBS-HSA intranasal bolus treatments were administered every other day to each group of mice, which were subsequently examined on days 14, 21, and 28 after bleomycin. On day 7 after bleomycin treatment, focal areas of
intense inflammation were observed (not shown), and a mixed inflammatory and fibrotic histological profile in the lungs was observed on days 14 and 21 after bleomycin treatment (Fig. 5, A and C). On day 28, the lungs of bleomycin-challenged mice were predominately fibrotic, with few inflammatory cells (Fig. 5E). Intranasal IL13-PE treatment from days 7–14 or from days 14–21 did not attenuate the inflammatory or fibrotic features in the lungs of bleomycin-challenged mice on days 14 and 21 (Fig. 5, B and D). However, a marked improvement in lung histology was observed in bleomycin-challenged mice that received intranasal IL13-PE from days 21–28 (day 28 histology shown in Fig. 5F). Blinded assessment of histological injury in whole lung sections from each group of mice on days 14, 21, and 28 after the i.t. bleomycin challenge showed that only the intranasal administration of IL13-PE from days 21–28 significantly attenuated the lung injury and fibrosis evoked in bleomycin-challenged mice (Fig. 5G). In addition, intranasal IL13-PE administration did not cause histologically evident lung injury in saline-challenged mice on days 14, 21, and 28 (not shown).

Intranasal administration of IL13-PE significantly reduced whole lung levels of proinflammatory cytokines and chemokines in bleomycin-challenged mice

Having observed that the intranasal IL13-PE therapy from days 21–28 after bleomycin challenge markedly reduced the amount of lung injury and fibrosis, we next examined the effect of this therapy on the elaboration of inflammatory mediators in the lung. In these experiments, groups of 10 mice received saline or bleomycin by an i.t. injection, and beginning on day 21, groups of five mice received either PBS-HSA or IL13-PE by intranasal bolus. A total of four IL13-PE or PBS-HSA intranasal bolus treatments were administered every other day to each group of mice, which were subsequently examined on day 28 after bleomycin or saline. Biochemical analysis of whole lung samples from saline- and bleomycin-challenged mice showed that hydroxyproline (a modified amino acid uniquely present in collagen (49)) was significantly decreased by the intranasal IL13-PE therapy in both groups of mice, but the greatest reduction was observed in the bleomycin-challenged group (Fig. 6A). However, IL13-PE therapy did not

Table 1. Temporal changes in immunoreactive levels of IFN-γ and IL-12 in whole lung homogenates at various times after i.t. bleomycin (0.15 U/kg) challenge in female CBA/J mice

<table>
<thead>
<tr>
<th>Time after Bleomycin (days)</th>
<th>IFN-γ (pg/mg protein)</th>
<th>IL-12 (pg/mg protein)</th>
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<tbody>
<tr>
<td>0</td>
<td>57 ± 8</td>
<td>45 ± 7</td>
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<tr>
<td>7</td>
<td>156 ± 23b</td>
<td>104 ± 7b</td>
</tr>
<tr>
<td>14</td>
<td>43 ± 6</td>
<td>61 ± 3</td>
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<tr>
<td>21</td>
<td>62 ± 7</td>
<td>53 ± 1</td>
</tr>
<tr>
<td>28</td>
<td>49 ± 3</td>
<td>54 ± 6</td>
</tr>
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</table>

*All cytokines were measured using specific ELISAs, and data are shown as the mean ± SEM from 5–10 mice at each time point after i.t. bleomycin challenge.

*b p < 0.001 vs time zero.
affect immunoreactive levels of bioactive TGF-β on day 28 (saline plus PBS-HSA, 2.3 ± 0.1 ng/mg protein; saline plus IL13-PE, 2.4 ± 0.2 ng/mg protein; bleomycin plus PBS-HSA, 6.7 ± 1.6 ng/mg protein; bleomycin plus IL13-PE, 6.2 ± 0.2 ng/mg protein). In addition, no changes in whole lung levels of immunoreactive IL-4, IL-13, IFN-γ, and IL-12 were observed in saline- and bleomycin-challenged mice that received IL13-PE compared with those in the appropriate group that received PBS-HSA (not shown). In contrast, ELISA of whole lung levels of TNF-α and IL-5 on day 28 showed that levels of these potent profibrotic cytokines (50) were significantly inhibited by the intranasal IL13-PE therapy in the bleomycin-challenged (Fig. 6B), but not the saline-challenged (not shown), mice. Furthermore, ELISA of KC (35), CCL3 (51, 52), and CCL5 (53) showed that the intranasal IL13-PE therapy from days 21–28 significantly reduced whole lung levels of these proinflammatory and profibrotic chemokines in bleomycin-challenged (Fig. 6C), but not saline-challenged (not shown), mice.

**Intranasal IL13-PE therapy targeted IL-4Rα and IL-13Rα2-positive lung cells**

IL13-PE has been shown to target cancer cells (32) and pulmonary fibroblasts (34) that exhibit increased expression of IL-13Rα2, a high affinity IL-13R that in its monomeric form appears to be a decoy receptor (54). RT-PCR analysis of whole lung samples from saline and bleomycin i.t. challenged groups on day 28 revealed that the IL13-PE therapy reduced, but did not abolish, the gene expression of IL-4Rα and IL-13Rα1 compared with that in the PBS-HSA control groups (Fig. 7). Interestingly, the gene expression for IL-13Rα2 was increased in two of three whole lung samples in the bleomycin group receiving IL13-PE therapy relative to that in the PBS-HSA control in which two of three lungs exhibited this IL-13R subunit.

Immunohistochemical analyses confirmed that IL-4Rα and IL-13Rα2 protein expression was markedly diminished in mononuclear cells and in interstitial areas of the lung in the bleomycin

**FIGURE 4.** Immunohistochemical localization of IL-4Rα (B, E, H, and K) and IL-13Rα2 (C, F, I, and L) in whole lung sections on days 7 (A–C), 14 (D–F), 21 (G–I), and 28 (J–L). Staining of the specific IL-4 and IL-13 receptor subunits appears purple in these tissue sections. The appropriate negative staining control sections for each time point after bleomycin challenge are depicted in A, D, G, and J. Magnification, ×400.
group that received the intranasal IL13-PE therapy (Fig. 8, A–F). BAL morphometric analysis confirmed that the numbers of mononuclear cells were similar in the lungs of the PBS-HSA- and IL13-PE-treated bleomycin groups (not shown). However, examination of the mononuclear cells present in the BAL samples revealed that none of these cells was in an activated state in IL13-PE-treated mice, which contrasted sharply with the highly activated "foamy" appearance of mononuclear cells in PBS-HSA-treated mice.

Further evidence that monocytes and macrophages were less activated in IL13-PE-treated mice on day 28 after bleomycin was observed in an ELISA analysis of BAL levels for the presence of two monocyte/macrophage-derived chemokines, specifically CCL6 (PBS-HSA, 19.8 ± 2.1 ng/mg protein; IL13-PE, 13.0 ± 2.0 ng/mg protein) and MIP-2 (PBS-HSA, 7.4 ± 0.5 ng/mg protein; IL13-PE, 2.6 ± 0.9 ng/mg protein) (35). Both profibrotic chemokines were significantly reduced by the intranasal IL13-PE treatment.

Flow cytometric analysis of whole lung cell populations after IL13-PE treatment on day 28 after bleomycin

Flow cytometric analysis of CD4-, CD8a-, CD19-, F4/80-, and pan-NK-positive cells in dispersed whole lungs from normal (SPF) and bleomycin-challenged mice is summarized in Fig. 9. Firstly, it was observed that the i.t. instillation of bleomycin 28 days previously caused a marked reduction in the percentage of lung cells expressing CD4 and CD8 cells compared with lung cell populations in normal, SPF mice. Secondly, the i.t. bleomycin challenge promoted a marked increase in the percentage of CD19-, F4/80-, and pan-NK-positive lung cells compared with lung cell populations in normal, SPF mice. Thirdly, it was noted that the intranasal IL13-PE therapy from days 21–28 after bleomycin challenge reduced the percentage of F4/80 and NK cells in the lung compared with that in the bleomycin group that received PBS-HSA over the same time period. Fourthly, IL13-PE therapy did not alter the percentage of CD19-positive
cells in dispersed lung samples compared with that in the control bleomycin group. Finally, the percentage of CD4- and CD8-positive cells in the IL-13-PE-treated bleomycin group was higher than that detected in the PBS-HSA-treated bleomycin group and actually mirrored the percentage of these cells detected in the normal, SPF group.

Discussion
Understanding the cellular and molecular mechanisms in the lung that ultimately lead to excessive pulmonary fibrotic conditions such as idiopathic interstitial pneumonia (IIP) has garnered considerable research investigation (55). Pulmonary exposure to the chemotherapeutic drug bleomycin leads to pulmonary fibrosis in humans and has been widely used in animals to model severe forms of IIP, such as UIP (39). Although it has been previously shown that IL-4 (37) and IL-13 (10) transcript and/or protein levels are increased following bleomycin challenge in mice, the temporal expression of IL-4R and IL-13R subunits during experimental bleomycin-induced pulmonary fibrosis has not been previously reported. Therefore, the present study addressed two major aims: 1) to examine the expression of IL-4R and IL-13R subunits during the development of bleomycin-induced interstitial fibrosis, and 2) to determine the role of IL-4- and IL-13-responsive cells in the pulmonary fibrotic response via selective targeting of these cells using a chimeric IL-13 immunotoxin. To date, few therapeutic interventions have been shown to exert an antifibrotic effect when administered over the time period that we examined in this model of interstitial fibrosis. It appears that IL-13-PE therapy was effective at this time because the receptor subunits that bind this chimeric immunotoxin were maximally expressed. Previous studies by Puri and colleagues (56–58) have shown that most notably the expression of IL-13Rα2 subunit confers susceptibility of tumor cells to the cytotoxic effects of IL-13-PE. At present, we suspect that IL-13-PE may be targeting cells in the fibrotic lung solely via this receptor subunit, but further studies are planned using IL-13Rα2-deficient mice (59, 60) to determine whether this is true. Thus, these findings demonstrate that targeting of IL-4- and/or IL-13-responsive cells in the lung effectively interrupted the established fibrotic response induced by an i.t. bleomycin challenge in mice. While IL-4 and IL-13 appear to interact in a divergent manner to mediate protective immunity during helminth infection and deleterious inflammatory responses during allergy (61), the importance of both IL-4 (62) and IL-13 (5) in the fibrotic process in various tissues has been established. Previous molecular and immunohistochemical analysis in whole lung samples has shown that IL-4 expression was augmented in mononuclear cells and macrophages localized to areas of active fibrosis between days 3 and 14 after the induction of bleomycin-induced lung injury, but levels of this cytokine decreased toward the control level after day 21 (37). Using quantitative PCR analysis, we observed a temporally dependent increase in IL-4 and IL-13 transcript expression after bleomycin challenge that peaked on day 28. In addition, IL-4 and IL-13 protein expression was present in areas of active fibrosis in whole lung histological sections, and the expression of both cytokines appeared to be maximal on day 28 after bleomycin challenge. With the immunohistochemical technique, IL-4 and IL-13 protein expression was predominately detected in macrophages and mononuclear cells near or in areas of active fibrosis. This finding was consistent with previous observations that IL-13 may be expressed by normal human alveolar macrophages as part of a homeostatic control process, but its production was increased in the presence of fibrotic lung disease (4). It should be noted that the importance of

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**FIGURE 7.** Effect of IL-13-PE therapy on whole lung gene expression of IL-4Rα, IL-13Rα1, and IL-13Rα2 in saline- and bleomycin-challenged mice that received intranasal PBS-HSA or IL-13-PE treatments from days 21–28 after the i.t. bleomycin challenge.
IL-4 in the fibrotic phase of the bleomycin model remains controversial. Huaux and colleagues (38) recently showed that IL-4 had a profibrotic role in this model, because IL-4-deficient (−/−) mice developed significantly less pulmonary fibrosis relative to IL-4 wild-type mice. However, Izbicki and colleagues (63) also examined IL-4−/− mice and concluded that IL-4 was not a critical profibrotic mediator during bleomycin-induced pulmonary fibrosis. In fact, their data suggest an important immunomodulatory role for IL-4 in this fibrosis model given that IL-4−/− mice exhibit increased, whereas IL-4 transgenic mice exhibit decreased, pulmonary fibrosis (63). Recent studies by Belperio and colleagues (10) demonstrated that IL-13, but not IL-4, was the major profibrotic mediator after bleomycin challenge in mice.

The lungs of bleomycin-challenged mice exhibited IL-4Rα and IL-13Rα2 subunits in interstitial areas containing pulmonary fibroblasts, but the greatest expression of both subunits (determined by immunohistochemical analysis) was clearly observed on mononuclear cells and macrophages, particularly on days 21 and 28, in this model. It is not immediately apparent why the expression of these receptor subunits was delayed in this model of pulmonary fibrosis. However, recent studies by Daines and Hershey (64) show that the IFN-γ mobilizes the intracellular stores of IL-13Rα2 to the cell surface of epithelial cells and monocytes. The bleomycin model of pulmonary fibrosis is characterized by an early (i.e., day 7 after instillation) increase in whole lung levels of IFN-γ and IL-12, and it may be this increase in Th1-type cytokines that subsequently up-regulates the expression of IL-13Rα2 on various cell types in the lung. Interestingly, we observed that IL-13Rα2 gene expression was increased in the lungs of IL-13-PE-treated mice. This finding is not readily explained at present, but we have observed previously that IL-13-PE targeted proliferating fibroblasts cells in a dose-dependent manner, but within the remaining fibroblasts not killed by IL13-PE, there was a dose-dependent increase in mRNA expression for IL-13Rα2 (65). We postulate that IL-13-PE may have a ligand effect in cells (before their death due to the accumulation of the truncated Pseudomonas exotoxin) that is consistent with the ability of IL-13 to induce IL-13Rα2 (59, 65).

Both IL-4 and IL-13 have potent activating effects on monocytes and macrophages that include enhanced cytokine, chemokine, and chemokine receptor expression (22). Resident macrophage and recruited monocyte activation is a well-described feature of clinical pulmonary fibrosis (66–68), and these cells contribute to the pathogenesis of pulmonary fibrosis due to bleomycin (69). Of major significance to the fibrotic response is TGF-β, a multifunctional cytokine involved in controlling critical cellular activities, including proliferation, differentiation, extracellular matrix production, and apoptosis (70). Lee and colleagues (6) recently showed that IL-13-induced fibrosis was ameliorated by treatment with the TGF-β antagonist. In the present studies IL-13-PE treatment did not affect whole lung levels of TGF-β, suggesting that IL-4- and IL-13-responsive cells were not the primary producers of this cytokine. However, IL-13-PE treatment significantly reduced the levels of CCL6, KC, and MIP-2, chemokines that are induced in mononuclear cells and macrophages (22) by IL-4 and IL-13. The whole lung levels of the profibrotic cytokines TNF-α and IL-5 (50, 71, 72) and profibrotic chemokines CCL3 and CCL5 (51, 52, 73) were significantly reduced by IL-13-PE treatment, and this reduction presumably reflects the fact that this chimeric immunotoxin significantly reduced the numbers of important cellular sources of these mediators, such as macrophages and NK cells.

Flow cytometric analysis showed that the intranasal IL-13-PE therapy from days 21–28 markedly reduced the percentage of F4/
FIGURE 9. Flow cytometric analysis of dispersed lung cells on day 28 after bleomycin in groups receiving PBS-HSA (□) or IL13-PE (○) intranasally. Mice received PBS-HSA or IL13-PE from days 21–28 after the i.t. bleomycin challenge. Lung cells from normal, SPF mice (□) were also subjected to flow cytometric analysis. All data represent the percentage of marker-positive cells. Results are representative of whole lung samples from five mice.

80-positive monocytes and NK cells in the lungs of bleomycin-challenged mice to the values observed in normal, SPF mice. The 80-positive monocytes and NK cells in the lungs of bleomycin-challenged mice to the values observed in normal, SPF mice.

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Acknowledgments

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