IL-12 attenuates bleomycin-induced pulmonary fibrosis

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Keane, Michael P., John A. Belperio, Marie D. Burdick, and Robert M. Strieter. IL-12 attenuates bleomycin-induced pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 281:L92–L97, 2001.—Interleukin (IL)–12 is a potent inducer of interferon (IFN)–γ. We postulated that IL-12 would attenuate bleomycin-induced pulmonary fibrosis. To test this hypothesis, we administered IL-12 or murine serum albumin to bleomycin-treated mice by daily intraperitoneal injection until day 12. Mice treated with IL-12 demonstrated decreased hydroxyproline levels compared with control treated mice. Furthermore, administration of IL-12 led to a time-dependent increase in both lung and bronchoalveolar lavage fluid IFN-γ. The antioxidant effect of IL-12 could be attenuated with simultaneous administration of neutralizing anti-IFN-γ antibodies. These findings support the notion that IL-12 attenuates bleomycin-induced pulmonary fibrosis via modulation of IFN-γ production.

Material and methods

IDIOPATHIC PULMONARY FIBROSIS (IPF) is a chronic and often fatal pulmonary disorder with prevalence rates of 27–29 cases per 100,000 (9). The incidence of IPF appears to be on the rise in certain parts of the world (10). Conventional treatment with immunosuppressive therapy has been disappointing, with objective response rates of <30% and a median survival of 5 yr. A recent study suggests that interferon (IFN)–γ may have a beneficial role in the treatment of IPF (43). One of the major inducers of IFN-γ production is interleukin (IL)–12, which is produced by activated macrophages and antigen-presenting cells and acts on NK and T cells (24). IL-12 has been described to have a variety of in vivo activities, including antitumor and antiangiogenic activities (8, 11, 37). These effects appear to be mediated through IFN-γ (8, 22, 37, 41).

Bleomycin sulfate has been used in rodents to initiate fibrotic lung lesions, which have many of the histological components of IPF (1, 4). Bleomycin administration results in a route-, dose-, and strain-dependent pulmonary inflammatory response characterized by increases in leukocyte accumulation, fibroblast proliferation, and collagen content. Although these pathological changes occur in a more rapid fashion than human IPF, the rodent pulmonary inflammatory response to intratracheal bleomycin challenge constitutes a representative model of human IPF. The hallmark of fibrosis is collagen deposition. Because collagen contains significant amounts of hydroxyproline, measurement of hydroxyproline is a good index of fibrosis (18, 19, 25, 26). In this study, we show that IL-12 attenuates bleomycin-induced pulmonary fibrosis. Furthermore, administration of IL-12 leads to a time-dependent increase in intrapulmonary IFN-γ. Moreover, the beneficial effects of IL-12 can be inhibited by simultaneous administration of anti-IFN-γ antibodies. These findings provide further support for IFN-γ as an inhibitor of fibrosis.

MATERIALS AND METHODS

Reagents. Murine IL-12 and IFN-γ were purchased from R&D Systems (Minneapolis, MN). Polyclonal anti-murine IFN-γ antibodies were produced by the immunization of rabbits with murine recombinant IFN-γ (R&D Systems) in multiple intradermal sites with complete Freund’s adjuvant. The specificity of the antibody was assessed by Western blot analysis and ELISA against a panel of other recombinant cytokines. The antibody was specific in our sandwich ELISA without cross-reactivity to a panel of cytokines, including IL-1 receptor antagonist protein, IL-1, IL-2, IL-4, IL-6, tumor necrosis factor–α, interferon-inducible protein-10, monokine induced by IFN-γ, and members of the C-X-C and C-C chemokine families (2, 3). The IFN-γ antibody is a neutralizing antibody as determined by its ability to block IFN-γ activity in an antiviral assay using L-929 cells infected with encephalomyocarditis virus (38). The “anti-protease” buffer for tissue homogenization consisted of 1× PBS with one Complete tablet (Boehringer Mannheim, Indianapolis, IN) per 50 ml. Animal model of pulmonary fibrosis. Female CBA/J mice (6–8 wk) were purchased from Jackson Laboratory (Bar Harbor, ME). We used CBA/J mice because they are a well-characterized inbred strain of mice which are susceptible to bleomycin-induced pulmonary fibrosis. Mice were maintained in specific pathogen-free conditions and provided with food and water ad libitum. To induce pulmonary fibrosis, mice were treated with intratracheal bleomycin (Blenoxane, a gift from Bristol Myers, Evansville, IN; 1 U/kg) on day 0 as previously described (18, 19). Control animals received only sterile saline as previously described (18, 19). Briefly, mice were anesthetized with 250 μl of 12.5 μg/ml ketamine injected intraperitoneally followed by intratracheal instillation of 0.025 U of bleomycin in 25 μl of sterile isotonic saline. Bleomycin-treated mice were given daily intraperitoneal injections of recombinant murine IL-12 [1 μg in 100 μl of 0.25%...
mouse serum albumin (MSA)] or MSA (100 μl of 0.25% MSA) until day 16. Mice were killed on days 2, 4, 8, 12, and 16 for ELISA and on day 12 for hydroxyproline assay. In separate experiments, mice were given daily intraperitoneal injections of IL-12 as described previously; on days 2, 4, 6, and 8, these IL-12-treated mice were given either anti-IFN-γ antibodies or normal rabbit serum by intraperitoneal injection. These mice were killed on day 12 for hydroxyproline measurement.

**Lung tissue preparation.** Bleomycin- or saline-treated lungs were homogenized and sonicated in anti-protease buffer as previously described (3, 18, 19, 31). Specimens were centrifuged at 900 g for 15 min, filtered through 1.2-μm Sterile Acrodiscs (Gelman Sciences, Ann Arbor, MI), and frozen at −70°C until thawed for assay by specific IFN-γ ELISA or Western blot analysis.

**IFN-γ ELISA.** Antigenic murine IFN-γ was quantitated using a modification of an ELISA as previously described (2, 3). The sensitivity of our ELISA is ≈50 pg/ml. Briefly, flat-bottomed 96-well microtiter plates (Nunc) were coated with 50 μl/well of the polyclonal anti-murine IFN-γ antibody (1 ng/μl in 0.6 M NaCl, 0.26 M H3BO4, and 0.08 N NaOH, pH 9.6) for 24 h at 4°C and then washed with PBS and 0.05% Tween 20 (wash buffer). Nonspecific binding sites were blocked with 2% BSA. Plates were rinsed and samples were added (50 μl/well), followed by incubation for 1 h at 37°C. Plates were then washed and 50 μl/well of the appropriate biotinylated polyclonal antibody (3.5 ng/μl in wash buffer and 2% FCS) were added for 45 min at 37°C. Plates were washed three times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) was added, and the plates were incubated for 30 min at 37°C. Chromogen substrate (DAKO, Carpinteria, CA) was then added, and the plates were incubated at room temperature to the desired extinction. Plates were read at 490 nm in an automated microplate reader (Bio-Tek Instruments, Winooski, VT). Standards were 1/2 log dilutions of recombinant IFN-γ from 100 ng to 1 pg/ml (50 μl/well).

**Western blot analysis of IFN-γ.** Western blot analysis was performed as described previously (19, 20). Total protein extracts were made by homogenizing lungs in TNE lysis buffer (20 mM Tris·HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, and 2.5 mM EDTA) supplemented with 2 ng/ml aprotinin and 35 ng/ml phenylmethylsulfonyl fluoride. Cell extracts were incubated on ice for 30 min, followed by centrifugation at 4°C for 30 min. Supernatants were then removed and assayed for total protein content using bicinchoninic acid protein assay reagents (Pierce, Rockford IL) and comparison to known amounts of bovine serum albumin. Total protein (1 μg) was loaded in each well of a 12% polyacrylamide gel, and extracts were subjected to SDS-PAGE. The separated proteins were transferred to polyvinylidene difluoride membrane (Pierce) by electrophoretic transfer overnight in Tris-glycine buffer (20 mM Tris and 150 mM glycine, pH 8.0, methanol added to a final concentration of 20% [vol/vol]). Blots were blocked in 5% skim milk in TBS-T buffer (10 mM Tris·HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) for 2 h at room temperature, followed by incubation in rabbit primary antibody serum against IFN-γ diluted 1:1,000 in blocking solution for 2 h at room temperature. Blots were washed for three 10-min washes in TBS-T and were incubated for 1 h at room temperature in goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules CA) at a 1:10,000 dilution. Blots were again washed for four 10-min washes in TBS-T, and proteins were visualized after incubation of the blots in SuperSignal chemiluminescent substrate solution according to the manufacturer’s protocol (Pierce) and exposure to XAR-5 film (Kodak, Rochester NY).

**Hydroxyproline assay.** Total lung collagen was determined by analysis of hydroxyproline as previously described (18, 19). Briefly, lungs were harvested on day 12 or 16 post-bleomycin administration and homogenized in 1 ml of PBS, pH 7.4, with a Tissue Tearor. One-half milliliter of each sample (both lungs) was then digested in 1 ml of 6 N HCl for 8 h at 120°C. Five microliters of citrate-acetate buffer (5% citric acid, 7.24% 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 n-propanol, 2 ml of H2O, and 16 ml of citrate-acetate buffer) were added to 5 μl of sample, and the samples were left at room temperature for 20 min. Next, 100 μl of Ehrlich’s solution [2.5 g of 4-(dimethylamino)-benzaldehyde (4-DMAB, Aldrich, Milwaukee, WI)], 9.3 ml of n-propanol, and 3.9 ml of 70% perchloric acid (Eastman Kodak) were added to each sample and the samples were incubated for 15 min at 65°C. Samples were cooled for 10 min and read at 550 nm on a Beckman DU 640 spectrophotometer (Fullerton, CA). Hydroxyproline (Sigma Immunochemicals, St. Louis, MO) concentrations from 0 to 400 μg/ml were utilized to construct a standard curve.

**Statistical analysis.** Data were analyzed on a Power Macintosh 7500 computer using the Statview 4.5 statistical package (Abacus Concepts, Berkeley, CA). Two-group comparisons were made using the unpaired t-test. Multiple groups were compared using ANOVA followed by Bonferroni post hoc test where appropriate. Data were considered statistically significant at P ≤ 0.05. All values are means ± SE.

**RESULTS**

**IL-12 attenuates bleomycin-induced pulmonary fibrosis.** We assessed whether administration of exogenous IL-12 by repeated intraperitoneal injection during bleomycin-induced pulmonary fibrosis would attenuate the fibrotic response. We used lung hydroxyproline levels as an index of total lung collagen and fibrosis. Administration of IL-12 on days 1–12 led to reduced total lung hydroxyproline on day 12 compared with control treated mice (Fig. 1).

**IL-12 administration leads to augmented intrapulmonary and bronchoalveolar lavage levels of IFN-γ.** Having shown that administration of IL-12 led to a reduction in bleomycin-induced pulmonary fibrosis, we were next interested to see whether IL-12 augmented intrapulmonary IFN-γ levels. We first measured levels of IFN-γ by specific ELISA in lung tissue from bleomycin-treated mice compared with controls. We found that there was a deficiency of IFN-γ in lung tissue from bleomycin-treated mice compared with controls. We next looked at IFN-γ levels from lung tissue of bleomycin-treated mice who received IL-12. Lungs were harvested on days 2, 4, 6, 8, 12, and 16 and assayed for IFN-γ by specific ELISA. Before lung harvest, bronchoalveolar lavage (BAL) was performed. Administration of IL-12 on days 1–16 led to a time-dependent increase in both intrapulmonary and BAL fluid IFN-γ levels (Fig. 3). This is consistent with the known effects of IL-12 as a potent inducer of IFN-γ from NK and T cells (24).
Antifibrotic effects of IL-12 are reversed by antibodies to IFN-γ. Having shown that IL-12 leads to both a reduction in pulmonary fibrosis and an increase in intrapulmonary IFN-γ, we were next interested in establishing that the antifibrotic effect of IL-12 was mediated through IFN-γ. IL-12-treated mice were given either anti-IFN-γ antibodies or normal rabbit serum (control) on days 2, 4, 6, and 8. We chose these time points based on the temporal increase in IFN-γ that we saw after administration of IL-12. IL-12 failed to attenuate bleomycin-induced pulmonary fibrosis when simultaneous neutralizing anti-IFN-γ antibodies were administered (Fig. 4A). This suggests that the antifibrotic actions of IL-12 are mediated through IFN-γ. This is consistent with previous studies that have demonstrated that IL-12 induces tumor regression and this effect is mediated through IFN-γ (8, 22, 37, 41). Furthermore, administration of anti-IFN-γ antibodies attenuates the IL-12-induced increase in IFN-γ levels, as assessed by Western blot analysis (Fig. 4B). We used Western blot analysis as opposed to an ELISA because the polyclonal anti-IFN-γ antibody used to treat the mice is the same antibody that we use in our ELISA.

DISCUSSION

Traditional approaches to the treatment of pulmonary fibrosis have involved immunosuppressive agents, with corticosteroids being the most commonly used (33). Despite objective response rates of no more than 30%, the standard of care remains corticosteroids either with or without a second immunosuppressive agent (33). Whereas experimental models have traditionally focused on attenuating the inflammatory response (32, 35, 42), more recent approaches have involved attenuation of epithelial injury (21) or the targeting of the transforming growth factor-β pathway and the associated fibrotic response (23).

In the present study, we assessed whether administration of exogenous IL-12 during bleomycin-induced pulmonary fibrosis would attenuate the fibrotic response. Administration of IL-12 on days 1–12 led to reduced total lung hydroxyproline on day 12 compared with control treated mice. Furthermore, we found that there was a relative deficiency of IFN-γ levels in bleomycin-treated lung tissue compared with controls and that administration of IL-12 led to a time-dependent reduction in pulmonary fibrosis.
increase in both intrapulmonary and BAL fluid IFN-γ levels from bleomycin-treated mice. This is consistent with the known effects of IL-12 as a potent inducer of IFN-γ from NK and T cells (24). Although we did not assess the predominant cellular source of IFN-γ in our study, we would speculate that IL-12 is inducing its expression from both NK and T cells.

IL-12 failed to attenuate bleomycin-induced pulmonary fibrosis when simultaneous neutralizing anti-IFN-γ antibodies were administered. This suggests that the antifibrotic actions of IL-12 are mediated through IFN-γ. This is consistent with previous studies that have demonstrated that IL-12 induces tumor regression and that this effect is mediated through IFN-γ (8, 22, 37, 41). Furthermore, the antitumor effects of IL-12 appear to be mediated through the induction of IFN-inducible chemokines, which result in tumor necrosis (17, 30).

IFN-γ is a known inhibitor of wound repair (34) and has been shown to attenuate fibrosis in bleomycin-induced pulmonary fibrosis (14, 15). IFN-γ can inhibit both fibroblast and chondrocyte collagen production in vitro, as well as decrease the expression of steady-state type I and III procollagen mRNA (5–7, 12, 13, 16). A recent study demonstrated a beneficial role for IFN-γ-1b in the treatment of IPF and suggested that this effect was mediated through inhibition of transforming growth factor-β1 and connective tissue growth factor (43). This would suggest that IFN-γ has the potential to inhibit several pathways involved in the fibrotic response. It has been shown that there is a deficiency of IFN-γ production in fibrosing interstitial lung diseases (27, 43). This suggests an underlying deficiency in the natural antifibrotic mechanisms in the lung in IPF. Moreover, in bleomycin-induced pulmonary fibrosis, there is a deficiency of IFN-γ and of IFN-inducible chemokines (18). In this study, we have shown that this deficiency of IFN-γ can be corrected by the administration of IL-12, with subsequent attenuation of fibrosis.

Furthermore, IL-12 has the potential to switch the response from a fibrotic T helper type 2 (Th2) phenotype to a more favorable type 1 (Th1) phenotype (36, 40). The realization that Th1 and Th2 cytokines are expressed by a variety of cells and that the function of these cytokines is different suggests that an imbalance in the expression of Th1 and Th2 cytokines may be important in dictating different immunopathological responses (28, 29). Th2 cytokines are predominantly involved in mediating allergic inflammation and chronic fibroproliferative disorders such as asthma, atopic dermatitis, IPF, and systemic sclerosis (28, 29). Thus it may be appropriate to define certain diseases in terms of the predominant cytokine profile.

Although animal models of pulmonary fibrosis have provided insight into a role for Th2 cytokines in the mediation of pulmonary fibrosis, recent studies have confirmed this profile in IPF. Lung tissue of patients with IPF have been examined for the presence of a Th1 vs. Th2 pattern of cytokine expression by in situ hybridization and immunolocalization of cytokine protein (39). Although there is evidence for the existence of both Th1 (characterized by the expression of IFN-γ) and Th2 (characterized by the expression of IL-4 and IL-5) cytokines in IPF lung tissue, the presence of Th2 cytokines predominated over the expression of IFN-γ (39). This pattern of cytokine expression may be related to the potential role for the humoral response in the pathogenesis of IPF or related to the inability of IL-12 and IFN-γ to tilt the balance that may favor an IL-4/IL-13-dependent profibrotic environment. In further support of an imbalance in the presence of Th2 cytokines compared with IL-12 and IFN-γ is the finding that IFN-γ levels are inversely related to the levels of type III procollagen in the BAL fluid of IPF patients (20). The levels of IFN-γ were especially correlated with patients that demonstrate progression of their pulmonary fibrosis by evidence of further deterioration of their pulmonary function (20). These findings suggest that the persistent imbalance in the expression of Th1 and Th2 cytokines in the lung may be a mechanism for the progression of pulmonary fibrosis. IL-12 has the ability to correct this abnormality by directly promoting a Th1 response and indirectly through the induction of IFN-γ, which itself promotes a Th1 response.

![Image](http://ajplung.physiology.org/).

**Fig. 4.** A: lung hydroxyproline levels on day 12 after administration of IL-12 (1 μg in 100 μl of 0.25% MSA ip daily) and normal rabbit serum (days 2, 4, 6, and 8) or IL-12 (1 μg in 100 μl of 0.25% MSA ip daily) and neutralizing anti-IFN-γ antibodies (days 2, 4, 6, and 8). Control mice received vehicle alone (100 μl of 0.025% MSA daily). All mice received intratracheal bleomycin on day 0. Values are means ± SE; n = 6 mice in each group. *P < 0.05. B: Western blot analysis of IFN-γ from bleomycin-treated mice which received either MSA alone (100 μg of 0.25% MSA ip every 24 h), IL-12 (1 μg in 100 μl of 0.25% MSA ip daily), and normal rabbit serum (days 2, 4, 6, and 8) or IL-12 (1 μg in 100 μl of 0.25% MSA ip daily) and neutralizing anti-IFN-γ antibodies (days 2, 4, 6, and 8).
IL-12 INHIBITS PULMONARY FIBROSIS

In summary, we have shown that the administration of IL-12 leads to increased IFN-γ production and a reduction in pulmonary fibrosis, suggesting that the serial pathway of IL-12 to IFN-γ represents an important pathway in attenuating the fibrotic response. Furthermore, it suggests that a Th1 cytokine profile is beneficial in attenuating the fibrotic response. Future studies will address the possibility that the antifibrotic effects of IL-12 may be mediated in part through the induction of interferon-inducible chemokines and the inhibition of angiogenesis.

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