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Protection from Fluorescein Isothiocyanate-Induced Fibrosis in IL-13-Deficient, but Not IL-4-Deficient, Mice Results from Impaired Collagen Synthesis by Fibroblasts[1]

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Intratracheal injection of FITC results in acute lung injury and progresses to fibrosis by day 21 postchallenge. In response to FITC, BALB/c mice produce IL-4 and IL-13 in the lung. To investigate whether IL-4 and/or IL-13 were important profibrotic mediators in this model, we examined the fibrotic response to FITC in mice that were genetically deficient in IL-4 (IL-4$^{-/-}$), IL-13 (IL-13$^{-/-}$), or IL-4 and IL-13 combined (IL-4/13$^{-/-}$). Baseline levels of collagen were similar in all mice. In response to FITC, both BALB/c and IL-4$^{-/-}$ mice developed fibrosis, whereas the IL-13$^{-/-}$ and IL-4/13$^{-/-}$ mice were significantly protected, as measured by total lung collagen levels and histology. Total leukocyte recruitment to the lung was similar in all four strains of mice when measured on days 7, 14, and 21 post-FITC. BALB/c mice showed prominent eosinophilia on day 7 that was absent in IL-4$^{-/-}$, IL-13$^{-/-}$, and IL-4/13$^{-/-}$ mice, suggesting that eosinophilia is not necessary for development of a fibrotic response. There were no significant differences in the percentages of any other leukocytes analyzed between the genotypes. Similarly, protection in IL-13$^{-/-}$ mice was not associated with alterations in cytokine or eicosanoid profiles. Interestingly, TGF-β1 production was not reduced in IL-13$^{-/-}$ mice. Analyses of fibroblasts isolated from the four genotypes demonstrated that although there were similar numbers of fibroblasts present in cultures of lung minces, fibroblasts from IL-13-deficient strains have reduced basal and stimulated levels of collagen production. IL-13Rα1 expression increases on fibroblasts during fibrotic responses in vivo, and IL-13 increases collagen synthesis in fibroblasts. Thus, IL-13 mediates its profibrotic actions through direct effects on fibroblast production of extracellular matrix. ***The Journal of Immunology,*** **2004, 172: 4068–4076.**

Animal model systems have been developed for the study of fibrotic processes in the lung. These model systems include the intratracheal instillation of chemotherapeutic agents (bleomycin) (1) or the instillation of particulate Ags such as silica (2) or FITC (3). FITC challenge results in acute lung injury that leads to patchy areas of inflammation and subpleural scarring, reflecting collagen deposition. In response to this injury, a variety of proinflammatory and profibrotic mediators are made that serve to modulate the fibrotic process (4). The fibrotic process in response to FITC can be viewed as an overlapping continuum. The initial deposition of FITC within the lung causes an acute injury that results in both vascular and alveolar permeability, which peaks between days 1–3 after the insult (4). The peak of the inflammatory response is day 7 post-FITC (4). By day 10 post-FITC, fibroproliferation is evident, and extracellular matrix deposition begins. This fibrotic phase continues through days 21–28 postinsult and is persistent at least through day 42 post-FITC (3).

A number of soluble factors have been implicated as profibrotic mediators. Among these are growth and differentiation factors such as TGF-β (5), proinflammatory cytokines such as TNF-α (6), lipid mediators such as leukotrienes (7), and type 2 (T2) cytokines such as IL-4 and IL-13 (8–10). T2 cytokines have been implicated in remodeling associated with granuloma formation (11, 12) as well as asthma (13–15). The T2 cytokines, IL-4 and IL-13, share many functional properties mediated by shared receptor components, yet have been described to have differing functions as well (16). IL-4 is known to promote fibroblast chemotaxis, proliferation, and collagen synthesis (17–22). IL-13 can also promote fibroblast proliferation and collagen synthesis (21–24). Similarly, IL-4 and IL-13 have both been shown to augment the ability of human mesenchymal cells to contract collagen gels (25) and the expression of adhesion molecules and profibrotic cytokines from fibroblasts (26). Studies using IL-4$^{-/-}$ animals or IL-4 neutralization have shown reduced granuloma formation in *Schistosoma japonicum*-infected mice (27) and reduced liver fibrosis in *Schistosoma mansoni*-infected mice (11), suggesting that IL-4 can promote the fibrotic-like granulomatous response. Similarly, IL-13 blockade has been shown to ameliorate hepatic fibrosis after *Schistosoma mansoni* infection (12). Neutralization of IL-13 after bleomycin stimulation ameliorates pulmonary fibrosis as well (28). Finally, recent studies using animals that overexpressed IL-13 under control of the Clara cell-specific CC10 promoter demonstrated increased subepithelial cell fibrosis, mucus hypersecretion, and eosinophilic inflammation reminiscent of asthma exacerbations (10). Further investigations by the same authors demonstrated the ability of IL-13 to promote transcription and activation of the profibrotic cytokine TGF-β1 (29), thus suggesting TGF-β1 activation as a central mechanism for the profibrotic effects of IL-13.

As mentioned above, numerous studies have suggested a profibrotic role for these T2 cytokines; however, there have been no

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studies to date to examine the progression of experimental fibrosis in the complete absence of IL-4 and/or IL-13. We chose to do this by examining the fibrotic response to FITC in IL-$4^{-/-}$, IL-$13^{-/-}$, and IL-$4/13^{-/-}$ mice. IL-$13^{-/-}$ and IL-$4/13^{-/-}$ mice were significantly protected from FITC-induced pulmonary fibrosis; however, IL-$4^{-/-}$ mice were not. The protection in IL-$13^{-/-}$ mice did not correlate with reduced inflammation. Surprisingly, protection did not correlate with reduced production of, or responsiveness to, TGF-β1. Fibroblasts from IL-$13^{-/-}$ mice produce lower basal levels of collagen than wild-type controls, whereas fibroblasts from IL-$4^{-/-}$ mice produced collagen levels equivalent to control values. IL-13 can stimulate collagen production in both wild-type and IL-$13^{-/-}$ fibroblasts; however, the overall collagen synthesis in IL-$13^{-/-}$ animals is still lower. IL-13Rα1 expression increases on fibroblasts during fibrotic responses in vivo. The fact that IL-$13^{-/-}$ mice are protected from FITC-induced fibrosis despite the presence of TGF-β1 suggests that IL-13 plays a crucial regulatory role in determining fibrotic responses in vivo.

Materials and Methods

Mice

IL-$4^{-/-}$ mice bred onto the BALB/c background and control BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The generation of IL-$13^{-/-}$ (30) and IL-$4/13^{-/-}$ (31) mice has been previously described. These genotypes were backcrossed onto the BALB/c background for eight generations and were bred at University of Michigan under specific pathogen-free conditions. All mice were given food and water ad libitum and monitored daily by University of Michigan Unit for Laboratory Animal Medicine veterinary staff. These experiments were approved by the university committee for the use and care of animals.

FITC inoculation

FITC inoculation was performed as previously described (4). Briefly, mice were anesthetized with sodium pentobarbital. The trachea was exposed and entered with a needle under direct visualization. FITC (21 mg; F-7250; Sigma-Aldrich, St. Louis, MO) was dissolved in 10 ml of sterile PBS, vortexed extensively, and sonicated for 30 s. This slurry was transferred to multiuse vials and vortexed extensively before each 50-μl aliquot was removed for intratracheal injection using a 26-gauge needle.

Lung collagen measurements

Total lung collagen levels were determined by harvesting lungs from mice on day 21 after FITC or saline administration. Animals were euthanized and perfused with 3 ml of normal saline before all five lung lobes were removed and snap-frozen in liquid nitrogen. Before analysis, lungs were homogenized in 1 ml of normal saline and spun at 2000 rpm for 10 min. Aliquots of lung homogenate (100 μl) were then assayed for total lung collagen levels and compared with a standard curve prepared from rat tail collagen using the Sircol collagen dye binding assay (Accurate, Westbury, NY) according to the manufacturer's instructions.

Histology

Animals were euthanized and perfused via the right ventricle with 5 ml of normal saline. Lungs were inflated with 1 ml of 10% neutral buffered formalin, removed, and fixed overnight in formalin before being dehydrated in 70% ethanol. Lungs were processed using standard procedures and embedded in paraffin. Three- to 5-μm sections were cut, mounted on slides, and stained with H&E or Masson's Trichrome Blue for collagen deposition.

Collagenase digestion to isolate lung leukocytes

Collagenase digestions can be used to analyze both resident and recruited populations of lung cells found in the alveolar space and interstitium. This procedure has been optimized to purify lung leukocytes (32). Lungs were excised, minced, and enzymatically digested for 30 min using 15 ml/lung of digestion buffer (RPMI 1640, 5% FCS, antibiotics, 1 mg/ml collagenase (Roche, Chicago, IL), and 30 μg/ml DNase (Sigma-Aldrich)). The cell suspension and undigested fragments were further dispersed by repeated passage through the bore of a 10-ml syringe without a needle. The total cell suspension was pelleted, and any contaminating erythrocytes were eliminated by lysis in ice-cold NH_4Cl buffer (0.829% NH_4Cl, 0.1% $KHCO_3$, and 0.0372% Na_2 EDTA, pH 7.4). The pellet was resuspended in 5 ml of complete medium (RPMI 1640, 5% FCS, 1% penicillin/streptomycin, and 1% L-glutamine) and dispersed by 20 passages through a 5-ml syringe. The dispersed cells were filtered through a Nytex filter (Tetko, Kansas City, MO) to remove clumps. The total volume was brought up to 10 ml with complete medium. An equal volume of 40% Percoll (Sigma-Aldrich) was added, and the cells were centrifuged at 3000 rpm for 30 min (room temperature) without a brake. The cell pellets were resuspended in complete medium, and leukocytes were counted on a hemocytometer in the presence of trypan blue. Cells were >90% viable by trypan blue exclusion. Cytospins of recovered cells were prepared for differential staining as described below. In addition, recovered leukocytes were analyzed by flow cytometry.

Lung homogenates

After perfusion of the lungs with 5 ml of normal saline via the right ventricle, individual lung lobes were dissected and homogenized in 1 ml of PBS containing protease inhibitors (Complete Protease tablets; Roche, Manheim, Germany). Cellular debris was removed by centrifugation at 3000 rpm for 10 min. Aliquots of lung homogenate were then assayed by specific ELISA for cytokines. In some experiments lipids were extracted from lung homogenates using Sep-Pak cartridges (Waters, Milford, MA) as previously described (33). Eicosanoids were then measured by specific enzyme immunoassay (EIA)[3] kits obtained from Cayman Chemicals (Ann Arbor, MI).

Differential staining

Cytospins of collagenase digestions were made by centrifuging 50,000 cells onto microscope slides using a Cytospin 3 (Shandon, Astmoore, U.K.). The slides were allowed to air-dry and were stained using a modified Wright-Giemsa (WG) stain. For WG staining, the slides were fixed/prestained for 2 min with a one-step, methanol-based WG stain (Harleco; EM Diagnostics, Gibbstown, NJ), followed by steps 2 and 3 of the Diff-Quik whole blood stain (Diff-Quik; Baxter Scientific, Miami, FL). This modification of the Diff-Quik stain procedure improves the resolution of eosinophils from neutrophils in the mouse. A total of 300 cells was counted from randomly chosen high power microscope fields for each sample. The differential percentage was multiplied by the total leukocyte number to derive the absolute numbers of monocyte/macrophages, neutrophils, and eosinophils per sample.

FACS analysis

Lung cells (1×10^6) from the collagenase digestions of individual animals were incubated for 15 min on ice in Fc block (BD PharMingen, San Diego, CA) before washing and centrifugation. Cells were stained in a 100-μl total volume with 1 μg of combinations of the following Abs (obtained from BD PharMingen unless otherwise noted): CD45 (YW62.3; Caltag Laboratories, Burlingame, CA), CD4 (RM4-4), CD8 (53-6.7), CD19 (1D3), or DX5. Stained samples were stored in the dark at 4°C until being analyzed on a flow cytometer (FACScan; BD Biosciences, Mountain View, CA). All samples were stained with CD45 to identify a leukocyte-specific gate. The absolute number of a type of leukocyte in the lungs was determined as the percentage of that cell type multiplied by the total number of cells (leukocytes) in the lung collagenase digestion.

Lung leukocyte culture

Lung leukocytes purified by collagenase digestion were cultured at 5×10^6/ml in complete RPMI 1640 medium containing 10% FCS, 1% penicillin-streptomycin, and 1% L-glutamine. Cell-free supernatants were collected after 24 h of culture and were analyzed by ELISA for production of cytokines.

ELISA and EIA

Measurements of IL-4, monocyte chemotactic protein-1 (MCP-1), IFN-γ, and IL-10 were performed on culture supernatants and lung homogenates using Opti-EIA kits from BD PharMingen according to the manufacturer's instructions. IL-13 ELISAs were purchased from R&D Systems (Minneapolis, MN). Sensitivity ranged from 2–10 pg/ml at the lower limit. Lung homogenates were analyzed by EIA for the production of PGE_2, cysteinyl leukotrienes, and leukotriene B_4 using commercially available kits from Cayman Chemicals.

[3] Abbreviations used in this paper: EIA, enzyme immunoassay; MCP-1, monocyte chemotactic protein-1; PAI-1, plasminogen activator inhibitor-1; αSMA, α-smooth muscle actin; WG, Wright-Giemsa.

Plasminogen activator inhibitor-1 (PAI-1)/luciferase assay for TGF-β1

PAIL cells (mink lung epithelial cells (MLECs-clone 32) stably transfected with an 800-bp fragment of the 5′ end of the human PAI-1 gene fused to the firefly luciferase reporter gene in a p19LUC-based vector containing the neomycin resistance gene from pMAMneo; a gift from Dr. D. Rifkin, Department of Cell Biology, New York University, New York, NY) were maintained in DMEM supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine, and G418 (geneticin effective concentration, 200 μg/ml). To perform the assay, cells were plated at 1×10^6 cells/well in a six-well plate. The following day, medium was replaced with DMEM supplemented with 0.1% BSA, penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine, and G418. Then, 250 μl of lung homogenate or recombinant TGF-β1 was added to each well. After a 24-h incubation, medium was removed, cells were rinsed with PBS, and 400 μl of cell lysis buffer (Promega, Madison, WI) was added to each well. Cells were scraped and transferred to a microcentrifuge tube placed on ice. After all samples were collected, tubes were vortexed and spun at 12,000 × *g* for 15 s at room temperature. Supernatants were transferred to a new tube and assayed using the Promega Luciferase Assay System according to the manufacturer's directions. To prepare lung homogenates, one set of lungs was dissolved in 2 ml of PBS containing protease inhibitors. For measurements of active TGF-β1, 250 μl of lung homogenates were used neat. To determine total TGF-β1 activity, 250 μl of lung homogenate was incubated with 3.3 μl of 12 N HCl for 10 min at room temperature, followed by the addition of 3.5 μl of 12 N NaOH. Samples were vortexed and added immediately to the PAIL cells.

Fibroblast isolation

Lungs were perfused free of blood, and individual lung lobes were removed to a petri dish. Using sterile technique, lung lobes were finely minced with scissors, and the lung mince was cultured for 10–14 days in complete DMEM containing 10% FCS and 1% penicillin-streptomycin. Fibroblasts were passaged by trypsinization when they reached 70% confluence and were always used before the third passage. To insure that there was no leukocyte contamination in fibroblast cultures that were to be stimulated with cytokines, cells were stained with anti-CD45 Abs coupled to magnetic beads (Miltenyi Biotech, Auburn, CA), and the CD45-deficient cells were negatively selected as the column flow-through (SuperMacs columns; Miltenyi Biotec).

mRNA analysis

Total mRNA was prepared using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. TGF-β1, TGF-β2, and TGF-β3 mRNA levels were determined using RNase protection assays (RiboQuant; BD PharMingen, San Diego, CA) according to the manufacturer's instructions. IL-13Rα1 expression was determined by RT-PCR using the Promega Access RT-PCR kit according to the manufacturer's instructions. Primer sequences were as follows: IL-13Rα1 sense, GAATTTGAGCGTCTCTGTCGAA; IL-13Rα1 antisense, GGTTATGC CAAATGCACTTGAG; β-actin sense, GTGGGGCTCCCCAGGCACCA; and β-actin antisense, GCTCGGCCGTGGTGGTGAAGC.

Western blotting

Cells were grown to 85–95% confluence and serum-starved for 24–48 h in 35-mm dishes. Cultures were exposed to treatments for 24 h, then were washed with ice-cold PBS, and 200 μl of cold RIPA lysis buffer (1% (w/w) Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.02 M EDTA, 0.05 M NaF, 0.002 M NA_3VO_4, and a 1/100 dilution of Protease Cocktail Set II (Calbiochem-Novabiochem, San Diego, CA)) was added to each sample. After a 2-min incubation on ice, the lysed sample was collected. Lysis samples were assayed for total protein concentration using the DC Protein Assay (Bio-Rad, Hercules, CA). Equal amounts of protein from each experimental condition were mixed with a 1:5 (v/v) ratio of 6× electrophoresis sample buffer (0.2 M EDTA, 40 mM DTT, 6% SDS, and 0.6 mg/ml pyronin, pH 6.8) and boiled for 7 min. SDS-PAGE was performed using a 4–12% polyacrylamide gradient gel. Protein samples were electrophoretically transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) and incubated with 5% nonfat milk and 0.1% Tween 20, pH 7.4, for at least 1 h. Collagen was detected using a 1/500 dilution of purified rabbit anti-mouse collagen type I (Cedarlane Laboratories, Hornby, Ontario, Canada) in 5% nonfat milk and 0.1% Tween 20 for 1 h, followed by two washes with PBS-Tween (0.1% Tween). The secondary Ab was an HRP-coupled goat anti-rabbit Ab (ImmunoPure Goat Anti-Rabbit IgG, (H+L) peroxidase conjugated; Pierce, Rockford, IL) used at a 1/10,000 dilution in 5% nonfat milk and 0.1% Tween 20, incubated for 1–2 h. The blot was then washed five times with PBS-Tween (0.1% Tween) before incubation with SuperSignal West Pico chemiluminescence substrate (Pierce) for 5 min and exposed to chemiluminescent-sensitive Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY). For other experiments, α-smooth muscle actin (αSMA) was detected after electrophoresis and transfer of a 4–20% polyacrylamide gradient gel using a monoclonal primary Ab (clone 1A4; DAKO, Carpinteria, CA) diluted 1/1000 in blocking buffer, followed by detection with an HRP-coupled secondary Ab (ImmunoPure goat anti-mouse IgG, (H+L) peroxidase conjugated; Pierce) diluted 1/10,000. In most experiments lanes represent cells pooled from three individual animals. Equal protein loading in each lane was confirmed by stripping the blots and reprobing them with anti-α-tubulin Ab (Sigma-Aldrich).

TGF-β1 responsiveness

Fibroblast responsiveness to TGF-β1 was measured in two ways. First, fibroblasts were serum-starved for 24 h before incubation with 2 ng/ml TGF-β1 for 24 h. Cells were then analyzed for collagen synthesis by Western blot analysis as described. TGF-β1 stimulates collagen synthesis in fibroblasts. Additionally, TGF-β1 signaling capacity was analyzed by transfecting fibroblast cultures with a reporter plasmid carrying a TGF-β1-responsive promoter (p3TP-LUX; a gift from Dr. J. Massague, Memorial Sloan-Kettering Cancer Center, Howard Hughes Medical Institute, New York, NY). Four million fibroblasts were transfected with 10 μg of plasmid DNA via electroporation at 250 V and 960 μF using a Gene Pulser (Bio-Rad). Transfected cells were then split into two cultures. Cultures were maintained in complete medium for the first 24 h posttransfection. Cultures were switched to serum-free medium containing 0.1% BSA with or without 10 ng/ml TGF-β1 for an additional 24 h before luciferase activity was measured in cell lysates. Results are reported as the fold induction of TGF-β1-stimulated luciferase activity over nonstimulated levels.

Statistics

Statistical significance was analyzed using the InStat 2.01 program (GraphPad, San Diego, CA) on a Power Macintosh G3. Student's *t* tests were run to determine *p* values when comparing two groups. When comparing three or more groups, ANOVA was performed with a post-hoc Bonferroni test to determine which groups showed significant differences. A value of $p < 0.05$ was considered significant.

Results

IL-4 and IL-13 are induced after FITC challenge

To determine whether IL-4 and IL-13 are induced in the lung in response to FITC challenge, lung leukocytes were prepared from BALB/c mice on days 0, 7, 14, and 21 post-FITC challenge and cultured at 5×10^6/ml in the presence or the absence of 5 μg/ml Con A for 24 h before harvest. IL-4 and IL-13 production were measured in cell-free supernatants using specific ELISAs. IL-13 was readily detectable in supernatants from lung leukocytes and remained elevated compared with baseline secretion measured from leukocytes purified from untreated mice throughout days 7–21 (Fig. 1). IL-4 was also elevated compared with baseline, but was present at much lower levels than IL-13. Stimulation of the lung leukocytes with Con A increased the production of both cytokines. IL-4 levels were stimulated ~4- to 5-fold at all time points, whereas IL-13 levels were stimulated 20- to 30-fold by Con A at all time points (not shown). Thus, IL-4 and IL-13 are both produced by lung leukocytes after FITC challenge; however, IL-13 is induced to a much greater extent.

IL-13$^{-/-}$ and IL-4/13$^{-/-}$ mice, but not IL-4$^{-/-}$ mice, are protected from FITC-induced pulmonary fibrosis

To determine whether the production of IL-4 and/or IL-13 was regulating the development of pulmonary fibrosis, we injected BALB/c, IL-13$^{-/-}$, IL-4$^{-/-}$, and IL-4/13$^{-/-}$ mice with either saline or FITC intratracheally on day 0. On day 21 postchallenge, mice were euthanized, and lungs were analyzed for collagen content as a surrogate measure of fibrosis. Baseline (saline-challenged) levels of lung collagen were not statistically different in any of the four genotypes tested (Fig. 2). After FITC challenge,

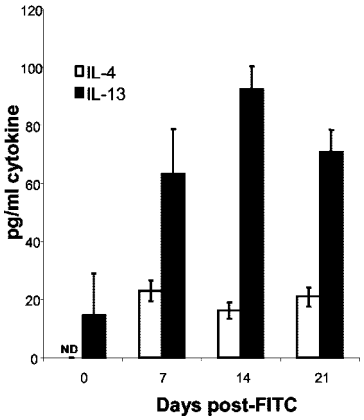

FIGURE 1. IL-13 and IL-4 are produced in the lung in response to FITC challenge. Mice were injected on day 0 with FITC. On days 0, 7, 14, and 21 post-FITC, lungs were harvested, and lung leukocytes were purified via collagenase digestion. Leukocytes were cultured at 5×10^6/ml for 24 h before cell-free supernatants were collected and measured by specific ELISA for IL-4 and IL-13. Values represent the mean ± SEM for samples from four individual mice. Data are representative of two identical experiments.

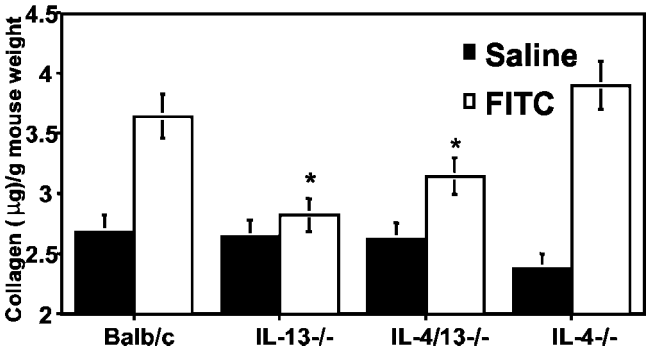

FIGURE 2. IL-$13^{-/-}$ mice, but not IL-$4^{-/-}$ mice, are protected from FITC-induced pulmonary fibrosis. Mice of all four genotypes were injected with FITC or saline on day 0. On day 21 postchallenge, lungs were harvested, and collagen content was measured by the Sircol assay to determine lung fibrosis. The level of collagen in saline-treated lungs was similar for all four genotypes (■). After FITC challenge (□), the levels of collagen increased dramatically in BALB/c and IL-$4^{-/-}$ mice. However, IL-$13^{-/-}$ and IL-4/$13^{-/-}$ mice were significantly protected from FITC-induced fibrosis compared with BALB/c mice ($p = 0.01$ for both IL-$13^{-/-}$ and IL-4/$13^{-/-}$ compared with BALB/c). Values represent the mean ± SEM of eight animals per group and are representative of three experiments.

BALB/c mice develop fibrosis, as measured by increased deposition of collagen similar to what has been reported previously (3). Similarly, the IL-$4^{-/-}$ mice showed no protection from the development of pulmonary fibrosis. In contrast, mice deficient in IL-13 or mice deficient in both IL-4 and IL-13 showed protection from the development of FITC-induced fibrosis that was statistically significant. There was no additive protection seen in the mice that were deficient in both IL-4 and IL-13 compared with the mice deficient in IL-13 alone.

We confirmed that leukocytes from IL-$4^{-/-}$ mice produced IL-13 at levels similar to those in wild-type mice at all time points after FITC challenge. Leukocytes from IL-$4^{-/-}$ mice made 58 ± 21 pg/ml IL-13 on day 7 post-FITC, 102 ± 14 pg/ml IL-13 on day 14 post-FITC, and 66 ± 7 pg/ml IL-13 on day 21 post-FITC ($n = 4$). These levels are similar to those measured in BALB/c lung leukocytes after FITC administration (Fig. 1).

The results of the collagen assay were confirmed by examining histology from FITC-treated mice of the four genotypes. Histologic sections were obtained from mice on day 21 post-FITC and were stained with H&E to identify inflammatory cells and with Masson's Trichrome to visualize mature collagen deposition. Both BALB/c and IL-$4^{-/-}$ mice show evidence of dense consolidation and fibrosis, whereas the IL-$13^{-/-}$ and IL-4/$13^{-/-}$ mice showed only isolated areas of inflammation and consolidation (Fig. 3).

Magnitude of inflammatory response is similar in all genotypes

To determine the degree of inflammation seen in the four genotypes after FITC administration, mice were injected with FITC on day 0 and harvested for collagenase digestion on days 7, 14, and 21 post-FITC. Total cell numbers were enumerated and compared with cell numbers from collagenase digestions of untreated mice (day 0). All genotypes of mice tested showed cellular influx to the lung in response to FITC inoculation. The inflammatory response was maximal on day 7, but remained slightly elevated on days 14 and 21 post-FITC (Fig. 4*A*). There was no statistical difference in the magnitude of the inflammatory response between any of the groups of mice tested. Thus, the protection seen in the IL-$13^{-/-}$ and IL-4/$13^{-/-}$ mice could not be attributed to diminished inflammation.

Eosinophilia does not correlate with fibrotic outcome

To determine the composition of the inflammatory cells that were recruited in the four genotypes of mice, leukocytes purified from collagenase digests were subjected to differential and flow cytometric analysis to determine cellular phenotype. Fig. 4*B* represents data from day 7 post-FITC, the peak of the inflammatory response. These data demonstrate that BALB/c mice develop marked eosinophilia in response to FITC challenge on day 7 postinoculation. The eosinophilia is transient and is gone by day 14 post-FITC (not shown). Interestingly, the IL-$13^{-/-}$, IL-$4^{-/-}$, and IL-4/$13^{-/-}$ mice all showed reduced eosinophilia on day 7 despite the fact that the IL-$4^{-/-}$ mice were not protected from fibrosis. We conclude that the genetic disruption of the T2 cytokines inhibits the development of transient eosinophilia in response to fibrotic challenge; however, the absence of eosinophils does not correlate with protection from fibrosis. To determine whether there were any differences in the composition of the lymphocytes that were recruited to the lung after FITC challenge, we subjected the collagenase-digested leukocytes to flow cytometric analysis. There was no statistical difference in the number of B, NK, $\gamma\delta$ T, $CD4^+$ $\alpha\beta$ T, or $CD8^+$ $\alpha\beta$ T cells within the lungs of any of the genotypes tested (not shown). Similarly, there were no significant differences in the composition of the inflammatory response among the various genotypes when tested on day 14 or 21 post-FITC (not shown).

Number of fibroblasts cultured from lung minces is similar in all four genotypes

To determine whether there were differences in the numbers of fibroblasts present in the lungs of FITC-treated mice, lung minces were prepared from mice of all four genotypes on day 7 post-FITC, the peak of inflammation. Lung minces from individual mice ($n = 4$) were cultured for 14 days and total numbers of mesenchymal cells were enumerated. BALB/c mice contained an average of 2.4 ± 0.5 million fibroblasts. IL-$13^{-/-}$ mice contained 2.8 ± 0.5 million, IL-4/$13^{-/-}$ mice contained 2.5 ± 0.6 million, and IL-$4^{-/-}$ mice contained 2.8 ± 0.3 million fibroblasts/lung. There were no statistical differences in the number of fibroblasts grown from lung minces of the different genotypes at any time point tested between days 0 and 21.

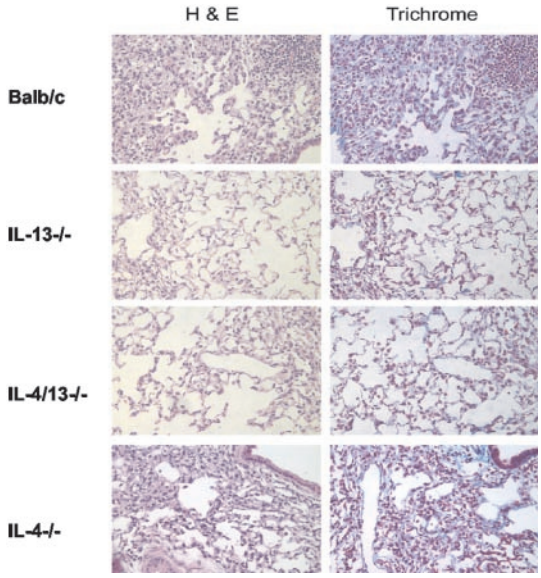

FIGURE 3. IL-13$^{-/-}$ and IL-4/13$^{-/-}$ mice have less histologic evidence of fibrosis than do BALB/c or IL-4$^{-/-}$ mice after FITC challenge. Mice of all four genotypes were injected on day 0 with FITC. On day 21, lungs were harvested for histological analysis. Panels on the *left* represent H&E staining of lung sections for all four genotypes. Panels on the *right* represent Masson's Trichrome staining, where the blue coloration indicates areas of collagen deposition. Panels shown are representative of the histology seen in six individual mice.

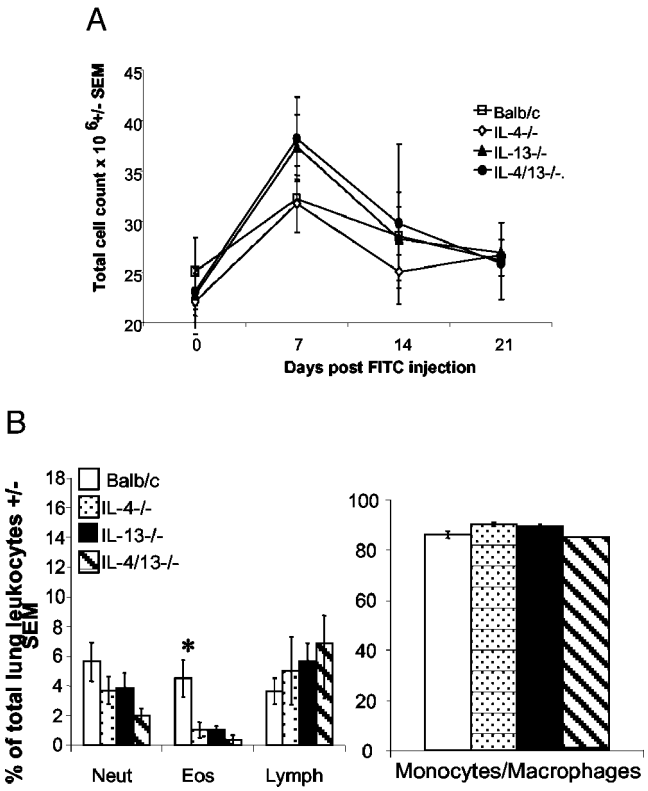

FIGURE 4. Protection in IL-13$^{-/-}$ mice cannot be explained by differences in inflammatory cell numbers or subtypes. *A*, Mice of all four genotypes were injected with FITC on day 0. On days 0, 7, 14, and 21, lungs were harvested for collagenase digestion and leukocyte purification ($n = 4$ mice/group). Day 7 is the peak of inflammation for all genotypes, but leukocyte cell numbers remain elevated throughout day 21 in all groups. There are no statistical differences between the number of inflammatory cells present within the lungs of the different mice at any of the time points tested. Data are representative of three identical experiments. *B*, Purified leukocytes on day 7 post-FITC were subjected to differential staining and analysis to identify leukocyte subpopulations. BALB/c mice show a transient eosinophilia ($p < 0.05$) on day 7 post-FITC that is absent in the T2-deficient genotypes. Flow cytometric analysis of the lymphocyte population identified no statistical differences in B, CD4$^+$ T, CD8$^+$ T, γδT, or NK cell numbers. This experiment was repeated twice with similar results.

Neither T1 cytokine profiles nor eicosanoid differences correlate with protection from FITC-induced fibrosis

We were next interested in determining whether there were notable differences in the profiles of eicosanoids or cytokines made in response to FITC in the susceptible vs resistant genotypes. Mice of all four genotypes were injected with FITC on day 0. On day 3 or 7 post-FITC, lungs were harvested, and lung homogenates were analyzed for cytokine levels by specific ELISA. Table I describes the levels of IFN-γ, IL-10, and MCP-1 that were present in the lungs of each genotype. There were no significant differences in the levels of IL-10 or MCP-1 present in the lungs of the four genotypes of mice. Interestingly, the level of IFN-γ was significantly greater in the BALB/c lungs than in the other three genotypes ($p < 0.01$); however, there was no statistical difference between the protected IL-13-deficient mice and the susceptible IL-4$^{-/-}$ mice. Furthermore, when IFN-γ was measured in leukocyte supernatants of cells purified from lungs on day 7 postfibrotic insult, there were no significant differences noted (BALB/c lung leukocytes secreted 790 ± 293 pg/ml compared with 1176 ± 506 pg/ml from IL-4$^{-/-}$ leukocytes and 715 ± 177 pg/ml from IL-13$^{-/-}$ leukocytes; $n = 4$.) Thus, IFN-γ production does not correlate with protection.

Our previous studies have demonstrated that endogenous leukotrienes can promote fibrotic responses within the lung (7). Likewise, endogenous PGs can limit fibrotic responses (33, 34). Therefore, we next questioned whether the levels of leukotrienes and PGs were different in the four genotypes of mice post-FITC. Mice of all four genotypes were injected with FITC on day 0. On day 7, lungs were harvested and homogenized, and lipids were extracted using Sep-Pak cartridges. Table I details the levels of cysteinyl leukotrienes, LTB_4, and PGE_2 that were measured in each genotype on day 7. Despite the fact that T2 cytokines have been reported to modulate eicosanoid production (35–44), there were no differences that could account for the pattern of protection seen in the various genotypes. Thus, differences in eicosanoid profiles do not correlate with protection in these genotypes.

IL-13$^{-/-}$ mice do not have diminished levels of TGF-β1

TGF-β1 is a critical profibrotic cytokine that is known to be able to induce fibrotic responses within the lung (45). It has been well documented that TGF-β1 can induce the transformation of fibroblasts to myofibroblasts (46–50) and can increase the production of extracellular matrix components in fibroblasts (51, 52). Recent work using IL-13-overexpressing mice has suggested that IL-13 exerts its profibrotic effects via increasing both the transcription and the activation of TGF-β1 (29). Therefore, we wanted to determine the level of TGF-β1 expression in IL-13$^{-/-}$ and IL-4/13$^{-/-}$ mice. For these analyses, BALB/c, IL-13$^{-/-}$, and IL-4/13$^{-/-}$ mice were injected with FITC on day 0. On days 0, 3, 7, 14, and 21 post-FITC, lungs were harvested for RNA analysis of TGF-β isoforms or were homogenized and assayed for TGF-β1 bioactivity using PAIL cells. Fig. 5*A* demonstrates the levels of total (acid-treated) TGF-β1 activity detected in lung homogenates from the various genotypes after FITC. There were no statistical differences between any of the strains. Likewise, when active

Table I. *Levels of cytokines and eicosanoid post-FITC*[a]

Genotypes	Cytokines					
	IFN-γ (pg/ml)	IL-10 (pg/ml)	MCP-1 (ng/ml)	LTB_4 (pg/ml)	CysLT (pg/ml)	PGE_2 (pg/ml)
BALB/c	9.4 ± 0.5	129 ± 11	1.4 ± 0.3	4.3 ± 0.3	9.8 ± 2.1	1.27 ± 0.3
IL-$4^{-/-}$	5.8 ± 0.1	112 ± 3.4	1.1 ± 0.4	3.7 ± 0.4	11.6 ± 3.3	2.06 ± 0.9
IL-$13^{-/-}$	4.3 ± 0.5	99 ± 2	1.9 ± 0.3	3.8 ± 1	11.5 ± 2.5	1.7 ± 0.7
IL-4/$13^{-/-}$	2.9 ± 0.3	112 ± 0.4	1.2 ± 0.3	2.9 ± 0.3	8.2 ± 0.1	4 ± 1

[a]Mice of all four genotypes were injected with FITC on day 0. On day 3 or 7 post-FITC, lungs were harvested. Lungs were homogenized and aliquoted. Cytokine measurements were made by ELISA. In other aliquots, lipids were extracted before levels of leukotrienes and eicosanoids were measured by specific EIA. All mediators were measured on day 7 post-FITC, except for MCP-1, which was measured on day 3, because our earlier experiments suggest that this mediator peaks between days 1 and 3 post-FITC (3). Data represent analysis of at least four mice per group and are representative of two experiments. LTB_4, Leukotriene B_4; CysLT, cysteinyl leukotriene.

TGF-β1 was analyzed by bioassay in non-acid-treated samples (Fig. 5*B*), the levels paralleled the pattern seen in total TGF-β1 activity, and there were no statistical differences between the groups. Similarly, the mRNA levels were not statistically different for TGF-β1, TGF-β2, and TGF-β3 when analyzed by RNase protection assay (Fig. 5*C*) on day 7. Additionally, RT-PCR analysis of TGF-β1 expression in lungs on day 14 post-FITC are not different (Fig. 5*D*). Thus, IL-$13^{-/-}$ mice are protected from FITC-induced pulmonary fibrosis despite abundant levels of TGF-β1.

TGF-β1 responsiveness is intact in IL-13-deficient fibroblasts

We next determined whether the responsiveness to TGF-β1 was diminished in IL-13-deficient fibroblasts. To ascertain whether TGF-β1 signaling pathways were functional in IL-$13^{-/-}$ fibroblasts, cells were grown from lung minces of unchallenged BALB/c and IL-$13^{-/-}$ mice. Four million primary fibroblasts pooled from three mice of each genotype were transiently transfected with the p3TP-LUX plasmid, which contains a TGF-β1-responsive promoter driving a luciferase reporter gene. Transfected cells were split into equal aliquots, and half were treated with 10 ng/ml TGF-β1. Cellular lysates were then analyzed for luciferase activity. TGF-β1 induced a 7.4 ± 2.2-fold increase in luciferase activity in BALB/c fibroblasts compared with a 15.2 ± 4.8-fold increase in IL-$13^{-/-}$ fibroblasts ($n = 3$; p = NS). Thus, there was no deficiency in TGF-β1 signaling in the IL-$13^{-/-}$ fibroblasts. TGF-β1 is well known to up-regulate collagen synthesis in fibroblast cultures; thus, we tested the effect of TGF-β1 on collagen production in BALB/c and IL-$13^{-/-}$ fibroblasts. Fig. 6*A* demonstrates that TGF-β1 augments the production of collagen I in BALB/c fibroblasts. Fig. 6*B* demonstrates that TGF-β1 is similarly able to augment collagen I production from IL-13-deficient fibroblasts. These data suggest that there are no defects in TGF-β1 signaling in fibroblasts from IL-13-deficient mice.

Myofibroblasts from IL-$13^{-/-}$ mice are deficient in collagen I expression

We next addressed whether the overall production of collagen was different among fibroblasts purified from the four genotypes of mice. Fibroblasts were grown from pooled lung minces ($n = 3$) of the four strains of mice as described in *Materials and Methods*. After 13 days of culture, cells were adhered to 35-mm dishes and were then serum-starved for 24 h. Some dishes of cells were treated with IL-13 at 10 ng/ml or were maintained in serum-free medium for an additional 48 h. Cellular lysates were prepared and analyzed for collagen I production by Western blot. Fig. 7*A* demonstrates that IL-$13^{-/-}$ and IL-4/$13^{-/-}$ fibroblasts from unchallenged mice produce lower levels of collagen I compared with BALB/c mice when equal amounts of protein from the cultures are compared. Densitometry of multiple experiments showed the production of collagen I by IL-$13^{-/-}$ fibroblasts was reduced 60–90% compared with that by BALB/c fibroblasts. This was also true when fibroblasts were isolated from day 7 FITC-treated mice (not shown). In contrast, fibroblasts from IL-$4^{-/-}$ mice produced equivalent collagen levels (never >12% different by densitometry) compared with BALB/c controls (Fig. 7*B*). Fig. 7*C* demonstrates that IL-13 could augment the production of collagen in both BALB/c and IL-$13^{-/-}$ fibroblasts. However, because of the overall lower levels of collagen at baseline in the IL-$13^{-/-}$ mice, even stimulated levels of collagen production were lower. Thus, an overall diminished production of collagen in the IL-$13^{-/-}$ mice is probably responsible for the protection from fibrosis seen in these mice in vivo.

IL-13Rα1 expression increases on fibroblasts during fibrotic responses

Our data demonstrate that IL-13 is a potent profibrotic cytokine that drives the production of collagen I from fibroblasts. Therefore, we hypothesized that IL-13R expression might increase on fibroblasts during fibrotic processes in vivo. We examined the expression of the high affinity, signal-transducing IL-13Rα1 chain on fibroblasts purified from wild-type mice at various time points post-FITC challenge. Fibroblasts were grown out of lung minces started on days 0, 1, 3, 7, 10, and 14 post-FITC. Fibroblasts were allowed to grow for 14 days in culture before RNA was harvested and analyzed by RT-PCR for the expression of IL-13Rα1. The levels of mRNA for IL-13Rα1 were increased by days 10 and 14 during the fibrotic response, a time point that correlates well with in vivo fibroproliferation post-FITC. We were unable to identify transcripts for IL-13Rα2 in these same cells by RT-PCR (not shown). Thus, fibroblast IL-13Rα1 expression increases during fibrotic processes in vivo, and the profibrotic effects of IL-13 on collagen I production are probably amplified.

Discussion

IL-$13^{-/-}$, but not IL-$4^{-/-}$, BALB/c background mice are protected from FITC-induced pulmonary fibrosis. There is no additive protection in IL-4/$13^{-/-}$ mice; thus, IL-4 does not substitute for the functions of IL-13 in this model. The protection in IL-13-deficient mice is not attributable to changes in inflammatory cell recruitment, inflammatory cell composition, fibroblast numbers, cytokine profiles, eicosanoid profiles, or TGF-β1 activation or signaling. Rather, the protection seen in IL-13-deficient mice is related to the collagen synthesis phenotype of the fibroblasts. Fibroblasts from IL-$13^{-/-}$ and IL-4/$13^{-/-}$ mice produce less collagen I than wild-type cells both at baseline and after FITC challenge. The expression of IL-13Rα1 increases on fibroblasts during fibrotic responses in vivo. Thus, fibrotic protection in IL-$13^{-/-}$ mice can be attributed to the lack of IL-13 activation of collagen secretion in fibroblasts from those animals. IL-$13^{-/-}$ animals are protected from fibrosis despite the presence of TGF-β1; thus, these

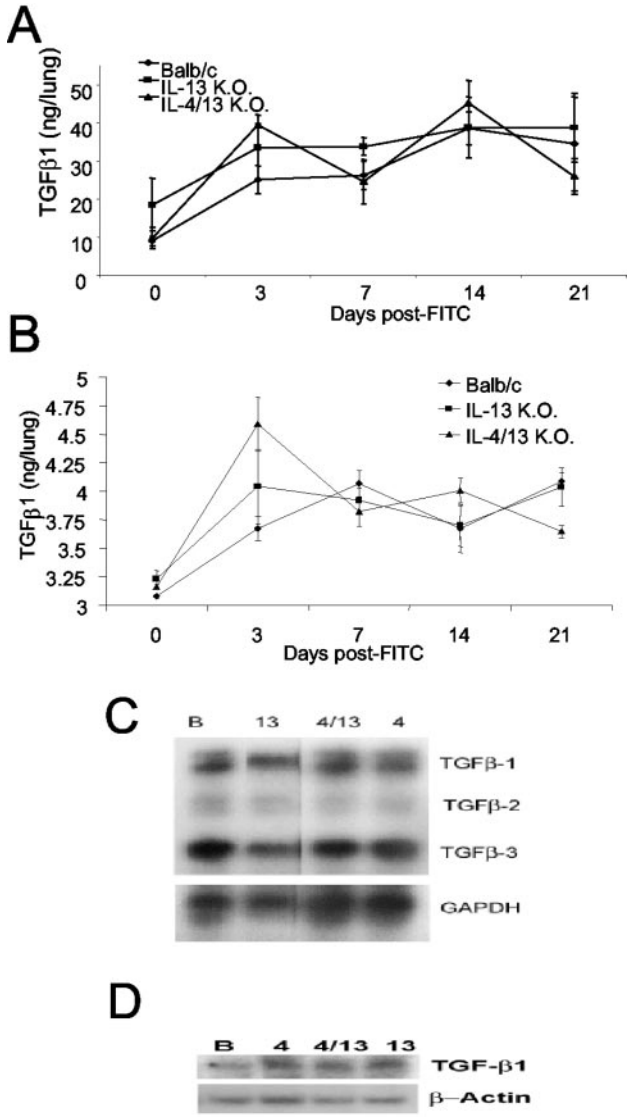

FIGURE 5. Levels of TGF-β1 in IL-13-deficient mice are not reduced compared with those in BALB/c mice. *A*, Mice were injected with FITC on day 0. On the indicated days post-FITC, lung homogenates were prepared in a total volume of 2 ml; 250 μl of the lung homogenate was acid-treated, then neutralized to activate latent TGF-β1 within the sample. These lung homogenates were then analyzed in a bioassay for TGF-β1 activity (PAIL cell assay). Levels of TGF-β1 rose post-FITC; however, there were no differences seen in the levels of total TGF-β1 measured between the susceptible BALB/c mice and the protected IL-13-deficient mice. *B*, When active TGF-β1 was measured from non-acid-treated lung homogenates, the levels were lower, but showed the same trends in all samples. Data in *A* and *B* represent the mean ± SEM for three animals per group measured in duplicate and are representative of two similar experiments. *C*, Mice were treated with FITC on day 0. On day 7, lungs were removed, and total lung RNA was analyzed for TGF-β1, -β2, and -β3 isoform expression by RNase protection analysis. Each lane represents RNA pooled from three lungs per group and represents two similar experiments. *D*, RT-PCR analysis for TGF-β1 in whole lung RNA isolated on day 14 post-FITC. Each lane represents RNA pooled from three lungs per group. The data shown represent two similar experiments.

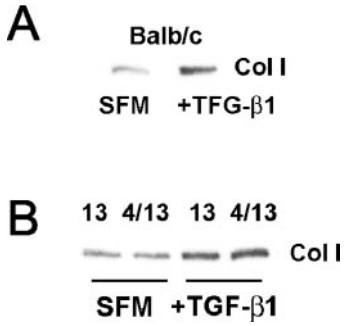

FIGURE 6. There are no defects in TGF-β1 responsiveness in IL-13-deficient fibroblasts. Fibroblasts were grown out of tissue minces (n = 3) of unchallenged BALB/c (*A*) or IL-13$^{-/-}$ and IL-4/13$^{-/-}$ (*B*) mice for 13 days. On day 13, fibroblasts were lifted by trypsin digestion and plated at equal confluence in 35-mm dishes. Cells were then serum-starved for 24 h before medium was changed, and cells were incubated for an additional 24 h in the presence of serum-free medium (SFM) or 2 ng/ml TGFβ1. Cellular lysates were prepared, and equal amounts of protein were loaded onto polyacrylamide gels for analysis of collagen I by Western blot. The blot in *B* was exposed for a longer period of time so that the unstimulated levels of collagen would be similar in *A* and *B*. The experiments were repeated four times with similar results.

results suggest that in vivo IL-13 plays an obligatory role in driving collagen production from fibroblasts.

Our studies involve IL-13- and IL-4/13-deficient mice. Previous studies involved transgenic mice overexpressing IL-13. These overexpression studies demonstrated that IL-13 increased subepithelial cell fibrosis, mucus hypersecretion, and eosinophilic inflammation reminiscent of changes associated with asthma and airway remodeling (10). Further investigations demonstrated that IL-13 increased the transcription and activation of TGF-β1 (29), thus suggesting that activation of TGF-β1 was a central mechanism for the profibrotic effects of IL-13. These experiments clearly demonstrated that overexpression of IL-13 (in the absence of a fibrotic stimulus) was associated with fibrotic changes in the lung. Our results confirm a crucial role for IL-13 in mediating fibrotic responses, but demonstrate a different mechanism from that found in earlier studies. IL-13$^{-/-}$ mice are significantly protected when administered a fibrotic challenge. Interestingly, these IL-13$^{-/-}$ mice are protected from FITC-induced pulmonary fibrosis despite abundant production of TGF-β1. Our results show that there were no differences in the levels of transcription of TGF-β1, TGF-β2, or TGF-β3 among any of the genotypes tested at any time point. No differences in the levels of active or latent TGF-β1 were detected in lung homogenates of any of the genotypes at any time point analyzed, and no differences in TGF-β1 signaling were identified. Thus, in this model, IL-13$^{-/-}$ mice do not show a defect in their ability to transcribe and activate this profibrotic cytokine. We propose that IL-13 plays a dominant, non-TGF-β1-dependent role in the regulation of fibroblast extracellular matrix production.

Our previous work has demonstrated that 5-lipoxygenase products of arachidonic acid metabolism (leukotrienes) are profibrotic molecules in pulmonary fibrosis (7), whereas the cyclooxygenase products of arachidonic acid metabolism (PGs) are protective lipid mediators in fibrotic responses (33, 34). T2 cytokines can influence the expression of enzymes regulating arachidonic acid metabolism (35–44). Table I demonstrates that the expression of cysteinyl leukotrienes, leukotriene B_4, and PGE_2 are similar despite the genotype of the mouse tested. Similar patterns of expression were seen on days 14 and 21 post-FITC as well (not shown). Thus, altered eicosanoid generation could not account for the protection in IL-13$^{-/-}$ mice.

Fibrotic diseases in the lung have been attributed to alterations in the balance of T1 vs T2 cytokines (53–55). Diseases such as asthma and fungal infections, which can develop fibrotic consequences, are often characterized by the overproduction of T2 relative to T1 cytokines. Thus, one possibility for the protection seen

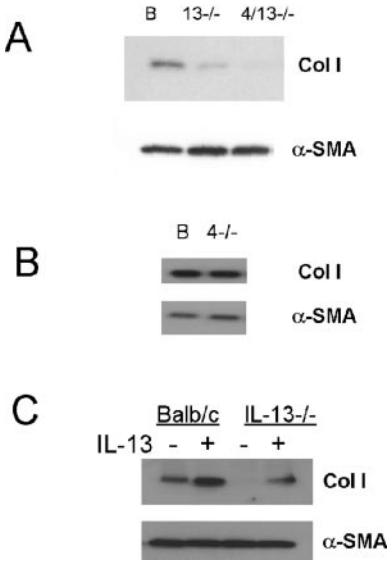

FIGURE 7. Fibroblasts from IL-13-deficient mice produce lower levels of collagen I than wild-type fibroblasts. Fibroblasts were grown for 13 days from lung minces of unchallenged mice as described in *Materials and Methods*. Equal numbers of cells were plated in 35-mm dishes and allowed to adhere overnight. The following day, the cells were placed into serum-free medium for 24–48 h before cellular lysates were made and analyzed for collagen I (col I) or αSMA expression by Western blot. *A*, Comparison of wild-type BALB/c (B) cells to cells from IL-13$^{-/-}$ and IL-4/13$^{-/-}$ strains. *B*, Comparison of fibroblasts from BALB/c mice to those from IL-4$^{-/-}$ mice. *C*, Comparison of the production of col I and α-SMA from BALB/c or IL-13$^{-/-}$ fibroblasts treated with serum-free medium or 10 ng/ml IL-13 for 48 h. In all wells data represent analysis of cells pooled from three mice. Data are representative of at least three independent experiments.

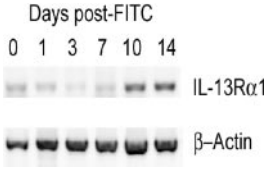

FIGURE 8. Expression of IL-13Rα1 increases on fibroblasts during fibrotic responses in vivo. BALB/c mice were injected with FITC on day 0. On days 0, 1, 3, 7, 10, and 14 post-FITC, lungs were harvested and minced, and fibroblasts were grown out of the lung minces for 14 days before total RNA was prepared and analyzed for IL-13Rα1 expression by RT-PCR. Each lane represents combined RNA from three animals per time point. Data are representative of two similar experiments.

in IL-13-deficient mice was that the fibrotic protection was due to a compensatory overproduction of T1 cytokines such as IFN-γ. However, no clear pattern of elevated IFN-γ could be identified in the protected IL-13$^{-/-}$ and IL-4/13$^{-/-}$ mice at any time point post-FITC.

Fibroblasts, specifically myofibroblasts, are the cell type responsible for the pathologic consequences of excess matrix production during fibrosis (50). Fibroblasts grown from lung minces in complete medium phenotypically resemble myofibroblasts, as evidenced by the high level expression of αSMA. IL-13- and IL-4/13-deficient cells produced less collagen I despite having equivalent levels of αSMA. These data suggest that IL-13 does not regulate myofibroblast differentiation, but, rather, regulates the effector function of these myofibroblasts in situ. One possibility to explain this finding is that fibroblasts from IL-13-deficient mice failed to express receptors for TGF-β1 or IL-13. However, our results suggest that the fibroblasts from IL-13-deficient mice are fully capable of responding to both TGF-β1 and IL-13 in vitro by increasing collagen I production. Diminished matrix production in IL-13$^{-/-}$ fibroblasts cannot be attributed to decreased fibrotic ligand responsiveness, but, rather, results from an absence of fibrotic ligand (IL-13) expression. Additionally, our data from wild-type mice demonstrate that IL-13Rα1 expression increases on fibroblasts/myofibroblasts throughout the progression of fibrotic disease (Fig. 8). Given that our data in Fig. 7 demonstrate that IL-13 is a potent inducer of extracellular matrix secretion in fibroblasts, increased receptor expression during fibrotic responses is likely to have important pathogenic consequences. Increased receptor expression probably leads to increased signaling, and thus the cycle of IL-13-driven matrix production would be expected to be amplified.

Our data are the first to demonstrate that signaling pathways leading to αSMA expression and collagen I expression are distinct. Previous studies have suggested that the increased collagen production in fibrotic responses is due to the increased synthesis of collagen by myofibroblasts (56). IL-13$^{-/-}$ fibroblasts are deficient in collagen I synthesis despite equivalent levels of αSMA expression. Furthermore, this observation suggests that neutralization of IL-13 may still be beneficial in fibrotic disorders where myofibroblast recruitment/proliferation is already underway.

In conclusion, our data demonstrate that IL-13 crucially regulates collagen I production by myofibroblasts. Signals from IL-13 increase the levels of collagen I protein expression independent of TGF-β1 signaling. The absence of IL-13 is sufficient to prevent collagen I synthesis, even in the presence of TGF-β1, both in vitro and in vivo. Blockade of IL-13 or IL-13R may represent a promising therapeutic target, because it may block the undesired effects of excess matrix production, presumably while leaving intact the ability of myofibroblasts to contract and close injured areas via αSMA-mediated processes. Furthermore, our results suggest that anti-IL-13 therapy would be unlikely to modulate the eicosanoid and cytokine cascades necessary for efficient wound repair. Thus, our results suggest that anti-IL-13 therapies may be very beneficial for the treatment of fibroproliferative lung disorders.

References

1. Lazo, J. S., D. G. Hoyt, S. M. Sebti, and B. R. Pitt. 1990. Bleomycin: a pharmacologic tool in the study of the pathogenesis of interstitial pulmonary fibrosis. *Pharmacol. Ther. 47:347*.
2. Klien, J. H., and I. Y. Adamson. 1989. Fibroblast inhibition and prostaglandin secretion by alveolar epithelial cells exposed to silica. *Lab. Invest. 60:808*.
3. Christensen, P., R. Goodman, L. Pastoriza, B. Moore, and G. Toews. 1999. Induction of lung fibrosis in the mouse by intratracheal instillation of fluorescein isothiocyanate is not T-cell dependent. *Am. J. Pathol. 155:1773*.
4. Moore, B., R. Paine III, P. Christensen, T. Moore, S. Sitterding, R. Ngan, C. Wilke, W. Kuziel, and G. Toews. 2001. Protection from pulmonary fibrosis in the absence of CCR2 signaling. *J. Immunol. 167:4368*.
5. Khalil, N., O. Bereznay, M. Sporn, and A. H. Greenberg. 1989. Macrophage production of transforming growth factor β and fibroblast collagen synthesis in chronic pulmonary inflammation. *J. Exp. Med. 170:727*.
6. Ortiz, L., J. Lasky, R. Hamilton, A. Holian, G. Hoyle, W. Banks, J. Peschon, A. Brody, G. Lungarella, and M. Friedman. 1998. Expression of TNF and the necessity of TNF receptors in bleomycin-induced lung injury in mice. *Exp. Lung Res. 24:721*.
7. Peters-Golden, M., M. Bailie, T. Marshall, C. Wilke, S. H. Phan, G. Toews, and B. Moore. 2002. Protection from pulmonary fibrosis in leukotriene-deficient mice. *Am J. Respir. Crit. Care Med. 165:229*.
8. Buttner, C., A. Skupin, T. Reimann, E. P. Rieber, G. Unteregger, P. Geyer, and K. H. Frank. 1997. Local production of interleukin-4 during radiation-induced pneumonitis and pulmonary fibrosis in rats: macrophages as a prominent source of interleukin-4. *Am. J. Respir. Cell Mol. Biol. 17:315*.
9. Fallon, P. G., E. J. Richardson, G. J. McKenzie, and A. N. McKenzie. 2000. Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. *J. Immunol. 164:2585*.
10. Zhu, Z., R. J. Homer, Z. Wang, Q. Chen, G. P. Geba, J. Wang, Y. Zhang, and J. A. Elias. 1999. Pulmonary expression of interleukin-13 causes inflammation,

mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J. Clin. Invest. 103:779*.
11. Cheever, A. W., M. E. Williams, T. A. Wynn, F. D. Finkelman, R. A. Seder, T. M. Cox, S. Hieny, P. Caspar, and A. Sher. 1994. Anti-IL-4 treatment of Schistosoma mansoni-infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egg-induced hepatic fibrosis. *J. Immunol. 153:753*.
12. Chiaramonte, M., D. Donaldson, A. Cheever, and T. Wynn. 1999. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2 dominated inflammatory response. *J. Clin. Invest. 104:777*.
13. Richter, A., S. M. Puddicombe, J. L. Lordan, F. Bucchieri, S. J. Wilson, R. Djukanovic, G. Dent, S. T. Holgate, and D. E. Davies. 2001. The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *Am. J. Respir. Cell Mol. Biol. 25:385*.
14. Blease, K., C. Jakubzick, J. Westwick, N. Lukacs, S. L. Kunkel, and C. M. Hogaboam. 2001. Therapeutic effect of IL-13 immunoneutralization during chronic experimental fungal asthma. *J. Immunol. 166:5219*.
15. Doucet, C., D. Brouty-Boye, C. Pottin-Clemenceau, G. W. Canonica, C. Jasmin, and B. Azzarone. 1998. Interleukin (IL) 4 and IL-13 act on human lung fibroblasts. Implication in asthma. *J. Clin. Invest. 101:2129*.
16. Chomarat, P., and J. Banchereau. 1998. Interleukin-4 and interleukin-13: their similarities and discrepancies. *Int. Rev. Immunol. 17:1*.
17. Fertin, C., J. F. Nicolas, P. Gillery, B. Kalis, J. Banchereau, and F. X. Maquart. 1991. Interleukin-4 stimulates collagen synthesis by normal and scleroderma fibroblasts in dermal equivalents. *Cell. Mol. Biol. 37:823*.
18. Gillery, P., C. Fertin, J. F. Nicolas, F. Chastang, B. Kalis, J. Banchereau, and F. X. Maquart. 1992. Interleukin-4 stimulates collagen gene expression in human fibroblast monolayer cultures: potential role in fibrosis. *FEBS Lett. 302:231*.
19. Postlethwaite, A. E., and J. M. Seyer. 1991. Fibroblast chemotaxis induction by human recombinant interleukin-4. Identification by synthetic peptide analysis of two chemotactic domains residing in amino acid sequences 70–88 and 89–122. *J. Clin. Invest. 87:2147*.
20. Postlethwaite, A., M. Holness, H. Katai, and R. Raghow. 1992. Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin-4. *J. Clin. Invest. 90:1479*.
21. Kraft, M., C. Lewis, D. Pham, and H. W. Chu. 2001. IL-4, IL-13, and dexamethasone augment fibroblast proliferation in asthma. *J. Allergy Clin. Immunol. 107:602*.
22. Morita, Y., M. Yamamura, M. Kawashima, T. Aita, S. Harada, H. Okamoto, H. Inoue, and H. Makino. 2001. Differential in vitro effects of IL-4, IL-10, and IL-13 on proinflammatory cytokine production and fibroblast proliferation in rheumatoid synovium. *Rheumatol. Int. 20:49*.
23. Oriente, A., N. S. Fedarko, S. E. Pacocha, S. K. Huang, L. M. Lichtenstein, and D. M. Essayan. 2000. Interleukin-13 modulates collagen homeostasis in human skin and keloid fibroblasts. *J. Pharmacol. Exp. Ther. 292:988*.
24. Leonardi, A., R. Cortivo, I. Fregona, M. Plebani, A. G. Secchi, and G. Abatangelo. 2003. Effects of Th2 cytokines on expression of collagen, MMP-1, and TIMP-1 in conjunctival fibroblasts. *Invest. Ophthalmol. Vis. Sci. 44:183*.
25. Liu, X., T. Kohyama, H. Wang, Y. K. Zhu, F. Q. Wen, H. J. Kim, D. J. Romberger, and S. I. Rennard. 2002. Th2 cytokine regulation of type I collagen gel contraction mediated by human lung mesenchymal cells. *Am. J. Physiol. 282:L1049*.
26. Doucet, C., D. Brouty-Boye, C. Pottin-Clemenceau, C. Jasmin, G. W. Canonica, and B. Azzarone. 1998. IL-4 and IL-13 specifically increase adhesion molecule and inflammatory cytokine expression in human lung fibroblasts. *Int. Immunol. 10:1421*.
27. Hirata, M., M. Kage, T. Hara, Y. Yoneda, M. Zhang, and T. Fukuma. 2001. *Schistosoma japonicum* egg granuloma formation in the interleukin-4 or interferon-γ deficient host. *Parasite Immunol. 23:271*.
28. Belperio, J. A., M. Dy, M. D. Burdick, Y. Y. Xue, K. Li, J. A. Elias, and M. P. Keane. 2002. Interaction of IL-13 and C10 in the pathogenesis of bleomycin-induced pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol. 27:419*.
29. Lee, C. G., R. J. Homer, Z. Zhu, S. Lanone, X. Wang, V. Koteliansky, J. M. Shipley, P. Gotwals, P. Noble, Q. Chen, et al. 2001. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor β_1. *J. Exp. Med. 194:809*.
30. McKenzie, G. J., A. Bancroft, R. K. Grencis, and A. N. McKenzie. 1998. A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Curr. Biol. 8:339*.
31. McKenzie, G. J., P. G. Fallon, C. L. Emson, R. K. Grencis, and A. N. McKenzie. 1999. Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses. *J. Exp. Med. 189:1565*.
32. Huffnagle, G., R. Strieter, T. Standiford, R. McDonald, M. Burdick, S. Kunkel, and G. Toews. 1995. The role of monocyte chemotactic protein-1 (MCP-1) in the recruitment of monocytes and $CD4^+$ T cells during a pulmonary *Cryptococcus neoformans* infection. *J. Immunol. 155:4790*.
33. Moore, B., M. Coffey, P. Christensen, S. Sitterding, R. Ngan, R. McDonald, S. Phare, M. Peters-Golden, R. Paine III, and G. Toews. 2000. GM-CSF regulates bleomycin-induced pulmonary fibrosis via a prostaglandin E_2 mechanism. *J. Immunol. 165:4032*.
34. Moore, B. B., M. Peters-Golden, P. J. Christensen, V. Lama, W. A. Kuziel, R. Paine III, and G. B. Toews. 2003. Alveolar epithelial cell inhibition of fibroblast proliferation is regulated by MCP-1/CCR2 and mediated by PGE_2. *Am. J. Physiol. 284:L342*.
35. Woods, J. M., K. J. Katschke, Jr., M. Tokuhira, H. Kurata, K. I. Arai, P. L. Campbell, and A. E. Koch. 2000. Reduction of inflammatory cytokines and prostaglandin E_2 by IL-13 gene therapy in rheumatoid arthritis synovium. *J. Immunol. 165:2755*.
36. Woods, J. M., M. Tokuhira, J. C. Berry, K. J. Katschke, Jr., H. Kurata, J. A. Damergis, Jr., K. Arai, and A. E. Koch. 1999. Interleukin-4 adenoviral gene therapy reduces production of inflammatory cytokines and prostaglandin E_2 by rheumatoid arthritis synovium ex vivo. *J. Invest. Med. 47:285*.
37. Noguchi, K., M. Shitashige, H. Watanabe, S. Murota, and I. Ishikawa. 1999. Interleukin-4 and interferon-γ inhibit prostaglandin production by interleukin-1β-stimulated human periodontal ligament fibroblasts. *Inflammation 23:1*.
38. Rey, A., F. Quartulli, L. Escoubet, P. Sozzani, D. Caput, P. Ferrara, and B. Pipy. 1999. IL-13 induces serine phosphorylation of $cPLA_2$ in mouse peritoneal macrophages leading to arachidonic acid and PGE_2 production and blocks the zymosan-induced serine phosphorylation of cPLA2 and eicosanoid production. *Biochim. Biophys. Acta 1440:183*.
39. Yu, C. L., M. H. Huang, Y. Y. Kung, C. Y. Tsai, Y. Y. Tsai, S. T. Tsai, D. F. Huang, K. H. Sun, S. H. Han, and H. S. Yu. 1998. Interleukin-13 increases prostaglandin E_2 (PGE_2) production by normal human polymorphonuclear neutrophils by enhancing cyclooxygenase 2 (COX-2) gene expression. *Inflamm. Res. 47:167*.
40. Spanbroek, R., M. Hildner, A. Kohler, A. Muller, F. Zintl, H. Kuhn, O. Radmark, B. Samuelsson, and A. J. Habenicht. 2001. IL-4 determines eicosanoid formation in dendritic cells by down-regulation of 5-lipoxygenase and up-regulation of 15-lipoxygenase 1 expression. *Proc. Natl. Acad. Sci. USA 98:5152*.
41. Profita, M., A. Sala, L. Siena, P. M. Henson, R. C. Murphy, A. Paterno, A. Bonanno, L. Riccobono, A. Mirabella, G. Bonsignore, et al. 2002. Leukotriene B4 production in human mononuclear phagocytes is modulated by interleukin-4-induced 15-lipoxygenase. *J. Pharmacol. Exp. Ther. 300:868*.
42. Montero, A., G. M. Nassar, S. Uda, K. A. Munger, and K. F. Badr. 2000. Reciprocal regulation of LTA_4 hydrolase expression in human monocytes by γ-interferon and interleukins 4 and 13: potential relevance to leukotriene regulation in glomerular disease. *Exp. Nephrol. 8:258*.
43. Hsieh, F. H., B. K. Lam, J. F. Penrose, K. F. Austen, and J. A. Boyce. 2001. T helper cell type 2 cytokines coordinately regulate immunoglobulin E-dependent cysteinyl leukotriene production by human cord blood-derived mast cells: profound induction of leukotriene C_4 synthase expression by interleukin 4. *J. Exp. Med. 193:123*.
44. Zaitsu, M., Y. Hamasaki, M. Matsuo, A. Kukita, K. Tsuji, M. Miyazaki, R. Hayasaki, E. Muro, S. Yamamoto, I. Kobayashi, et al. 2000. New induction of leukotriene A_4 hydrolase by interleukin-4 and interleukin-13 in human polymorphonuclear leukocytes. *Blood 96:601*.
45. Sime, P. J., Z. Xing, F. L. Graham, K. G. Csaky, and J. Gauldie. 1997. Adenovector-mediated gene transfer of active transforming growth factor-β1 induces prolonged severe fibrosis in rat lung. *J. Clin. Invest. 100:768*.
46. Roy, S. G., Y. Nozaki, and S. H. Phan. 2001. Regulation of α-smooth muscle actin gene expression in myofibroblast differentiation from rat lung fibroblasts. *Int J. Biochem. Cell Biol. 33:723*.
47. Thannickal, V. J., D. Y. Lee, E. S. White, Z. Cui, J. M. Larios, R. Chacon, J. C. Horowitz, R. M. Day, and P. E. Thomas. 2003. Myofibroblast differentiation by TGF-β1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *J. Biol. Chem.*
48. Vaughan, M. B., E. W. Howard, and J. J. Tomasek. 2000. Transforming growth factor-β1 promotes the morphological and functional differentiation of the myofibroblast. *Exp. Cell. Res. 257:180*.
49. Desmouliere, A., A. Geinoz, F. Gabbiani, and G. Gabbiani. 1993. Transforming growth factor-β1 induces α-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J. Cell Biol. 122:103*.
50. Gabbiani, G. 1981. The myofibroblast: a key cell for wound healing and fibrocontractive diseases. *Prog. Clin. Biol. Res. 54:183*.
51. Fine, A., and R. H. Goldstein. 1987. The effect of transforming growth factor-β on cell proliferation and collagen formation by lung fibroblasts. *J. Biol. Chem. 262:3897*.
52. Fine, A., C. F. Poliks, B. D. Smith, and R. H. Goldstein. 1990. The accumulation of type I collagen mRNAs in human embryonic lung fibroblasts stimulated by transforming growth factor-β. *Connect. Tissue Res. 24:237*.
53. Lukacs, N. W., C. Hogaboam, S. W. Chensue, K. Blease, and S. L. Kunkel. 2001. Type 1/type 2 cytokine paradigm and the progression of pulmonary fibrosis. *Chest 120:5S*.
54. Lucey, D. R., M. Clerici, and G. M. Shearer. 1996. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. *Clin. Microbiol. Rev. 9:532*.
55. Spellberg, B., and J. E. Edwards, Jr. 2001. Type 1/type 2 immunity in infectious diseases. *Clin. Infect. Dis. 32:76*.
56. Phan, S. H. 2002. The myofibroblast in pulmonary fibrosis. *Chest 122:286S*.