Airway Fibroblasts in Asthma Manifest an Invasive Phenotype

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***Rationale*: Invasive cell phenotypes have been demonstrated in malignant transformation, but not in other diseases, such as asthma. Cellular invasiveness is thought to be mediated by transforming growth factor (TGF)-β1 and matrix metalloproteinases (MMPs). IL-13 is a key T_H2 cytokine that directs many features of airway remodeling through TGF-β1 and MMPs.**

***Objectives*: We hypothesized that, in human asthma, IL-13 stimulates increased airway fibroblast invasiveness via TGF-β1 and MMPs in asthma compared with normal controls.**

***Methods*: Fibroblasts were cultured from endobronchial biopsies in 20 subjects with mild asthma (FEV_1: 90 ± 3.6% pred) and 17 normal control subjects (FEV_1: 102 ± 2.9% pred) who underwent bronchoscopy. Airway fibroblast invasiveness was investigated using Matrigel chambers. IL-13 or IL-13 with TGF-β1 neutralizing antibody or pan-MMP inhibitor (GM6001) was added to the lower chamber as a chemoattractant. Flow cytometry and immunohistochemistry were performed in a subset of subjects to evaluate IL-13 receptor levels.**

***Measurements and Main Results*: IL-13 significantly stimulated invasion in asthmatic airway fibroblasts, compared with normal control subjects. Inhibitors of both TGF-β1 and MMPs blocked IL-13–induced invasion in asthma, but had no effect in normal control subjects. At baseline, in airway tissue, IL-13 receptors were expressed in significantly higher levels in asthma, compared with normal control subjects. In airway fibroblasts, baseline IL-13Rα2 was reduced in asthma compared with normal control subjects.**

***Conclusions*: IL-13 potentiates airway fibroblast invasion through a mechanism involving TGF-β1 and MMPs. IL-13 receptor subunits are differentially expressed in asthma. These effects may result in IL-13–directed airway remodeling in asthma.**

Keywords: airway remodeling; interleukin-13; transforming growth factor-β; matrix metalloproteinase

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The mechanisms contributing to airway remodeling in asthma are not completely understood. Previous studies have implicated an important role for IL-13 in the initiation of signaling within the airway fibroblast to direct a number of profibrogenic functions, such as cell proliferation and migration.

What This Study Adds to the Field

This study demonstrates a novel mechanism of airway remodeling in asthma whereby IL-13 stimulates invasion of airway fibroblasts through a simulated basement membrane. IL-13–induced invasion of asthmatic airway fibroblasts is dependent on matrix metalloproteinases and transforming growth factor-β.

IL-13 directs many of the features of airway remodeling, including subepithelial airway fibrosis. Transgenic overexpression of IL-13 in the murine lung results in an asthma-like phenotype characterized by inflammation, mucus cell metaplasia, and subepithelial fibrosis (1). These studies further demonstrated that IL-13 stimulated lung fibrosis through activation of transforming growth factor (TGF)-β1 requiring matrix metalloproteinase (MMP)-9 (2). We have shown that IL-13 induced lung fibroblast secretion of profibrotic growth factors and increased proliferation of airway fibroblasts isolated from subjects with mild asthma (3, 4).

The effects of IL-13 are mediated by receptor subunits: IL-4 receptor α (IL-4Rα), IL-13 receptor α 1 (IL-13Rα1), and IL-13 receptor α 2 (IL-13Rα2). IL-13Rα1 binds IL-13 with low affinity but complexes with cell surface IL-4Rα, which binds IL-13 with high affinity. IL-13Rα2 has been hypothesized to serve as a decoy receptor for IL-13 signaling (5). Deficiency of IL-13Rα2 has been shown to augment IL-13–induced inflammation and airway remodeling in the murine lung (6).

Cellular locomotion plays a vital role in the normal wound repair process. Fibroblasts must actively migrate to the site of injury to facilitate healing (7). In airway remodeling in asthma, chronic inflammation and exposure to environmental insults mediate the process of deranged wound-healing, resulting in areas of epithelial damage and thickening of the subepithelial basal lamina. Increased numbers of airway fibroblasts in the submucosa contribute to the progression of subepithelial fibrosis (8). Several factors are known to stimulate lung fibroblast motility, including TGF-β1 (9). However, it is unknown whether IL-13 facilitates increased fibroblast motility in asthma by serving as a chemoattractant.

Many of the fibroblast responses that occur in airway and pulmonary fibrosis are apparent in the interaction between cancer cells and stromal fibroblasts. These fibroblasts actively produce proinvasive factors that facilitate metastasis (10). Fibroblast production of TGF-β1 has been implicated as a key proinvasive factor in colon, breast, and squamous carcinomas (11, 12). In

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addition, proteolytic enzymes, prominently MMP-2 and -9, are required for malignant invasion and metastasis (13). Invading cancer cells use these enzymes to degrade extracellular matrix in basement membranes (14), and the expression levels of MMPs are modulated with malignant phenotypes (15, 16).

A model of invasion of lung fibroblasts across a simulated basement membrane, Matrigel, can be used with an assay developed by Albini and coworkers (17). In the present study, we used the Matrigel assay to investigate the hypothesis that IL-13 directs airway remodeling by stimulating increased airway fibroblast invasiveness in asthma compared with normal control subjects. We further hypothesized that the relative expression levels of the IL-13 receptor subunits in asthma may alter responses to IL-13. We demonstrated that IL-13Rα2 expression in airway fibroblasts from subjects with asthma was suppressed compared with that of normal patients. Some of the results of these studies have been reported previously in the form of abstracts (18, 19).

METHODS

Details of bronchoscopy, cell culture, immunohistochemistry, flow cytometry, and ELISA experiments are provided in the online supplement.

Subjects

Thirty-seven subjects were recruited from the general Durham, North Carolina, community. The subjects with asthma fulfilled criteria for asthma (20) exhibiting a provocative concentration of methacholine resulting in a 20% fall in the FEV_1 (PC_{20} FEV_1) of less than 8 mg/ml and reversibility, as demonstrated by at least a 12% and 200 ml increase in the FEV_1 or the FVC with inhaled albuterol. All subjects with asthma were atopic, as demonstrated by at least one positive skin test to common North Carolina allergens associated with a correlative clinical history. All subjects with asthma had been diagnosed with asthma for at least 1 year before participating in the study. All normal subjects had no clinical history of atopy and were skin test negative. We required that subjects used no controller medications, but did not discontinue any medications. Thus, only subjects on as-needed short-acting β_2 agonists only were recruited. Exclusion criteria included post-bronchodilator FEV_1 less than 50% predicted; inpatient status; upper or lower respiratory tract infection within 1 month of the study; use of inhaled corticosteroids, leukotriene modifiers, or theophylline preparations within 4 weeks of the study; use of long-acting β_2 agonists within 2 weeks of the study; smoking history greater than five pack-years or any cigarette use within the previous 2 years; and significant nonasthma pulmonary disease or other medical problems. All subjects provided consent in this Institutional Review Board–approved protocol.

Matrigel Invasion Assay

Airway fibroblasts were grown to 90% confluence in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 5% CO_2 and 37°C. The cells were resuspended in serum-free media (SFM) and seeded onto Matrigel (BD Biosciences, San Jose, CA) 24-well transwell plates (8-μm pore size) at 100,000 cells per insert. The cells were incubated in the presence of SFM as control or 5–100 ng/ml IL-13 (ProSpec, Rehovot, Israel) in the lower compartment of the transwell in the presence or absence of 10 μM pan-MMP inhibitor (GM6001; Calbiochem, San Diego, CA) or 10 μg/ml TGF-β or IL-13 neutralizing antibody or mouse IgG control antibody (R&D Systems, Minneapolis, MN) for 48 hours. After the incubation, the apical supernatants were collected, the inserts were scrubbed with cotton swabs, and the lower sides of the inserts were stained using a Diff-Quik kit (Fisher Scientific, Kalamazoo, MI). The Matrigel membranes were cut from the inserts, mounted onto slides, and the stained invading cells were counted by visual inspection under ×10 magnification.

Statistical Analysis

Analyses were performed using JMP statistical software (SAS, Cary, NC). The mean values from each condition were determined, because each condition for each subject was performed in duplicate or triplicate. All data that were not normally distributed were analyzed using nonparametric Wilcoxon analysis. Data are expressed as means ± SEM, and significance is denoted by $P < 0.05$. Analyses were performed using single linear regression of the $\log_{(2)}$-transformed methacholine PC_{20} data.

RESULTS

Subjects

Subject characteristics are shown in Table 1. The subjects with asthma were atopic and considered of mild severity, because their mean percentage predicted FEV_1 was 90 ± 3.6%. The subjects with asthma exhibited significant airway hyperresponsiveness (AHR) with a methacholine PC_{20} FEV_1 of 1.17 ± 0.49 mg/ml. The normal subjects were nonatopic and exhibited normal airway responsiveness, with a methacholine PC_{20} FEV_1 of greater than 16 mg/ml ($P = 0.0001$). The subjects with asthma used albuterol as needed, and no controller medications. The normal subjects took no chronic medications.

IL-13 Stimulates Increased Airway Fibroblast Invasion in the Matrigel Assay

To link IL-13 to airway remodeling and airway fibroblast motility, we tested whether IL-13 may be a chemotactic stimulus for airway fibroblast invasion. We used the Matrigel assay to measure invasiveness of airway fibroblasts in the presence of IL-13 or SFM as untreated control in the lower chamber of the apparatus. The cells were incubated in the Matrigel assay for 48 hours. Representative photomicrograph images at ×10 magnification of invading airway fibroblasts in the Matrigel assay are shown in Figure 1, with airway fibroblasts from a subject with asthma on the upper row (Figures 1A and 1B) and a normal control subject on the lower row (Figures 1C and 1D). Untreated control assays appear on the left of the figure (Figures 1A and 1C) and IL-13–treated invading airway fibroblasts are shown on the right (Figures 1B and 1D). For all Matrigel experiments, invading airway fibroblasts are counted under ×10 magnification on a light microscope. We observed no significant difference in airway fibroblast invasion between the asthma and normal control groups at baseline (202.5 ± 77.1 cells in subjects with asthma compared with 113.8 ± 30.5 in normal control subjects; $P = 0.30$) (Figure 2A). However, the addition of IL-13 resulted in a significant increase in mean numbers of asthmatic airway fibroblasts invading through the Matrigel filters (302.7 ± 109.8 cells with IL-13 compared with 202.5 ± 77.1 cells at baseline; $P = 0.04$) (Figure 2B). In addition, IL-13 induced a significant fold change in airway fibroblast invasion in asthma compared with normal control subjects (2 ± 0.3-fold in subjects with asthma compared with 1 ± 0.1-fold change for normal control subjects; $P = 0.005$) (Figure 2C). Dose–response experiments incorporating a broad range of concentrations of IL-13

TABLE 1. SUBJECT CHARACTERISTICS

	Asthma (*n* = *20*)	Controls (*n* = *17*)	*P* Value
Sex	9M:11F	5M:12F	0.33
Age	28 ± 2	27 ± 2	0.55
Ethnicity*	1A:5AA:13W	2A:5AA:10W	0.74
FEV_1, L	3.72 ± 0.15	3.47 ± 0.17	0.29
FEV_1, % pred	90 ± 3.6	102 ± 2.9	0.01
FVC, L	4.41 ± 0.20	4.04 ± 0.21	0.21
FEV_1/FVC, % pred	84 ± 0.8	86 ± 1.1	0.12
PC_{20}, mg/ml†	1.17 ± 0.49	>16	0.0001
Medications	Albuterol	None	

* A = Asian; AA = African-American; W = White.

† The provocative concentration of methacholine resulting in a 20% fall in FEV_1.

for incubation of airway fibroblasts within the Matrigel assay revealed that only the highest concentrations tested (50 ng/ml and 100 ng/ml IL-13) significantly stimulated airway fibroblast invasion in asthma compared with the effects of the lower concentrations of IL-13 (0 ng/ml, 5 ng/ml, and 10 ng/ml; $P < 0.03$) (Figure 3A). Furthermore, only the 50 ng/ml concentration of IL-13 induced a significantly increased invasive effect in asthma compared with the normal control fibroblasts at that same concentration (460.3 ± 125.5 cells in subjects with asthma compared with 109.6 ± 60.1 cells in normal control subjects; $P = 0.04$). Therefore, all further experiments for this study were conducted using the concentration of 50 ng/ml of IL-13. The effect of IL-13 on airway fibroblast invasion in asthma was demonstrated to be specific as a neutralizing antibody specific for inhibition of IL-13 signaling significantly reduced invasiveness of airway fibroblasts isolated from subjects with asthma (187.1 ± 50 cells with media alone compared with 64.9 ± 26.6 cells with IL-13 antibody; $P = 0.02$), but had no effect on cells isolated from normal, healthy control subjects (99.7 ± 21.9 cells with media alone compared with 89 ± 30.1 cells with IL-13 antibody; $P = 0.35$) (Figure 3B). The effect of the IL-13 neutralizing antibody on airway fibroblast invasion in the untreated control asthmatic airway fibroblasts Matrigel assay was not significant ($P = 0.38$), and the inhibitor had no significant effect on the untreated normal control group ($P = 0.88$) (data not shown). Taken together, these data indicate that IL-13 stimulates airway fibroblasts to invade Matrigel and that this effect is specific for asthmatic airway fibroblasts.

IL-13–induced Airway Fibroblast Invasion Is Blocked by Inhibition of MMPs and TGF-β1

IL-13 has been shown to stimulate expression of TGF-β1 and MMPs in the murine lung as a mechanism of tissue fibrosis (2). Because mechanisms involving both MMPs and TGF-β1 have been implicated in invasion of stromal fibroblasts in the context of cancer metastasis (14), we used inhibitors for TGF-β1 or MMPs, respectively, to demonstrate whether these mediators play a role in IL-13–induced airway fibroblast invasion in asthma using the Matrigel assay. The addition of the neutralizing antibody significantly reduced the effect of IL-13 to stimulate invasion of asthmatic airway fibroblasts into the Matrigel (266.4 ± 103 cells with media alone compared with 144.6 ± 46.5 cells with TGF-β1 antibody; $P = 0.04$); however, we observed no effect of the TGF-β1 neutralizing antibody to inhibit IL-13–induced airway fibroblast invasion for the cells isolated from normal control subjects (133.7 ± 35.2 cells with media alone compared with 141 ± 70.7 cells with TGF-β1 antibody; $P = 0.46$) (Figure 4A). No significant reduction in either baseline or IL-13–induced airway fibroblast invasion was observed after incubation of cells with IgG isotype control antibody (*see* Figure E1 in the online supplement). A similar pattern of inhibition was observed when airway fibroblasts were assayed in the presence of a broad-spectrum MMP inhibitor and IL-13 in the Matrigel assay for 48 hours. IL-13–induced asthmatic airway fibroblast invasion was significantly reduced by the pan-MMP inhibitor (262 ± 135.1 cells with media alone compared with 156.7 ± 72.8 cells with MMP inhibitor; $P = 0.02$), but airway fibroblast invasiveness in cells isolated from normal control subjects was unaffected by the small molecule pan-MMP inhibitor (174.9 ± 81 cells with media alone compared with 169.2 ± 89.7 cells with MMP inhibitor; $P = 0.77$) (Figure 4B). The effect of either the TGF-β1 neutralizing antibody or the pan-MMP inhibitor on airway fibroblast invasion in the untreated control asthmatic airway fibroblasts Matrigel assay was not significant ($P = 0.29$ and $P = 0.24$, respectively), and again, neither inhibitor had any significant effect on the untreated normal control group ($P = 0.15$ and $P = 0.82$, respectively) (data not shown). Taken together, these data suggest that asthmatic airway fibroblasts invade the Matrigel with a mechanism requiring MMPs and that IL-13–directed airway fibroblast invasion in asthma may require TGF-β1 signaling.

IL-13 Stimulates MMP-2 and TGF-β1 Secretion in Airway Fibroblasts

To connect IL-13 signaling in airway fibroblasts with MMP and TGF-β1 production, we performed ELISA to measure MMP-1, MMP-2, and TGF-β1 secretion by airway fibroblasts after exposure to 50 ng/ml IL-13 for 24 or 48 hours. Compared with untreated control, IL-13 significantly stimulated MMP-2 secretion at 24 hours in asthmatic airway fibroblasts (197.8 ± 72 ng/ml for IL-13 compared with 164.4 ± 62.7 ng/ml for control; $P = 0.02$), but no significant effect of IL-13 was observed in normal control airway fibroblasts (111.8 ± 28.7 ng/ml for IL-13 compared with 102.7 ± 37.4 ng/ml for control; $P = 0.55$) (Figure 5A). No significant stimulation of MMP-1 was observed after

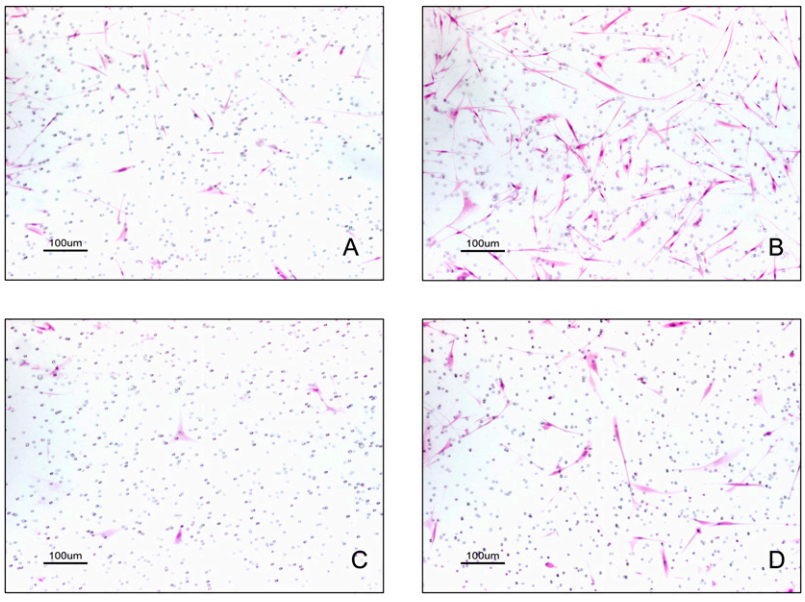

Figure 1. Photomicrographs indicating airway fibroblast invasion through the Matrigel membrane. (*A*) Image of airway fibroblasts isolated from a representative subject with asthma invading Matrigel toward serum-free media alone as untreated control. (*B*) Image of airway fibroblasts isolated from the same representative subject with asthma as *A* invading Matrigel toward IL-13 (50 ng/ml) in serum-free media. (*C*) Image of airway fibroblasts isolated from a representative normal control subject invading Matrigel toward serum-free media alone as untreated control. (*D*) Image of airway fibroblasts isolated from the same representative normal control subject as *C* invading Matrigel toward IL-13 (50 ng/ml) in serum-free media.

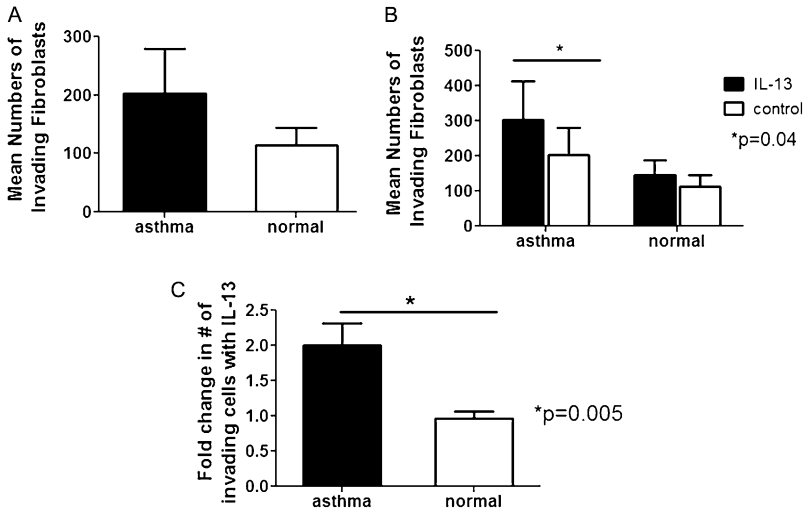

Figure 2. IL-13 stimulates airway fibroblast invasion of Matrigel in asthma. (*A*) Airway fibroblast invasiveness at baseline. No significant difference in invasion between groups was observed for the unstimulated airway fibroblasts ($P = 0.30$). (*B*) Airway fibroblasts were investigated in the Matrigel assay with 50 ng/ml IL-13 or serum-free media (as a negative control) in the lower chamber of the transwell for a 48-hour incubation period. IL-13 exerts a significant increase in mean numbers of invading fibroblasts in asthma compared with unstimulated control ($P = 0.04$); however, no effect of IL-13 was observed within the normal control subjects ($P = 0.59$). (*C*) IL-13 induces a significant increase in airway fibroblast invasion in asthma. Compared with the normal control subjects, IL-13 significantly induced airway fibroblast invasion in asthma ($P = 0.005$). Data are expressed as fold change in the mean numbers of airway fibroblasts invading the Matrigel as normalized to the negative control. Subjects with asthma (n = 20; FEV_1: 90 ± 3.6%) and normal control subjects (n = 17; FEV_1: 102 ± 2.9%), mean ± SEM.

IL-13 treatment in either asthmatic or normal control samples (data not shown). In addition, IL-13 significantly stimulated TGF-β1 secretion at 48 hours in asthmatic airway fibroblasts compared with untreated control (389.4 ± 224.5 pg/ml for IL-13 compared with 199.6 ± 89.6 pg/ml for control; $P = 0.02$), but no significant effect of IL-13 on TGF-β1 production was observed in normal control airway fibroblasts (184.9 ± 61.4 pg/ml for IL-13 compared with 162.2 ± 52.5 pg/ml for control; $P = 0.09$) (Figure 5B).

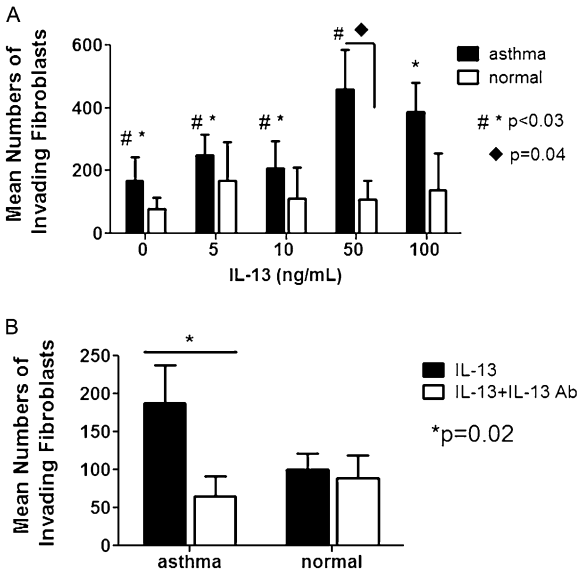

Figure 3. IL-13 stimulates airway fibroblast invasion in asthma. (*A*) Airway fibroblasts were investigated in the Matrigel assay with a range of concentrations of IL-13 (5–100 ng/ml) or serum-free media (0 ng/ml) in the lower chamber of the transwell for a 48-hour incubation period. The 50 ng/ml concentration of IL-13 induced significantly increased invasion in asthma compared with normal control fibroblasts (*solid diamond*: $P = 0.04$). Both the 50 ng/ml (#) and the 100 ng/ml (*) concentrations of IL-13 induced significantly increased numbers of invading cells in asthma compared with the 0, 5, and 10 ng/ml concentrations of IL-13 ($P < 0.03$). No significant differences in invasion in response to IL-13 were observed for the normal control fibroblasts ($P > 0.08$ for all comparisons). Subjects with asthma (n = 8; FEV_1: 85 ± 5.2%) and normal control subjects (n = 5; FEV_1: 105 ± 3%), mean ± SEM. (*B*) IL-13 neutralizing antibody blocked IL-13–induced airway fibroblast invasion in asthma. Significant reduction in IL-13–induced (50 ng/ml) airway fibroblast invasion was observed in response to 10 μg/ml IL-13 neutralizing antibody in asthma ($P = 0.02$), but not in the normal control fibroblasts ($P = 0.35$) or in the unstimulated (negative control) airway fibroblast invasion ($P = 0.38$) (data not shown). Subjects with asthma (n = 7; FEV_1: 78 ± 5.3%) and normal control subjects (n = 5; FEV_1: 108 ± 6.4%), mean ± SEM.

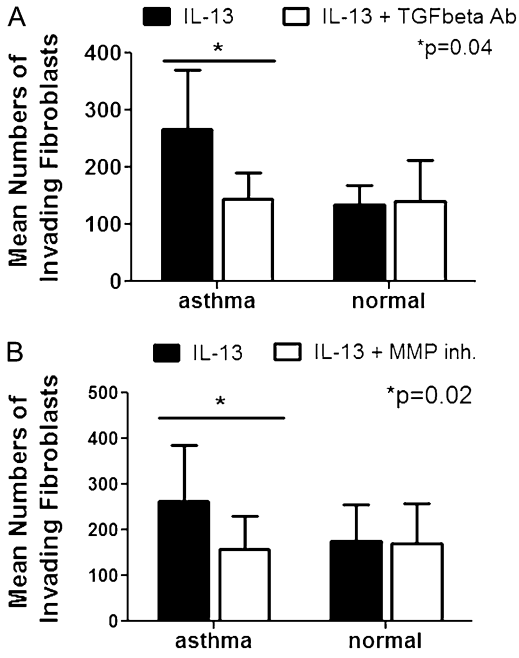

Figure 4. Transforming growth factor (TGF)-β neutralizing antibody and pan-matrix metalloproteinase (MMP) inhibitor block IL-13–induced airway fibroblast invasion in asthma. (*A*) Significant reduction in IL-13–induced (50 ng/ml) airway fibroblast invasion was observed in response to 10 μg/ml TGF-β1 neutralizing antibody in asthma ($P = 0.04$), but not in the normal control fibroblasts ($P = 0.15$) or in unstimulated (negative control) asthmatic airway fibroblast invasion ($P = 0.29$) (data not shown). (*B*) A pan-MMP inhibitor (GM6001; 10 μM) elicited significant reduction in asthmatic airway fibroblast invasion in response to 50 ng/ml IL-13 ($P = 0.02$); however, no effect was observed in the normal control fibroblasts ($P = 0.82$). Subjects with asthma (n = 13; FEV_1: 92 ± 4.8%) and normal control subjects (n = 9; FEV_1: 108 ± 4.0%), mean ± SEM.

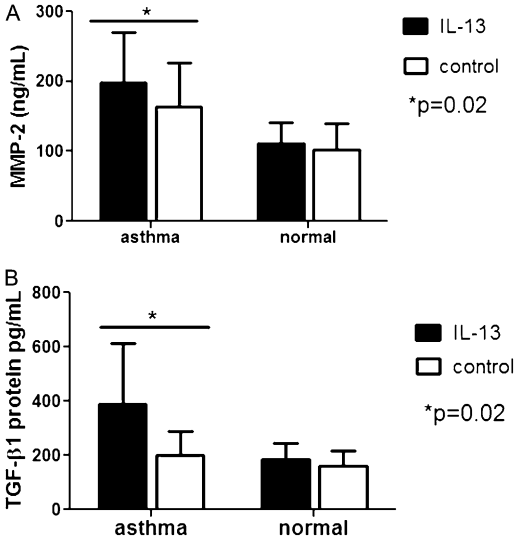

Figure 5. IL-13 significantly stimulates matrix metalloproteinase (MMP)-2 and transforming growth factor (TGF)-β1 secretion by airway fibroblasts in asthma. A subset of airway fibroblasts was grown to confluence in 10% fetal bovine serum and Dulbecco's modified Eagle medium. Cells were rendered quiescent by incubating in serum-free media for 24 hours before incubating with 50 ng/ml IL-13 in serum-free media or serum-free media alone as untreated control for a 24-hour (*A*, MMP-2) or 48-hour (*B*, TGF-β1) time course. Cell culture supernatants were collected and acid-treated for measurement of total TGF-β1 or diluted ×5 for measurement of total MMP-2 using colorimetric sandwich ELISA (R&D Systems, Minneapolis, MN). Subjects with asthma (n = 9; FEV_1: 88 ± 7.1%) and normal control subjects (n = 6; FEV_1: 102 ± 4.9%), mean ± SEM.

IL-13–induced Airway Fibroblast Invasion Inversely Correlates with Methacholine PC_{20}

Because airway remodeling may ultimately lead to diminished lung function in patients with asthma (21), we assessed the relationship between IL-13–induced invasion of airway fibroblasts in asthma and various measurements of lung function. We used single linear regression to determine the existence of significant correlations between the mean number of airway invading fibroblasts with IL-13 and FEV_1% predicted, FEV_1/FVC% predicted, or methacholine PC_{20} measurements in patients. We found no significant association between IL-13–induced airway fibroblast invasion in asthma or normal control in relation to FEV_1% predicted or FEV_1/FVC% predicted. However, methacholine PC_{20} was significantly inversely correlated with mean numbers of IL-13–induced airway fibroblast invasion in asthma ($r = -0.62$; $P = 0.0005$) (Figure 6).

IL-13R Subunits Expression Is Elevated at Baseline in Asthma

Because IL-13 signaling has been shown to direct airway remodeling in asthma, we sought to determine the relative expression levels of the three IL-13 receptor subunits at baseline in a subset of both subjects with asthma and normal control subjects. Sections of biopsies from these subjects were evaluated using immunohistochemistry with antibodies specific for IL-13Rα2, IL-13Rα1, or IL-4Rα. As shown in Figures 7A, 7D, and 7G, expression levels for all three IL-13R subunits are significantly higher in airway tissue in subjects with asthma compared with normal control subjects (IL-13Rα1: 3.5 ± 1.3% in asthma compared with 0.5 ± 0.2% in normal; IL-13Rα2: 6.1 ± 1.4% in asthma compared with 2.9 ± 0.8% in normal; IL-4Rα: 21.3 ± 4.3% in asthma compared with 10.5 ± 2.5 in normal; $P < 0.05$). Staining for IL-13Rα1 and IL-13Rα2 expression (Figures 7B, 7C, 7H, and 7I) was most prominent in airway epithelial cells and inflammatory cells. Positive staining for IL-4Rα was observed in airway epithelial cells, smooth muscle bundles, and inflammatory cells (Figures 7E and 7F).

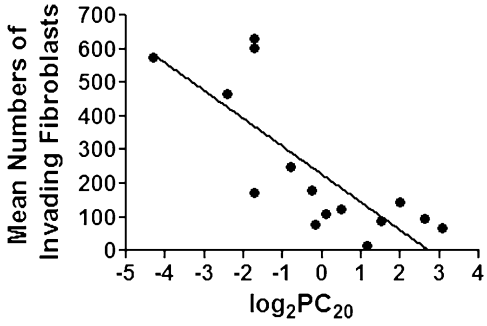

Figure 6. Airway fibroblast invasion is correlated with methacholine PC_{20}. Single linear regression of log_2-transformed methacholine PC_{20} data from subjects with asthma reveals significant and inverse correlation with mean numbers of invading airway fibroblasts in the Matrigel assay after incubation with 50 ng/ml IL-13 for 48 hours ($r = -0.62$; $P = 0.0005$). Subjects with asthma (n = 15, FEV_1: 91 ± 4%). Data points are mean numbers of IL-13–induced invading fibroblasts from duplicate or triplicate experiments for each subject.

Cell Surface Expression of IL-13Rα2 Is Suppressed in Asthma

To determine the degree to which the observed baseline expression of the IL-13 receptor subunits may be attributed to cell surface expression on airway fibroblasts, *ex vivo* cultures of these cells from human subjects with asthma and normal control subjects were evaluated using flow cytometry. At baseline, airway fibroblast IL-13Rα2 cell surface expression levels were significantly reduced in subjects with asthma compared with normal control subjects (2,044 ± 487 median fluorescence intensity [MFI] in subjects with asthma compared with 10,596.3 ± 4,966 MFI in normal control subjects; $P = 0.04$). In contrast, the median fluorescence intensity of both IL-13Rα1 and IL-4Rα on airway fibroblasts was not significantly different between subjects with asthma and control subjects (6,666.5 MFI in subjects with asthma compared with 6,454.7 MFI in normal control subjects, $P = 0.96$ for IL-13Rα1; 1,991.8 MFI in subjects with asthma compared with 2,120 MFI in normal control subjects, $P = 0.88$ for IL-4Rα) (Figure 8).

DISCUSSION

The invasion of fibroblasts into the submucosal region of the airway in patients with asthma is one of several key features of airway remodeling (22). In a normal response, fibroblasts are thought to be drawn to the subepithelial compartment of the airway after injury or environmental insult, and these cells begin wound healing through deposition of extracellular matrix proteins and interaction with inflammatory cells (23). In asthma, this wound healing and remodeling response in the airway becomes deranged and uncontrolled, leading to increased numbers of fibroblasts invading the submucosa and contributing to subepithelial fibrosis with diminished lung function over time (24). The numbers of myofibroblasts localized to the region correlates with basement membrane thickening (25, 26), and the thickness of the subepithelial layer and the reticular basement membrane has been shown to be associated with severity of asthma (27).

In the present study, we show for the first time that airway fibroblasts isolated directly from patients with asthma and stimulated with IL-13 as a chemoattractant invade a simulated

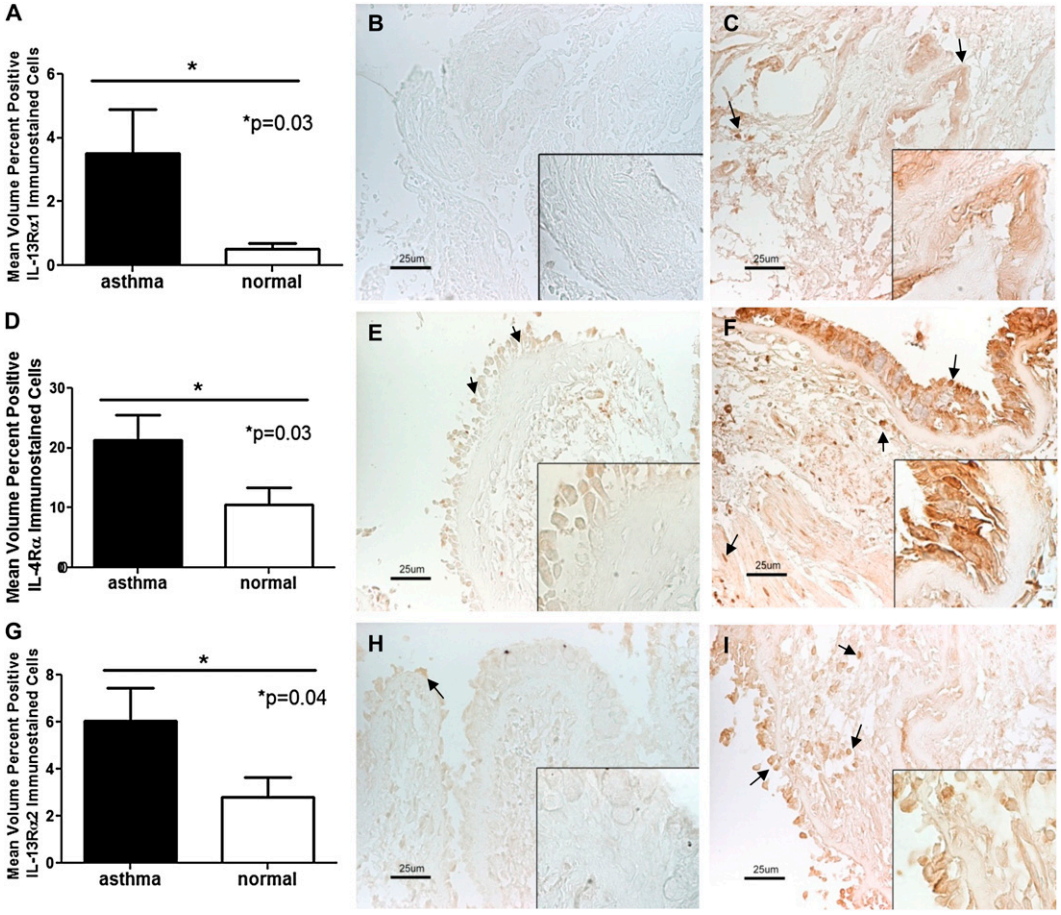

Figure 7. IL-13 receptor subunits expression is significantly augmented in asthmatic tissue. We observed significantly elevated expression of each of the IL-13 receptor subunits (IL-13Rα1, IL-13Rα2, and IL-4Rα) in asthma compared with normal control ($P < 0.05$). (*A*) IL-13Rα1, (*D*) IL-4Rα, and (*G*) IL-13Rα2 immunohistochemical staining of airway biopsy sections from eight subjects with asthma and eight normal subjects. Data are expressed as mean volume percent positive IL-13Rα1, IL-4Rα, or IL-13Rα2 immunostained cells ± SEM. Representative images of IL-13 receptor subunit immunostaining in normal control (*B*, *E*, *H*) and asthmatic (*C*, *F*, *I*) airway biopsies. Note positive staining in epithelial cells, inflammatory cells, and smooth muscle bundles (*arrows*).

basement membrane in significantly greater numbers than those isolated from normal control subjects. We also demonstrate that the IL-13–induced airway fibroblast invasion response in asthma is mediated by MMPs and TGF-β1. Our data show that both MMPs and TGF-β1 were required for the IL-13–induced asthmatic airway fibroblast invasion, and airway fibroblast secretion of both MMP-2 and TGF-β1 was significantly induced by IL-13, suggesting that both MMPs and TGF-β1 are critical mediators of the IL-13 pathway for invasion in asthma. We postulate that in the Matrigel assay, IL-13 acts a chemoattractant for airway fibroblast invasion. As an IL-13 chemical gradient is established within the transwell plate, the higher concentration of IL-13 in the lower well stimulates invasion of airway fibroblasts. The cytokine binds to the IL-13 receptors on the cell surface of the airway fibroblast, initiating secretion of TGF-β1 and MMPs capable of degrading Matrigel. IL-13–induced TGF-β1 expression in airway fibroblasts may result in phenotypic transition of the fibroblasts to activated myofibroblasts, which have been demonstrated in the oncology literature to be proinvasive (11, 12). Lee and colleagues (2) demonstrated that targeted transgenic overexpression of IL-13 in the murine lung resulted in significantly increased expression of MMP-9 mRNA, and IL-13–induced activation of TGF-β1 *in vivo* was mediated by an MMP-9–dependent mechanism. Thus, our findings reported here are supported by these data in that IL-13 stimulates airway fibroblast invasion and airway remodeling in asthma through a mechanism that requires the action of MMPs and TGF-β1.

Our data also highlight a significant and fundamental difference between subjects with asthma and normal healthy subjects because airway fibroblasts isolated from subjects with asthma invade the Matrigel with greater numbers and respond to IL-13 with significant differences. One possible explanation for the difference in responses to IL-13 between asthmatic and normal control airway fibroblasts may be differential expression levels of the IL-13 receptor subunits on these cells in the two groups of subjects. IL-13 signaling in the airway fibroblast is mediated through a complex of receptor subunits (IL-13Rα1 and IL-4Rα)

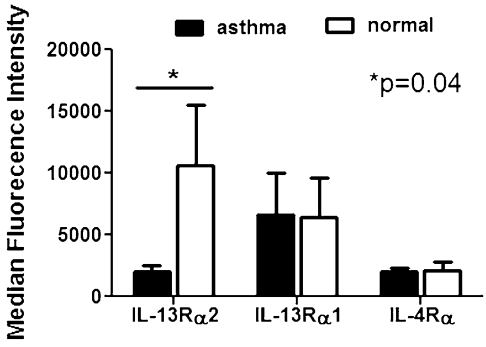

Figure 8. Cell surface receptor expression of IL-13Rα2, IL-13Rα1, and IL-4Rα on airway fibroblasts as measured by flow cytometry. Median fluorescence intensity ± SEM of each of the IL-13 receptor subunits was evaluated using flow cytometry in airway fibroblasts from eight subjects with asthma (FEV_1: 83 ± 5.1%) and six normal subjects (FEV_1: 104 ± 3.7%). At baseline, airway fibroblast cell surface expression of IL-13Rα2 is significantly reduced in asthma compared with normal control ($P = 0.04$), whereas expression of IL-13Rα1 and IL-4Rα is not significantly different between subject groups.

localized to the cell-surface (5). Although some evidence has shown that IL-13 may signal through IL-13Rα2 to induce TGF-β1 and fibrosis in the colon, IL-13Rα2 is generally thought to act as a decoy receptor to negatively regulate IL-13-signaling (28, 29). Deficiency of IL-13Rα2 in the murine lung has been shown to increase IL-13–induced airway inflammation and features of airway remodeling (6). Recent reports from research on asthma and airway remodeling lend support to the role of IL-13Rα2 acting as a nonsignaling, inhibitory receptor. Townley and coworkers (30) used IL-13Rα2-IgGFc fusion protein as a blocking pretreatment to inhibit IL-13–stimulated sensitization to methacholine in the murine airway. The authors found that IL-13Rα2 pretreatment was more effective than salmeterol–fluticasone in preventing IL-13–induced bronchial hyperresponsiveness (30). Also, pulmonary expression of IL-13 was reported to play a role in esophageal tissue remodeling, because mice that overexpressed IL-13 in the lung, and were also genetically deficient for IL-13Rα2, developed significantly more severe esophageal remodeling than IL-13Rα2–sufficient mice (31). In the present study, we demonstrate that in human asthma, airway fibroblast cell-surface expression of IL-13Rα2 is suppressed compared with normal control subjects. However, the airway fibroblast cell-surface levels of IL-13Rα1 and IL-4Rα were not significantly changed in subjects with asthma relative to normal control subjects. Our data indicating that significantly higher levels of IL-13Rα2 are expressed overall in asthmatic airway tissue compared with normal control subjects is somewhat in contrast with our data using airway fibroblasts in primary cultures *ex vivo*. However, the IL-13Rα2 airway tissue data take into account all cell types that stained positive for IL-13Rα2, including mesenchymal cells, and particularly epithelial cells, which showed marked positive staining for IL-13α2. Our *ex vivo* cultures of airway fibroblasts have been well-characterized to be primarily of a fibroblast phenotype, with little (<5%) α-smooth muscle actin–positive staining. We recognize that exposure to mediators, such as IL-13, or even the Matrigel itself, may alter the phenotype of fibroblasts; therefore, work is ongoing to characterize further the phenotype of these cells during the invasion process. Taken together, these data suggest that low expression levels of IL-13Rα2 in human asthmatic airway fibroblasts may contribute to airway remodeling through a lack of regulation of IL-13 signaling, thus stimulating profibrotic processes, such as fibroblast proliferation and invasion.

IL-13 and IL-13 receptor subunit expression has been implicated in cellular invasion in cancer and rheumatoid arthritis. Fujisawa and colleagues (32) used the Matrigel assay to demonstrate that invasion of pancreatic cancer cells increased significantly after stimulation with IL-13 and transfection with *IL-13Rα2.* The authors also linked the IL-13–induced invasion response in pancreatic cancer to increased levels of MMP-9, -12, and -14 (32). In lymphoma cells, IL-13 has been shown to induce MMP-10 expression, and invasion of these cells in the Matrigel assay was demonstrated to be dependent on MMP-10 activity (33). Thus, these observations are consistent with the present study and suggest that IL-13 and expression of IL-13 receptor subunits modulates cellular invasiveness in a variety of cell types during the pathogenesis of cancer and other diseases, such as asthma.

We also show that IL-13–induced asthmatic airway fibroblast invasion is significantly and inversely correlated with methacholine $\log_2 PC_{20}$, an index of AHR. Production of IL-13 and infiltration of IL-13–postive cells in the submucosal region of the airway has been associated with AHR (34, 35). In addition to T_H2 lymphocytes, IL-13 is secreted by many cell types in the asthmatic airway, including macrophages (36), mast cells (37), airway smooth muscle cells (38), and airway epithelial cells (39). Our histologic examination of endobronchial biopsy airway tissue sections from these patients indicated the presence of inflammatory cells that may be capable of producing IL-13 and stimulating airway fibroblast invasion. Expression of extracellular matrix by infiltrating airway myofibroblasts in the subepithelial region of the airway has been associated with AHR and may contribute to airway wall thickening and airway lumen narrowing leading to altered airway compliance and AHR (40). Invasion of airway fibroblasts into the submucosal region of the airway is likely to occur early in the repair response because these fibroblasts are drawn to areas of IL-13 secretion in the airway after epithelial damage and inflammation.

The patients included in the present study were subjects with mild asthma whose disease was controlled without the use of corticosteroids. We have demonstrated that IL-13 differentially increases fibroblast proliferation in this mild population (4). Subjects with severe asthma are a logical extension of this work to determine if their fibroblasts demonstrate more of an invasive phenotype. A previous report from our laboratory demonstrated that procollagen I expression was significantly increased in subjects with severe asthma, compared with subjects with mild asthma or normal control subjects after exposure to platelet-derived growth factor-BB (PDGF-BB) (41). The effect of PDGF-BB in severe asthma was attributed to significantly increased levels of the PDGF receptor-β at baseline on the severe asthmatic fibroblasts compared with the mild asthmatic and normal control fibroblasts. This study did not explore the effect of IL-13 on procollagen I expression in asthma. Therefore, ongoing studies in our laboratory will determine if IL-13 stimulates increased collagen deposition in the airways of both mild and severe subjects with asthma and if this effect is associated with increased airway fibroblast invasion and AHR.

In conclusion, we have demonstrated that IL-13 significantly stimulates human asthmatic airway fibroblast invasion in a Matrigel assay compared with that observed for normal control subjects. The mechanism of IL-13–induced airway fibroblast invasion in human asthma required both MMPs and TGF-β1, and we have also shown that airway fibroblasts cultured from subjects with asthma expressed reduced levels of IL-13Rα2 at baseline compared with normal control subjects. Goals of future studies include determining the direct effect that IL-13 receptor subunits expression may have on airway fibroblast invasion through knockdown of IL-4Rα, knockdown of specific MMPs, and characterization of the gene expression profile of invading fibroblasts to define further this feature of airway remodeling.

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