

Interleukin-13 induces collagen type-1 expression through matrix metalloproteinase-2 and transforming growth factor- $\beta$ 1 in airway fibroblasts in asthma

Rafael Firszt, MD,<sup>a</sup> Dave Francisco, MSc,<sup>b</sup> Tony D. Church, BSc,<sup>b</sup> Joseph M. Thomas, MSc<sup>b</sup>, Jennifer L. Ingram, PhD,<sup>b</sup> Monica Kraft, MD<sup>b</sup>

From <sup>a</sup>University of Utah, Department of Pediatrics, Division of Allergy, Immunology and Rheumatology and <sup>b</sup>Duke University Medical Center, Division of Pulmonary, Allergy and Critical Care, Department of Medicine, Durham, North Carolina.

Reprint requests: Monica Kraft, MD, Duke University Medical Center, Medical Sciences Research Building 1, Room 275, Durham, NC, 27710, USA. Email:monica.kraft@duke.edu

Supported by NIH-NHLBI HL-05-009

**Highlight Summary Sentence:**

IL-13 potentiates collagen production in a TGF- $\beta$ 1-dependent manner providing insight into the mechanism involved in airway remodeling in asthma.

**ABSTRACT**

**Background:** Airway remodeling is a feature of asthma that contributes to loss of lung function. One of the central components of airway remodeling is subepithelial fibrosis. Interleukin-13 (IL-13) is a key TH2 cytokine and is believed to be the central mediator of allergic asthma including remodeling, but the mechanism driving the latter has not been elucidated in human asthma.

**Objective:** We hypothesized that IL-13 stimulates collagen type-1 production by the airway fibroblast in a MMP- and TGF- $\beta$ 1-dependent manner in human asthma as compared to healthy controls.

**Methods:** Fibroblasts were cultured from endobronchial biopsies in 14 subjects with mild asthma and 13 normal controls that underwent bronchoscopy. Airway fibroblasts were treated with various mediators including IL-13 and specific MMP-inhibitors.

**Results:** IL-13 significantly stimulated collagen type-1 production in asthma as compared to normal controls. Inhibitors of MMP-2 significantly attenuated collagen production in asthma but had no effect in normal controls. IL-13 significantly increased total and active forms of TGF- $\beta$ 1, and this activation was blocked using an MMP-2 inhibitor. IL-13 activated endogenous MMP-2 in asthma patients as compared to normal controls.

**Conclusion:** In an *ex-vivo* model, IL-13 potentiates airway remodeling through a mechanism involving TGF- $\beta$ 1 and MMP-2. These effects provide insights into the mechanism involved in IL-13-directed airway remodeling in asthma.

**Key Words:** *Asthma, collagen, airway fibroblasts, Interleukin-13, airway remodeling*

Abbreviations:

$\alpha$ -SMA: Alpha-Smooth Muscle Actin

*COL1A1*: Collagen type 1  $\alpha$ -1

*COL1A2*: Collagen type 1  $\alpha$ -2

*COL3A1*: Collagen type 3  $\alpha$ -1

DMEM: Dulbecco's Modified Eagle's Medium

FBS: fetal bovine serum

FEV1: forced expiratory volume in one second

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

IL-13: Interleukin-13

IL-4: Interleukin-4

MMPs: Matrix Metalloproteinases

MMPi: Matrix Metalloproteinase inhibitor

*MMP2*: Matrix Metalloproteinase-2

MMP2i: Matrix Metalloproteinase-2 inhibitor

RLU: relative luciferase unit

RT-PCR: reverse transcriptase polymerase chain reaction

PC<sub>20</sub>FEV1: provocative concentration that causes the FEV1 to fall by 20%

TH2: T-helper 2

TIMP: Tissue Inhibitor of Metalloproteinase

TGF- $\beta$ 1: Transforming Growth Factor- $\beta$ 1

TGF- $\beta$ RII: TGF- $\beta$  Receptor II

## Introduction

Asthma is a chronic inflammatory disease of the airways, where specific inflammatory phenotypes are dominated by T-helper 2 (TH2) cytokines. Interleukin-13 (IL-13), which is primarily produced by T lymphocytes, is considered to be the central mediator of allergic asthma [1]. This chronic TH2-driven inflammation causes tissue injury that is believed to cause structural changes and airway remodeling in asthma [2]. Airway remodeling contributes significantly to airway dysfunction and persistent clinical symptoms in patients with asthma [3]. Furthermore, a significant proportion of patients with severe asthma develop progressive and persistent airflow limitation over time, providing further evidence that remodeling plays a role in impairment of lung function [4]. However, the direct effects of TH2 cytokines on the remodeling process have not been extensively studied in human disease.

One of the key characteristics of airway remodeling is subepithelial fibrosis caused by deposition of extracellular collagen and other matrix components [5]. The primary collagen within the airway extracellular matrix is type-I collagen; however, type-III and type-V are also major constituents of the subepithelial basement membrane [6]. This collagen is produced by fibroblasts and myofibroblasts, and the degree of fibrosis correlates with increased numbers of these cells in asthma [7].

Collagen deposition in tissues is believed to be controlled by matrix metalloproteinases (MMPs) and their corresponding tissue inhibitors of metalloproteinases (TIMPs). MMPs are a family of enzymes characterized by a common zinc ion at their active site. In general, MMPs and TIMPs are thought to be involved in the normal maintenance of extracellular matrix; however, they have also been shown to be involved in inflammation and cell-cell signaling [8]. Various cells are involved in producing the different MMPs. For example, macrophages, but not fibroblasts, produce MMP-9, whereas MMP-2 is produced by variety of cells, including fibroblasts.[9] Zheng, *et al.* reported that, MMP-2 and MMP-9 were elevated in the BAL fluids of IL-13 transgenic mice [10].

Transforming growth factor-beta1 (TGF- $\beta$ 1) is produced by many cells within the lung, including fibroblasts, and is a well-characterized mediator of tissue fibrosis [11]. TGF- $\beta$ 1 mRNA has been shown to be increased in moderate-to-severe asthmatics, and its expression was shown to be directly related to subepithelial fibrosis [12]. Previous reports have shown that IL-13 functions through TGF- $\beta$ 1 to induce a pro-fibrotic response [13-18]. Mechanisms involved in stimulating TGF- $\beta$ 1 have included both an increase in latent production and/or increased activation through an MMP-dependent mechanism. In IL-13 transgenic mice, MMP-9 was shown to play an important role in activating TGF- $\beta$ 1, demonstrating that MMPs may be an important link between IL-13 and TGF- $\beta$ 1 [14]. In addition, IL-13-induced fibrosis was significantly reduced in MMP-9-deficient mice.

Despite the evidence that IL-13 is involved in airway remodeling in murine models of asthma, its effects have yet to be demonstrated in human subjects. For the present study, we chose an *ex vivo* method of analyzing airway fibroblasts isolated directly from well-characterized patients with asthma to test our hypothesis that IL-13 modulates collagen production by airway fibroblasts in asthma and that MMPs and TGF- $\beta$ 1 are critical mediators in this process. Our findings have therapeutic implications given that IL-13 antagonists for humans are now under study for treatment of asthma.

## **METHODS**

### **Subject Recruitment**

Twenty-seven research participants were recruited from the general Research Triangle, North Carolina community. The asthmatics fulfilled criteria for asthma by exhibiting a provocative concentration of methacholine resulting in a 20% fall in the forced expiratory volume in one second (PC<sub>20</sub> FEV<sub>1</sub>) of < 8 mg/ml and reversibility, as demonstrated by at least a 12% and 200 ml increase in the FEV<sub>1</sub> or the forced vital capacity (FVC) with inhaled albuterol [19]. All participants provided informed consent in this Duke Institutional Review Board-approved protocol. Additional details are available in the online supplement.

### **Bronchoscopy**

Subjects underwent bronchoscopy with endobronchial biopsy as previously described [20].

### **Airway Fibroblast Culture**

Endobronchial tissue sections were placed on collagen-coated plates and fibroblasts were cultured as previously described [21]. As numerous passages can induce cellular metaplasia and senescence, only cells from the first three passages were used for experimentation [22].

### **Mediator Exposure**

When the cells were at confluence for seven days, media was changed, and cells were incubated in serum-free media for 24 hours. Cells were subsequently treated with and without IL-13 (ProSpec, Rehovot, Israel; 50ng/ml) for 48 hours, respectively. In addition, cells were treated with various MMP-inhibitors (MMPIs) and TGF- $\beta$ RII neutralizing antibody. For detailed description see online supplement.

### **Quantitative Real-time RT-PCR**

Total RNA from airway fibroblasts was extracted using Trizol after exposure to mediators. Relative mRNA expression was calculated by normalization of all expression levels to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and then compared to untreated control cells by the  $\Delta\Delta C_T$  method.[23]. See online supplement for additional details.

## **ELISA**

Supernatants from cultured airway fibroblasts were collected at 48 hours after mediator treatment in asthma and normal subjects. Measurement of collagen type-1 protein was performed by using a commercial ELISA kit according to manufacturer's protocol (MD Bioproducts, St. Paul, MN). Results are reported as a ratio of IL-13-treated cells to untreated fibroblasts. Measurement of TGF- $\beta$ 1 protein was performed using a commercial ELISA kit according to manufacturer's protocol (R&D, Minneapolis, MN).

## **MMP-2 Activation Assay**

Supernatants from cultured airway fibroblasts were collected at 48 hours after IL-13 (50 ng/ml) treatment in asthma and normal subjects and compared to untreated fibroblasts (baseline). Measurement of MMP-2 activity was performed by using a commercial activity assay system according to manufacturer's protocol (GE Healthcare, Buckinghamshire, UK). Results are reported as a ratio of IL-13-treated cells to untreated fibroblasts. For additional details, see online supplement.

## **Luciferase Activity**

Supernatants from cultured airway fibroblasts were collected at 48 hours after IL-13 (50 ng/ml) treatment in asthma and normal subjects. Measurement of active TGF- $\beta$ 1 was performed using a CCL64 mink lung epithelial cell line (generous gift from Dr. S. Mukherjee, Duke University) with a PAI1 luciferase reporter system This assay has been previously used and is sensitive and specific for TGF- $\beta$  [24, 25]. Luciferase

activity was developed according to protocol instructions (Perkin Elmer, Waltham, MA) and measured using a luminometer (BMG Labtech, Ortenberg, Germany). Results are expressed as relative luciferase units (RLU).

### **Western Blot Analysis**

Cell lysates from cultured airway fibroblasts were collected at 48 hours after IL-13 (50 ng/ml) treatment and probed for  $\alpha$ -Smooth Muscle Actin ( $\alpha$ -SMA), Desmin and GAPDH. The blot was developed by enhanced chemiluminescence, documented with the Kodak Image Station 4000 MM PRO and quantified using Carestream Molecular Image software (version 5.0). The western blot analysis was performed in 6 asthma and 4 normal control subjects demonstrating consistent bands at the correct molecular weights to confirm reproducibility. Densitometry analysis of Western blots was performed with the use of PDI Imageware System. Results are reported as ratio IL-13 treated cells to untreated fibroblasts. For additional details, see online supplement.

### **Statistical Analysis**

Statistical evaluation was performed using JMP statistical software (SAS, Cary, NC). The mean values from each condition were determined as each condition was performed in triplicates. Data are expressed as means  $\pm$  SEM. All data were compared using a 2-tailed Wilcoxon rank sum test. Significance is denoted by  $P < 0.05$ .

## Results

### Subjects

Subject characteristics are shown in **Table 1**. Asthmatic subjects were mild as defined by the NAEPP criteria [26].

### Collagen gene expression by airway fibroblasts

We evaluated *COL1A1*, *COL1A2* and *COL3A1* gene expression by airway fibroblasts in subjects with asthma and healthy controls in response to various mediators. We observed similar levels of mRNA expression in airway fibroblasts derived from subjects with asthma and from normal subjects at baseline, with no treatment, for collagen I and III (data not shown). IL-13 exposure significantly increased expression of *COL1A2* (Fold change between IL-13-treated compared to untreated fibroblasts:  $1.56 \pm 0.1$  in asthma compared to  $0.808 \pm 0.23$  in normal subjects,  $p < 0.05$  asthma versus normal subjects and within the asthma group) (**Figure 1**). However, there was no significant change in expression for *COL1A1* or *COL3A1* when comparing asthmatic subjects to normal subjects or within the asthma group (**Figure 1**).

Secondly, we treated these airway fibroblasts with a pan-MMP inhibitor in combination with IL-13 for 48 hours to evaluate whether MMPs are necessary for IL-13-induced *COL1A2* expression. This pan-MMP inhibitor blocks MMP-1,-2,-8, and-9. Our results demonstrate that the pan-MMP inhibitor significantly attenuated the IL-13-induced *COL1A2* expression (Fold change from IL-13 + pan-MMP inhibitor to IL-13 treated fibroblasts:  $0.48 \pm 0.13$  within asthma compared to  $0.93 \pm 0.13$  in normal subjects,  $p < 0.05$  comparing asthma vs. normal controls and within the asthma group) (**Figure 2**).

Since MMP-2 is produced by fibroblasts and is presumably inhibited by the pan-MMP inhibitor, we next evaluated whether MMP-2 may be involved in our observed IL-13-induced *COL1A2* expression in airway fibroblasts. Therefore, we co-treated airway fibroblasts with IL-13 and an inhibitor specific for MMP-2 for 48 hours. Once again, we found that *COL1A2* expression was significantly attenuated after co-incubating the

fibroblasts with both IL-13 and a specific MMP-2 inhibitor (Fold change from fibroblasts treated with IL-13 + MMP-2 inhibitor to IL-13 alone:  $0.38 \pm 0.11$  in asthma compared to  $1.10 \pm 0.13$  in normal subjects,  $p < 0.01$  between asthma and normal subjects and within the asthma group) (**Figure 2**). There was no significant effect on *COL1A2* expression following exposure of the cells to specific MMP-1 or MMP-3 inhibitors (data not shown). Although MMP-9 was shown to be involved in murine models of airway remodeling, we did not evaluate its effects in this model since we were unable to detect any measurable MMP-9 expression in the airway fibroblasts in this study (data not shown).

To determine whether TGF- $\beta$ 1 was involved in *COL1A2* expression, we incubated airway fibroblasts with a TGF- $\beta$ R2 neutralizing antibody (20  $\mu$ g/ml) in combination with IL-13 (50 ng/ml) for 48 hours. Using real time RT-PCR, our results show that *COL1A2* mRNA expression was significantly attenuated when TGF- $\beta$ R2 neutralizing antibody was added to the airway fibroblast cultures in the presence of IL-13 (Fold change from IL-13 + TGF- $\beta$ R2 antibody to IL-13-treated fibroblasts:  $0.47 \pm 0.17$  in asthma compared to  $1.22 \pm 0.11$  in normal subjects,  $p < 0.05$  between asthma and normal subjects and within asthma) (**Figure 2**). This effect was not seen using a goat IgG control antibody (data not shown).

### Collagen protein expression by airway fibroblasts

In order to evaluate whether gene expression correlated with increased collagen I protein expression, we used a commercial ELISA kit to quantify the amount of collagen protein in airway fibroblast cell culture supernatants. We incubated the fibroblasts with IL-13 for 48 hours and compared the ratio of collagen protein expression to untreated fibroblasts for both asthma and normal subjects. Although group means at baseline were not statistically different, there was individual variability within each group (Online Supplement, **Figure 7**). Therefore, the data are presented as a ratio to untreated fibroblasts. The ratio of collagen in the IL-13-treated fibroblasts as compared to the untreated fibroblasts was significantly elevated in asthma patients as compared to normal subjects (Ratio of collagen protein in IL-13-treated to untreated fibroblasts:  $1.29 \pm 0.14$  in asthma

compared to  $0.88 \pm 0.12$  in controls,  $p < 0.05$  between asthma and normal subjects and within the asthma group) (**Figure 3**).

Next, we evaluated whether an MMP-2 inhibitor would have similar effects in attenuating the amount of collagen protein expression in airway fibroblast cell culture supernatants using the same ELISA. We found that treating airway fibroblasts with an MMP-2 inhibitor and IL-13 together resulted in a significant reduction in the amount of collagen protein as compared to those fibroblasts treated with IL-13 alone, particularly in asthma subjects as compared to normal subjects (Ratio of collagen expression after exposure to IL-13 + MMP-2 inhibitor relative to IL-13 alone for collagen protein:  $0.39 \pm 0.11$  in asthma compared to  $0.97 \pm 0.04$  in controls,  $p < 0.05$  between asthma and normal subjects and within the asthma group) (**Figure 3**).

### **MMP-2 Activation**

MMP-2 is an enzyme that is secreted as a pro-enzyme requiring activation in order to function. Using an MMP-2 activity assay, we evaluated whether IL-13 treatment resulted in increased MMP-2 activation. Our results show that IL-13 significantly activated MMP-2 in airway fibroblast cell culture supernatants from asthma subjects compared to normal subjects after treating airway fibroblasts with IL-13 for 48 hours (Ratio of MMP-2 activity in IL-13-treated fibroblasts as compared to untreated fibroblasts:  $1.20 \pm 0.08$  in asthma subjects compared to  $0.76 \pm 0.09$  in normal subjects;  $p < 0.05$  between asthma and normal subjects and within the asthma group) (**Figure 4**). Therefore, our results indicate that IL-13 is likely involved in activating MMP-2 in airway fibroblasts in asthma.

### **TGF- $\beta$ 1 Expression and Activation**

To determine whether IL-13 (50 ng/ml) influenced both the total amount of TGF- $\beta$ 1, as well as the endogenously active form of TGF- $\beta$ 1, we incubated airway fibroblasts with IL-13 at 50 ng/ml for 48 hours. Using a TGF- $\beta$ 1 ELISA, we demonstrated that IL-13 significantly increased both total amount and the endogenously active form of TGF- $\beta$ 1 versus untreated fibroblasts in asthma subjects as compared to normal

subjects at 48 hours (Ratio of total TGF $\beta$ 1 from IL-13-treated fibroblasts to untreated fibroblasts:  $1.49 \pm 0.13$  in asthma compared to  $0.67 \pm 0.26$  in normal subjects; Ratio of active TGF- $\beta$ 1 from IL-13-treated to untreated fibroblasts:  $1.30 \pm 0.15$  in asthma compared to  $0.88 \pm 0.05$  in normal subjects,  $p < 0.05$  between asthma and normal controls and within the asthma group) (**Figure 5A**).

To further confirm the potential of airway fibroblasts to produce bioactive TGF $\beta$ 1 in response to IL-13, we utilized MLEC cells stably transfected with PAI-1-luciferase reporter to assay cell supernatants from cultures of airway fibroblasts from subjects with asthma or normal control subjects. Confluent airway fibroblasts were incubated with IL-13 (50 ng/ml) or serum-free media for control for 48 hours, and the cell supernatants were assayed for luciferase production. IL-13 stimulated significantly increased levels of bioactive TGF $\beta$ 1, as represented by relative luciferase units (RLU), in airway fibroblasts in asthma as compared to untreated control fibroblasts ( $124.88 \pm 26.6$  RLU for IL-13-treated fibroblasts compared to  $105.94 \pm 16.7$  RLU for untreated control fibroblasts,  $p = 0.04$  within the asthma group). No effect of IL-13 was observed in airway fibroblasts isolated from normal control subjects ( $103.14 \pm 8.0$  RLU for IL-13-treated fibroblasts compared to  $106.71 \pm 6.0$  RLU for untreated control cells) (**Figure 5B**).

Furthermore, to determine whether MMP-2 may be responsible for activating TGF- $\beta$ 1, we incubated airway fibroblasts with a specific MMP-2 inhibitor in combination with IL-13 and found that MMP-2 inhibition attenuated the activation of TGF- $\beta$ 1 in asthma subjects as compared to normal subjects at 48 hours (Ratio of active TGF $\beta$ 1 from IL-13 treated to untreated fibroblasts:  $0.69 \pm 0.07$  in asthma subjects compared to  $0.99 \pm 0.08$  in normal subjects,  $p < 0.05$  between asthma and normal subjects and within the asthma group) (**Figure 5A**).

### **Expression of $\alpha$ -SMA**

Using immunoblotting (**Figure 6A**) of cell culture lysates treated with IL-13 (50 ng/ml), we found that IL-13 increased protein production of both  $\alpha$ -SMA and desmin, two markers of myofibroblast phenotype, in asthmatic airway fibroblasts compared to untreated fibroblasts. No increase in production of  $\alpha$ -SMA or desmin

was observed in airway fibroblasts isolated from normal control subjects. Densitometry analysis indicated a significant increase in IL-13-induced  $\alpha$ -SMA production by airway fibroblasts in asthma as compared to normal controls (Ratio of  $\alpha$ -SMA in IL-13-treated fibroblasts as compared to untreated fibroblasts:  $1.96 \pm 0.42$  in asthma subjects compared to  $0.91 \pm 0.07$  in normal subjects;  $p=0.03$  between asthma and normal) (**Figure 6B**).

## Discussion

In this study, we present evidence that IL-13 induces *COL1A2* gene expression by bronchial fibroblasts from subjects with mild asthma compared to healthy control subjects. Interestingly, IL-13 had no effect on *COL1A1* expression or *COL3A1* expression. In addition, we show that IL-13 increases the amount of secreted mature type-1 collagen protein in the cell culture supernatants from airway fibroblasts derived from asthma patients. The assay used in our study does not discriminate between COL1A1 and COL1A2 and reflects total amount of collagen protein present.

Normal collagen protein is composed of two  $\alpha 1$  chains and one  $\alpha 2$  chain [27]. These data suggest that enhanced collagen production may be controlled through expression of *COL1A2* exclusively or that a different mediator is responsible for inducing *COL1A1* expression. Alternatively, COL1A1 may be constitutively expressed and control of collagen production may be through COL1A2. However, in a study by Plante *et al.* [28], these authors demonstrated that IL-4, another major TH2 cytokine, significantly induced *COL1A1* gene expression. The authors did not analyze *COL1A2* expression.

The mechanism by which IL-13 may promote airway fibrosis is largely unknown, although work in murine models suggest that it likely functions through activating TGF- $\beta$ 1 in an MMP-dependent manner.[14, 29]. To test this effect, we wanted to examine the primary MMPs that have been associated with asthma including MMP-1, MMP-2, MMP-3 and MMP-9 [30]. In our study, we were unable to detect gene expression of MMP-9 in airway fibroblasts (data not shown), and therefore, we did not study its effect using our experimental model. In our results, only MMP-2 inhibition was found to have a significant effect on reducing IL-13 induced collagen type-1 production in airway fibroblasts. Furthermore, we demonstrate that IL-13 significantly increased MMP2 activation in asthmatic airway fibroblasts compared to normal controls. The effects of IL-13 are mediated by its receptor subunits: IL-13R $\alpha$ 1, IL-13R $\alpha$ 2 and IL-4R $\alpha$ . Previous work from our laboratory has shown that airway fibroblast cell surface expression of IL-13R $\alpha$ 2 in asthma is suppressed compared with expression on airway fibroblasts from normal control subjects, while IL-13R $\alpha$ 1 and IL-4R $\alpha$

were not significantly changed [31]. IL-13R $\alpha$ 2 is thought to act as a decoy receptor to negatively regulate IL-13 signaling, and therefore, its relative deficiency may explain enhanced airway fibroblast responsiveness to IL-13 [32]. TGF- $\beta$ 1 is one of the most studied pro-fibrotic cytokines [33]. The potent activity of TGF- $\beta$ 1 is regulated at the post-transcriptional level by a latency-associated protein (LAP), which keeps TGF- $\beta$ 1 in an inactive state. Dissociation of TGF- $\beta$ 1 from LAP is required to release biologically active TGF- $\beta$ 1. In our experiments, we demonstrate that IL-13 increases both the total amount as well as the activated form of TGF- $\beta$ 1 in cell culture supernatants from airway fibroblasts in asthma patients at 48 hours using both ELISA and a functional bio-assay specific for active form of TGF- $\beta$ 1. One of the potential roles for MMP-2 may be to trigger release of TGF- $\beta$ 1, a well-known mediator in fibrosis, from its latency associated protein (LAP) allowing it to stimulate fibrosis [33]. This mechanism has been previously shown to occur with MMP-9 as well as other agents such as plasmin [29, 34]. We show that activation of TGF- $\beta$ 1 was attenuated following treatment with a specific MMP-2 inhibitor. Once activated, TGF- $\beta$ 1 binds primarily to TGF- $\beta$  RII, which then recruits the type-I receptor for downstream signaling [35]. TGF- $\beta$ 1 has also been shown to augment IL-13 signaling by dampening expression of IL-13R $\alpha$ 2 [36]. This would potentially allow fibroblasts to overcome IL-13's auto-regulatory pathway and remain in a state of persistent responsiveness to IL-13. To further study the effects of TGF- $\beta$ 1 in this pathway, the addition of a TGF- $\beta$  RII neutralizing antibody significantly decreased the IL-13-induced expression of *COL1A2*, also suggesting that TGF- $\beta$ 1 is involved in this pathway downstream of IL-13. One of the mechanisms by which IL-13 may lead to increased collagen deposition is through changing the phenotype of normal airway fibroblasts to the more synthetic myofibroblast. As we demonstrate qualitatively in our experiments, IL-13 induced expression of both  $\alpha$ -SMA and desmin in our asthmatic airway fibroblasts, two known markers for myofibroblasts. In our previous work, we have shown that IL-13 potentiates airway fibroblast invasion in asthma subjects, a feature associated with myofibroblast differentiation, through a mechanism involving TGF- $\beta$ 1. Other investigators have also implicated TGF- $\beta$ 1 as a critical mediator of myofibroblast differentiation and activation [37, 38]. The increased expression of  $\alpha$ -SMA

and desmin suggests that the airway fibroblasts in asthma subjects in response to IL-13 change their phenotype to myofibroblasts, though this mechanism requires further investigation.

Limitations of our study include evaluation of mediators only relevant to airway fibroblasts, and clearly other cells and their products are involved in airway remodeling. In addition, all of our findings were based on mild asthmatic patients that may behave differently, or have reduced remodeling signal as compared to patients with more severe asthma. Also, we studied fibroblasts isolated from proximal airways which may not behave in the same fashion as distal airway fibroblasts.

Our study demonstrates that IL-13 induces collagen type -1 production in airway fibroblasts cultured directly from mild asthmatic patients compared to healthy controls. We also show that IL-13 likely induces collagen production in an MMP-2-dependent manner which is a novel finding in human asthma. We show that MMP-2 likely induces collagen production by activating latent TGF- $\beta$ 1, and this mechanism may be a major contributor to the pathogenesis of airway fibrosis seen in this disease. There have been recent advances in the development of therapeutic monoclonal antibodies directed against IL-13 in poorly controlled asthma. Two recent studies found that using IL-13 monoclonal antibodies improved airway function in these patients [39, 40]. While this is an exciting development in an area that desperately needs better therapies to treat severe asthma, our findings also provide some insight that interventions leading to a decrease in IL-13-induced TGF- $\beta$ 1 activation could be beneficial in reducing airway remodeling in asthmatic patients. The use of such an IL-13 inhibitor or a therapeutic MMP-2 inhibitor may achieve this goal and provide a starting point for further study. Thus, there may be therapeutic options available for a process that to date has had none, particularly in the patients with more severe asthma.

## REFERENCES

1. Wynn TA. IL-13 effector functions. *Annu Rev Immunol* 2003; 21: 425-456.
2. Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. *J Clin Invest* 1999; 104: 1001-1006.
3. James AL, Wenzel S. Clinical relevance of airway remodelling in airway diseases. *Eur Respir J* 2007; 30: 134-155.
4. Bumbacea D, Campbell D, Nguyen L, Carr D, Barnes PJ, Robinson D, Chung KF. Parameters associated with persistent airflow obstruction in chronic severe asthma. *Eur Respir J* 2004; 24: 122-128.
5. Roche W, Williams J, Beasley R, Holgate S. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989; 333: 520-524.
6. Chakir J, Laviolette M, Boutet M, Laliberte R, Dube J, Boulet LP. Lower airways remodeling in nonasthmatic subjects with allergic rhinitis. *Lab Invest* 1996; 75: 735-744.
7. Brewster CE, Howarth PH, Djukanovic R, Wilson J, Holgate ST, Roche WR. Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1990; 3: 507-511.
8. Kelly EA, Jarjour NN. Role of matrix metalloproteinases in asthma. *Curr Opin Pulm Med* 2003; 9: 28-33.
9. Ohbayashi H. Matrix metalloproteinases in lung diseases. *Curr Protein Pept Sci* 2002; 3: 409-421.
10. Zheng T, Zhu Z, Wang Z, Homer RJ, Ma B, Riese RJ, Jr., Chapman HA, Jr., Shapiro SD, Elias JA. Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema. *J Clin Invest* 2000; 106: 1081-1093.
11. Lee CG, Kang HR, Homer RJ, Chupp G, Elias JA. Transgenic modeling of transforming growth factor-beta(1): role of apoptosis in fibrosis and alveolar remodeling. *Proc Am Thorac Soc* 2006; 3: 418-423.
12. Ohno I, Nitta Y, Yamauchi K, Hoshi H, Honma M, Woolley K, O'Byrne P, Tamura G, Jordana M, Shirato K. Transforming growth factor beta 1 (TGF beta 1) gene expression by eosinophils in asthmatic airway inflammation. *Am J Respir Cell Mol Biol* 1996; 15: 404-409.
13. Lee CG, Cho SJ, Kang MJ, Chapoval SP, Lee PJ, Noble PW, Yehualaeshet T, Lu B, Flavell RA, Milbrandt J, Homer RJ, Elias JA. Early Growth Response Gene 1-mediated Apoptosis Is Essential for Transforming Growth Factor  $\beta$ 1-induced Pulmonary Fibrosis. *J Exp Med* 2004; 200: 377-389.

14. Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Kotliansky V, Shipley JM, Gotwals P, Noble P, Chen Q, Senior RM, Elias JA. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). *J Exp Med* 2001; 194: 809-821.
15. Fulkerson PC, Fischetti CA, Hassman LM, Nikolaidis NM, Rothenberg ME. Persistent effects induced by IL-13 in the lung. *Am J Respir Cell Mol Biol* 2006; 35: 337-346.
16. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signaling through the IL-13[alpha]2 receptor is involved in induction of TGF-[beta]1 production and fibrosis. *Nat Med* 2006; 12: 99-106.
17. Fichtner-Feigl S, Young CA, Kitani A, Geissler EK, Schlitt HJ, Strober W. IL-13 signaling via IL-13R alpha2 induces major downstream fibrogenic factors mediating fibrosis in chronic TNBS colitis. *Gastroenterology* 2008; 135: 2003-2013, 2013 e2001-2007.
18. Zuo L, Fulkerson PC, Finkelman FD, Mingler M, Fischetti CA, Blanchard C, Rothenberg ME. IL-13 induces esophageal remodeling and gene expression by an eosinophil-independent, IL-13R alpha 2-inhibited pathway. *J Immunol* 2010; 185: 660-669.
19. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, November 1986. *Am Rev Respir Dis* 1987; 136: 225-244.
20. Pham DN, Chu HW, Martin RJ, Kraft M. Increased matrix metalloproteinase-9 with elastolysis in nocturnal asthma. *Annals of Allergy, Asthma, and Immunology* 2003; 90: 72-78.
21. Kraft M, Lewis C, Pham D, Chu HW. IL-4, IL-13, and dexamethasone augment fibroblast proliferation in asthma. *J Allergy Clin Immunol* 2001; 107: 602-606.
22. Dube J, Chakir J, Laviolette M, Saint Martin S, Boutet M, Desrochers C, Auger F, Boulet LP. In vitro procollagen synthesis and proliferative phenotype of bronchial fibroblasts from normal and asthmatic subjects. *Lab Invest* 1998; 78: 297-307.
23. Voynow JA, Fischer BM, Malarkey DE, Burch LH, Wong T, Longphre M, Ho SB, Foster WM. Neutrophil elastase induces mucus cell metaplasia in mouse lung. *American journal of physiology Lung cellular and molecular physiology* 2004; 287: L1293-1302.

24. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB. An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal Biochem* 1994; 216: 276-284.
25. Mukherjee S, Giamberardino C, Thomas JM, Gowdy K, Pastva AM, Wright JR. Surfactant protein A modulates induction of regulatory T cells via TGF-beta. *J Immunol* 2012; 188: 4376-4384.
26. Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report 2007. *J Allergy Clin Immunol* 2007; 120: S94-138.
27. Last JA, Reiser KM. Collagen biosynthesis. *Environ Health Perspect* 1984; 55: 169-177.
28. Plante S, Semlali A, Joubert P, Bissonnette E, Laviolette M, Hamid Q, Chakir J. Mast cells regulate procollagen I (alpha 1) production by bronchial fibroblasts derived from subjects with asthma through IL-4/IL-4 delta 2 ratio. *J Allergy Clin Immunol* 2006; 117: 1321-1327.
29. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000; 14: 163-176.
30. Demedts IK, Brusselle GG, Bracke KR, Vermaelen KY, Pauwels RA. Matrix metalloproteinases in asthma and COPD. *Current opinion in pharmacology* 2005; 5: 257-263.
31. Ingram JL, Huggins MJ, Church TD, Li Y, Francisco DC, Degan S, Firszt R, Beaver DM, Lugogo NL, Wang Y, Sunday ME, Noble PW, Kraft M. Airway Fibroblasts in Asthma Manifest an Invasive Phenotype. *Am J Respir Crit Care Med* 2011; 183: 1625-1632.
32. Mentink-Kane MM, Wynn TA. Opposing roles for IL-13 and IL-13 receptor  $\alpha 2$  in health and disease. *Immunol Rev* 2004; 202: 191-202.
33. Malavia NK, Mih JD, Raub CB, Dinh BT, George SC. IL-13 induces a bronchial epithelial phenotype that is profibrotic. *Respiratory research* 2008; 9: 27.
34. Khalil N, Corne S, Whitman C, Yacyshyn H. Plasmin regulates the activation of cell-associated latent TGF-beta 1 secreted by rat alveolar macrophages after in vivo bleomycin injury. *Am J Respir Cell Mol Biol* 1996; 15: 252-259.
35. Boxall C, Holgate ST, Davies DE. The contribution of transforming growth factor-beta and epidermal growth factor signalling to airway remodelling in chronic asthma. *Eur Respir J* 2006; 27: 208-229.

36. Zhou X, Hu H, Balzar S, Trudeau JB, Wenzel SE. MAPK Regulation of IL-4/IL-13 Receptors Contributes to the Synergistic Increase in CCL11/Eotaxin-1 in Response to TGF-beta1 and IL-13 in Human Airway Fibroblasts. *J Immunol* 2012.
37. Petrov VV, Fagard RH, Lijnen PJ. Stimulation of Collagen Production by Transforming Growth Factor-β1 During Differentiation of Cardiac Fibroblasts to Myofibroblasts. *Hypertension* 2002; 39: 258-263.
38. Casey TM, Eneman J, Crocker A, White J, Tessitore J, Stanley M, Harlow S, Bunn JY, Weaver D, Muss H, Plaut K. Cancer associated fibroblasts stimulated by transforming growth factor beta1 (TGF-beta 1) increase invasion rate of tumor cells: a population study. *Breast Cancer Res Treat* 2008; 110: 39-49.
39. Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, Harris JM, Scheerens H, Wu LC, Su Z, Mosesova S, Eisner MD, Bohen SP, Matthews JG. Lebrikizumab treatment in adults with asthma. *N Engl J Med* 2011; 365: 1088-1098.
40. Piper E, Brightling C, Niven R, Oh C, Faggioni R, Poon K, She D, Kell C, May RD, Geba GP, Molino NA. A phase II placebo-controlled study of tralokinumab in moderate-to-severe asthma. *Eur Respir J* 2013; 41: 330-338.

**Table 1: Participant Characteristics**

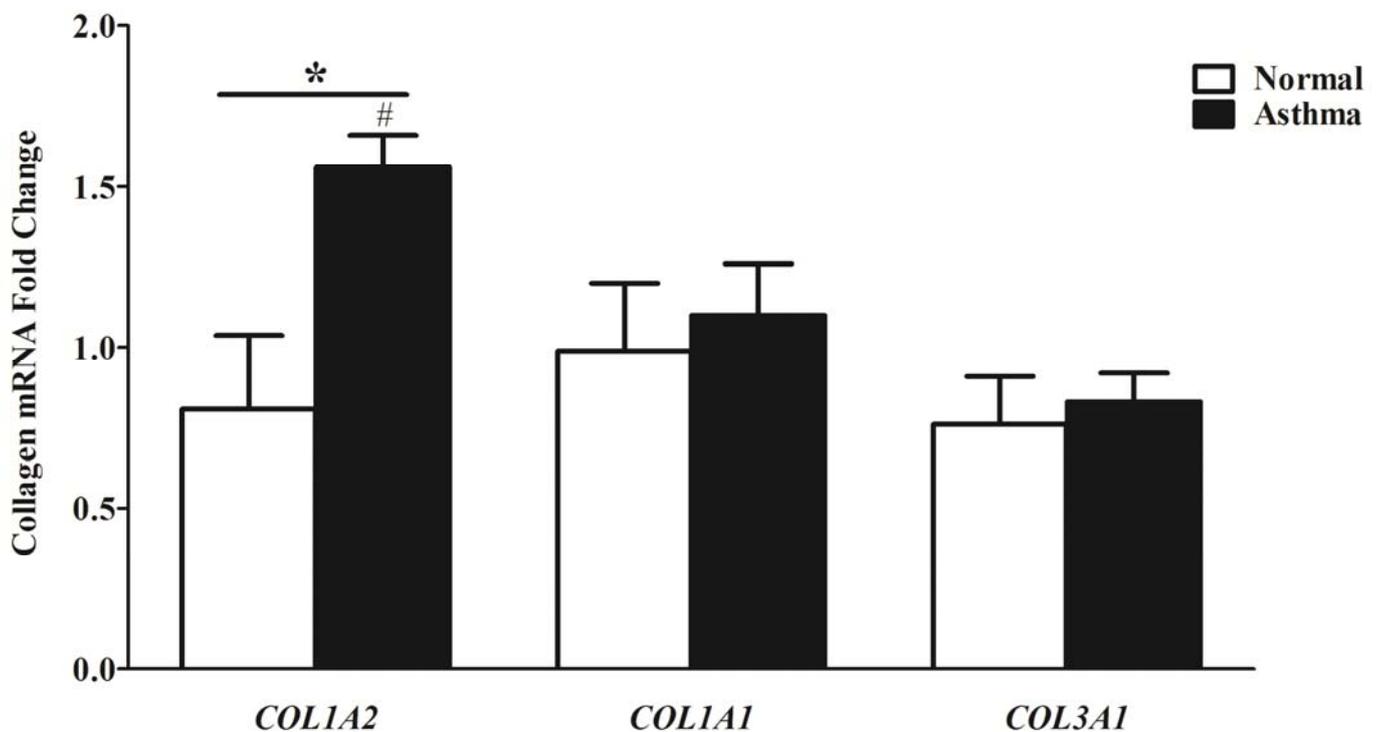
	<u>Asthma (14)</u>	<u>Healthy Controls (13)</u>	<u>p Value</u>
<b>Gender (F/M)</b>	8/6	8/5	p=NS
<b>Age</b>	25 ± 2	29 ± 2	p=NS
<b>FEV1 (L)</b>	3.87 ± 0.2	3.78 ± 0.1	p=NS
<b>FEV1 (% pred.)</b>	98 ± 4	102 ± 3	p=NS
<b>FVC (L)</b>	4.83 ± 0.2	4.5 ± 0.2	p=NS
<b>FVC (% pred.)</b>	98 ± 3	101 ± 2	p=NS
<b>FEV1/FVC</b>	0.80 ± 0.02	0.84 ± 0.01	p=0.03
<b>PC20 (mg/ml) †</b>	0.5 ± 0.01	> 16	p=0.0001

†The provocative concentration of methacholine resulting in a 20% fall in FEV1.

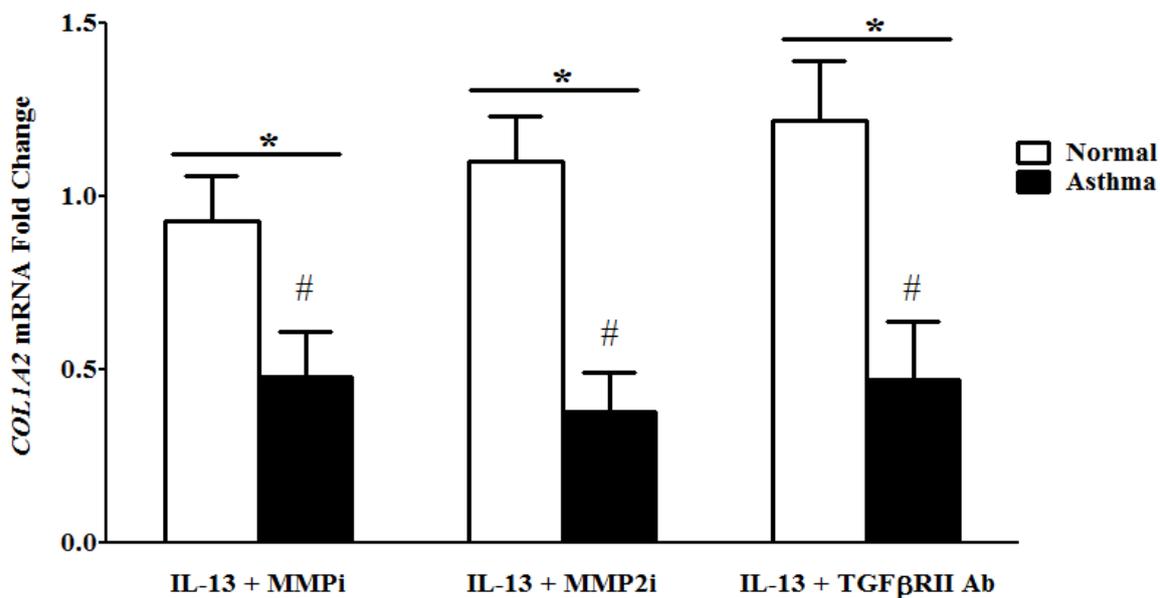
NS= not significant

## FIGURE LEGENDS

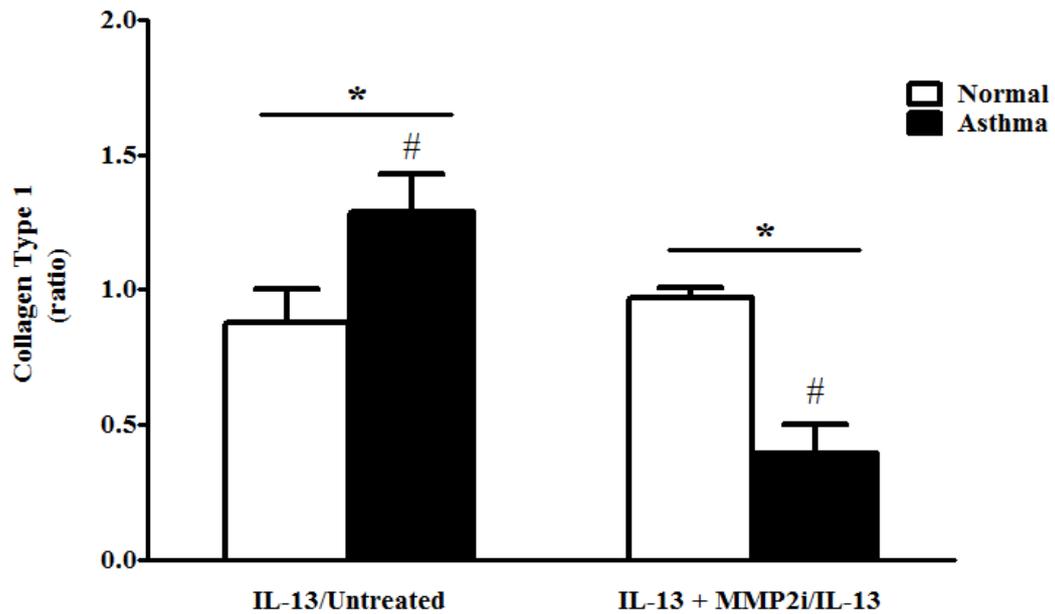
**Figure 1.** *COL1A2* mRNA expression was significantly induced by IL-13 (50 ng/ml) in asthma. *COL1A2*, *COL1A1* and *COL3A1* mRNA expression was measured by quantitative real-time RT-PCR in airway fibroblasts isolated from asthmatic (n=15; closed bars) and normal controls (n= 15; open bars). Results are expressed as fold change from untreated fibroblasts. \* $p < 0.01$  between asthma and normal controls; # $p < 0.01$  within asthmatic subjects.



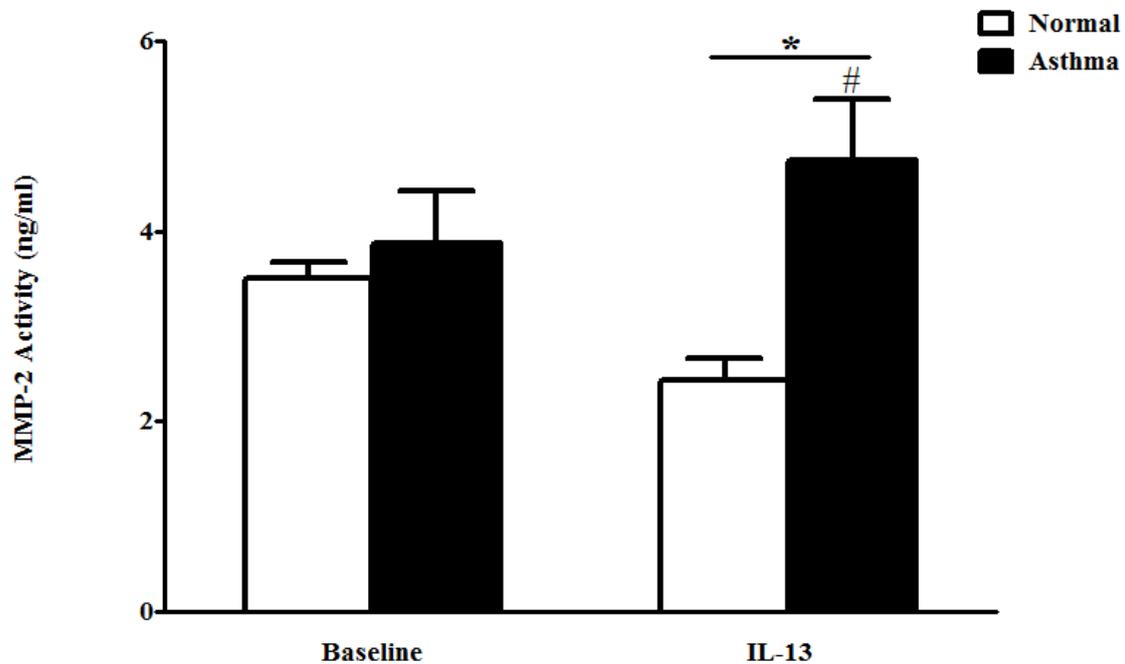
**Figure 2.** Significant attenuation in IL-13-induced *COL1A2* mRNA expression was observed at 48 hours when an MMP inhibitor (MMPi, 10  $\mu$ M), a specific MMP2i inhibitor (MMP2i, 40  $\mu$ M) and a TGF- $\beta$ RII neutralizing antibody (20  $\mu$ g/ml), respectively, were added to IL-13-treated airway fibroblasts in asthma subjects (n=15; closed bars) compared to normal subjects (n=15; open bars) and within asthma patients alone as measured by real-time RT-PCR. Results are expressed as fold change from fibroblasts from each group treated with IL-13 alone. \* $p < 0.05$  between asthma and normal subjects; # $p < 0.05$  within the asthma group for each condition.



**Figure 3.** Collagen type-1 protein was induced by IL-13 (50 ng/ml), depicted as the ratio of IL-13-treated airway fibroblasts to untreated fibroblasts, at 48 hours in asthma (n=18) as compared to normal controls (n=12) and within asthma alone. MMP-2 inhibition significantly attenuated IL-13-induced collagen type-1, depicted as the ratio of collagen type I production following treatment with IL-13 + MMP2-inhibitor fibroblasts as compared to treatment with IL-13 alone, in asthma subjects as compared to normal subjects and within the asthma group. \* $p < 0.05$  between asthma and normal controls; # $p < 0.05$  within asthmatic subjects for each condition.

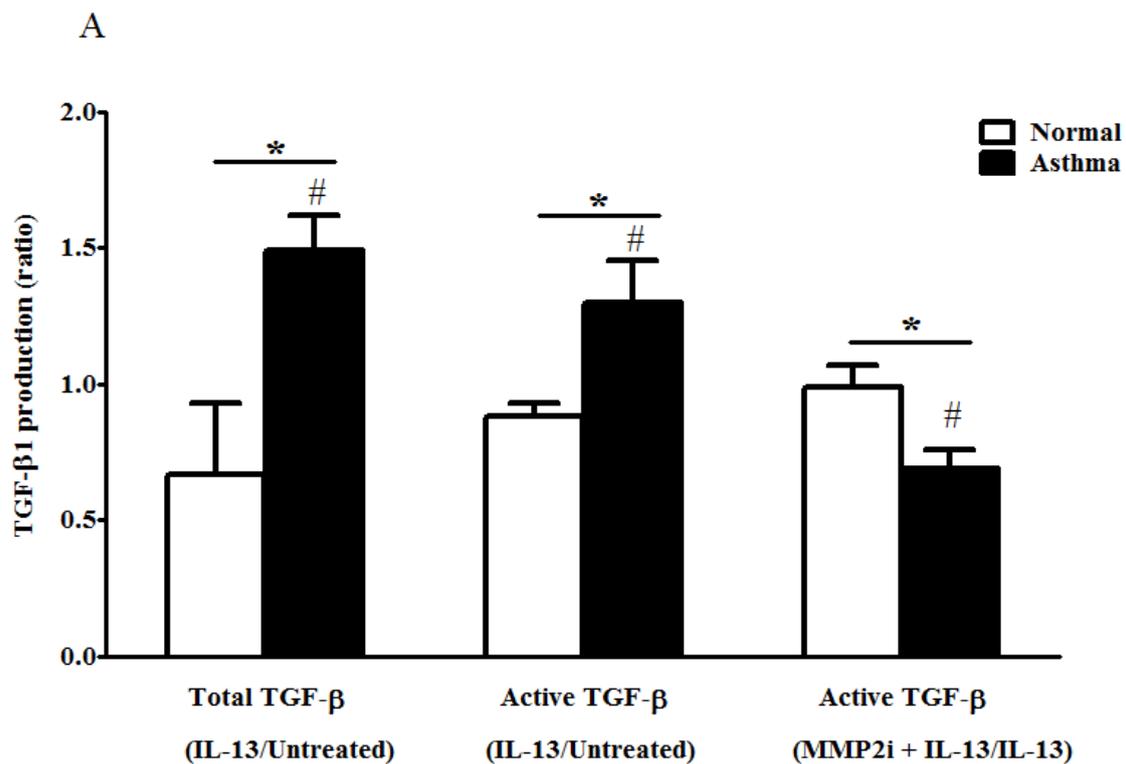


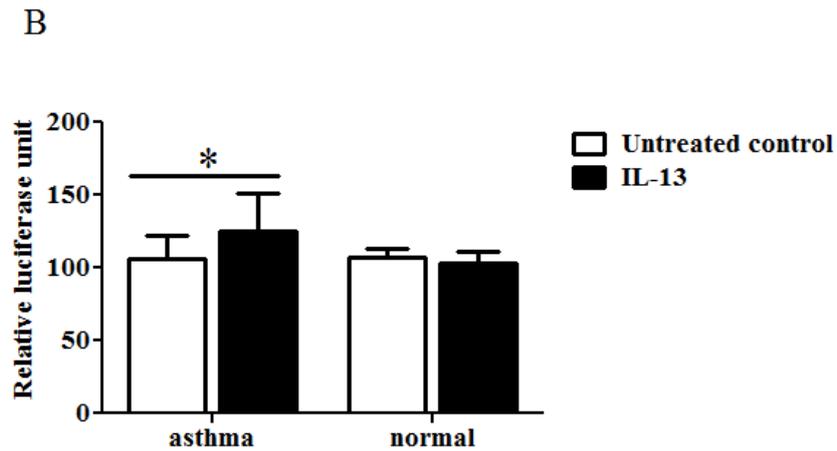
**Figure 4.** MMP-2 activity in normal and asthmatic fibroblasts at baseline and after treatment with IL-13. MMP-2 activity is induced after treatment with IL-13 (50 ng/ml) for 48 hours in asthma (n=6) as compared to normal controls (n=4) and within asthma alone, as measured by an MMP-2 activation assay. \* $p < 0.05$  between asthma and normal controls; # $p < 0.05$  within asthmatic subjects for the IL-13 condition.



**Figures 5A and 5B.** IL-13 stimulates active TGF- $\beta$ 1 production by asthmatic airway fibroblasts. A: Total and endogenously active forms of TGF- $\beta$ 1 from airway fibroblast cell culture supernatants were increased from baseline following stimulation with IL-13 (50 ng/ml) at 48 hours in asthma subjects (n=9; closed bars) as compared to normal subjects (n=7; open bars) and within the asthma group, depicted as the ratio TGF $\beta$ 1 production in fibroblasts treated with IL-13 as compared to untreated fibroblasts. MMP-2 inhibition blocked TGF- $\beta$ 1 activation in fibroblasts treated with IL-13 in asthma subjects compared to normal subjects and within the asthma group, depicted as the ratio of TGF $\beta$ 1 activation after treatment with IL-13 + MMP2i as compared to treatment with IL-13 alone (IL-13 + MMP2i/IL-13). B: Bioactive TGF- $\beta$ 1 levels, as represented by luciferase activity (relative luciferase units – RLU), in airway fibroblast cell culture supernatants were elevated following

exposure to IL-13 (50 ng/ml) at 48 hours in asthma subjects (n=9; closed bars) as compared to untreated control. \* $p < 0.05$  between asthma and normal subjects; # $p < 0.05$  within asthmatic subjects for each condition.





**Figures 6A and 6B.** Expression of  $\alpha$ -SMA and desmin proteins in cell culture lysates from airway fibroblasts was increased in asthma patients following treatment with IL-13. Cell lysates were prepared from airway fibroblasts from either asthma or normal subjects after exposure to IL-13 (50 ng/ml) or serum-free media (untreated control) for 48 hours. A: Representative western blot of myofibroblast markers in airway fibroblasts in asthma or normal subjects. Expression of GAPDH indicates loading control. B: Quantification of data shown in 6A: (mean of 6 asthma and 4 normal controls  $\pm$  SEM). Data are expressed as the ratio of the intensity of bands following IL-13 to untreated control. \* $p < 0.05$  between asthma and normal subjects.

