ORIGINAL RESEARCH

Role of Matrix Metalloproteinases-1 and -2 in Interleukin-13–Suppressed Elastin in Airway Fibroblasts in Asthma

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Abstract

Elastin synthesis and degradation in the airway and lung parenchyma contribute to airway mechanics, including airway patency and elastic recoil. IL-13 mediates many features of asthma pathobiology, including airway remodeling, but the effects of IL-13 on elastin architecture in the airway wall are not known. We hypothesized that IL-13 modulates elastin expression in airway fibroblasts from subjects with allergic asthma. Twenty-five subjects with mild asthma (FEV_1, 89 ± 3% predicted) and 30 normal control subjects (FEV_1, 102 ± 2% predicted) underwent bronchoscopy with endobronchial biopsy. Elastic fibers were visualized in airway biopsy specimens using Weigert's resorcin-fuchsin elastic stain. Airway fibroblasts were exposed to IL-13; a pan-matrix metalloproteinase (MMP) inhibitor (GM6001); specific inhibitors to MMP-1, -2, -3, and -8; and combinations of IL-13 with MMP inhibitors in separate conditions in serum-free media for 48 hours. Elastin (*ELN*) expression as well as MMP secretion and activity were quantified. Results of this study show that elastic fiber staining of airway biopsy tissue was significantly associated with methacholine PC_{20} (i.e., the provocative concentration of methacholine resulting in a 20% fall in FEV_1 levels) in patients with asthma. IL-13 significantly suppressed *ELN* expression in asthmatic airway fibroblasts as compared with normal control fibroblasts. The effect of IL-13 on *ELN* expression was significantly correlated with postbronchodilator FEV_1/FVC in patients with asthma. MMP inhibition significantly stimulated *ELN* expression in patients with asthma as compared with normal control subjects. Specific inhibition of MMP-1 and MMP-2, but not MMP-3 or MMP-8, reversed the IL-13–induced suppression of *ELN* expression. In asthma, MMP-1 and MMP-2 mediate IL-13–induced suppression of *ELN* expression in airway fibroblasts.

Keywords: asthma; fibroblast; interleukin-13; elastin; matrix metalloproteinase

Airway remodeling is a heterogeneous set of tissue responses to chronic inflammation in asthma, resulting in airway narrowing and irreversible airflow obstruction (1). Over time, the cumulative burden of structural changes can result in accelerated lung function decline, beginning as early as childhood. Evidence has linked structural airway changes to extracellular matrix deposition, including subepithelial fibrosis. In contrast, additional studies demonstrate that matrix loss, specifically the loss of elastin, also occurs within the spectrum of remodeling, manifested as exaggerated airways collapsibility (2, 3). Elastin loss can also account for the reduction in lung elastic recoil recently observed in asthma (3, 4).

Elastin is fundamentally important to lung architecture and is directly responsible for airway distensibility (for review *see* Refs. 5, 6). A decrease in lung elastin can affect airway mechanics by decreasing airway stiffness and increasing airway narrowing.

(Received in original form July 23, 2014; accepted in final form May 28, 2015)

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This work was supported by National Institutes of Health/National Heart, Lung and Blood Institute grants 2R01 HL064619–071A and HL-05–009.

Author Contributions: Conception and design of experiments: J.L.I., D.S., M.E.S., and M.K. Acquisition, analysis, and interpretation of data: J.L.I., D.S., T.D.C., D.F., K.H., R.W.S., M.G., A.M., R.F., N.L.L., L.Q., M.E.S., and M.K. Drafting the manuscript for important intellectual content: J.L.I., D.S., R.F., M.E.S., and M.K.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 54, Iss 1, pp 41–50, Jan 2016
Copyright © 2016 by the American Thoracic Society
Originally Published in Press as DOI: 10.1165/rcmb.2014-0290OC June 13, 2015
Internet address: www.atsjournals.org

Clinical Relevance

Our results suggest that IL-13 contributes to airway obstruction and loss of elastin in asthma through a process requiring matrix metalloproteinase (MMP)-1 and MMP-2 to suppress elastin mRNA expression. These data suggest a novel role for MMPs in elastin gene regulation.

Furthermore, as the tethering effect of elastic fibers is lost, airway smooth muscle may become predisposed to exaggerated shortening (7). Elastic fibers are required to maintain airway patency during respiration; both superficial and deeper elastin network layers can be disrupted in subjects with asthma (2, 3, 8). We have observed the loss of airway elastic recoil in the airways of subjects with asthma (9). Similarly, biopsies of asthmatic airways demonstrate elastin matrix loss (3). Loss of elastin may be due to reduced expression, enhanced destruction, or both. We have demonstrated that loss of elastin in asthma was associated with increased matrix metalloproteinase (MMP)-9 expression (3). We hypothesize that loss of elastin in tissue may be responsible for the airways collapsibility and loss of elastic recoil observed in asthma (3, 4) and that the action of MMPs may modulate this process.

The airway fibroblast is a key cell type that links inflammation to structural airway changes in the lung. These cells, along with airway smooth muscle cells, produce elastin, alter lung architecture after injury, stimulate inflammation, and respond to cytokines relevant to asthma such as IL-4, IL-13, and transforming growth factor-β1 (10–13). Specific fibroblast responses include mitogen-stimulated proliferation, invasion, and collagen production (12–14). IL-13, a critical effector cytokine in asthma, mediates its effects through several cells, including airway fibroblasts, and plays central roles in airway obstruction, airways hyperresponsiveness (AHR), mucus metaplasia, inflammatory cell infiltration, and fibrosis (15, 16). Transgenic murine models of IL-13 overexpression exhibit extensive airway remodeling (17).

In human asthma, airway fibroblasts link inflammation to structural airway changes and are crucial to elastin matrix regulation. We have made the novel observation that IL-13 suppresses elastin gene expression in airway fibroblasts isolated directly from human subjects, and this effect is significant in subjects with asthma as compared with healthy control subjects. The mechanisms by which IL-13 regulates elastin gene expression in human asthma are unknown, but in this study we hypothesize that MMPs mediate IL-13–induced suppression of matrix expression in airway fibroblasts isolated directly from subjects with asthma.

Materials and Methods

Subject Recruitment

Fifty-five subjects were recruited from the Denver, CO, and Durham, NC, communities. The subjects with asthma fulfilled criteria for asthma (18) exhibiting a provocative concentration of methacholine resulting in a 20% fall in FEV_1 (PC_{20}) of <8 mg/ml and reversibility, as demonstrated by at least a 12% and 200-ml increase in FEV_1 or FVC with inhaled albuterol. All subjects provided consent. This protocol was approved by the Duke University and National Jewish Health Institutional Review Board.

Bronchoscopy

Subjects underwent bronchoscopy with endobronchial biopsy as previously described (3, 14).

Airway Tissue Staining

Biopsy tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with Weigert's resorcin-fuchsin stain to visualize elastic fibers as previously described (19). Images of stained airway tissue sections were imported into ImageJ software (National Institutes of Health, Bethesda, MD). Relative quantities of elastic fiber staining were determined by calculating the percentage of staining within the total tissue area.

Fibroblast Culture

Endobronchial tissue was placed on collagen-coated plates, and airway fibroblasts were cultured as previously described with modifications (14). Because numerous passages can induce cellular senescence, only cells from the first three passages were used for experimentation (20). Normal and asthmatic airway fibroblasts were cultured and passaged under identical conditions.

Mediator Exposure

Airway fibroblasts were plated at 40,000 cells/well onto non–collagen coated 12-well plates as previously described (14) and incubated until 7 days past confluence. At baseline, these cells express α-smooth muscle actin and secrete collagen type 1, indicating a myofibroblast phenotype (13). After 24 hours in serum-free media, mediators were added, and cells were incubated for 48 hours. Conditions included 50 ng/ml IL-13 (ProSpec, Rehovot, Israel) and 10 μM MMP inhibitors (GM6001 [a broad-spectrum inhibitor of MMPs], MMP Inhibitor I, MMP-2 Inhibitor-1, and MMP-3 Inhibitor-1; Calbiochem, San Diego, CA). Combinations of IL-13 plus GM6001 and IL-13 plus inhibitors of MMP-1, -2, -3, and -8 were also studied. Chosen mediators were based on our previous work and other literature that suggested significant effects upon elastin synthesis or destruction (3, 5).

Quantitative Real-Time PCR

Quantitative real-time PCR was performed to demonstrate relative elastin (*ELN*) mRNA levels by normalization to glyceraldehyde-3-phosphate dehydrogenase mRNA levels in exposed cells relative to unexposed cells using the $\Delta\Delta C_T$ method. Data are shown as mean percentage of fold change from the unexposed control.

Western Blot Analyses

Cell lysates from cultured airway fibroblasts were collected at 48 hours after IL-13 (50 ng/ml) or serum-free media (untreated control) treatments. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-human elastin and β-actin antibodies (Santa Cruz Biotechnology, Dallas, TX). The blots were developed by enhanced chemiluminescence, documented with the Kodak Image Station 4000 MM PRO, and quantified using Carestream Molecular Image software (version 5.0).

MMP-1 and Tissue Inhibitor of Metalloproteinase-1 and -2 ELISAs and MMP-1 Activity Assay

Supernatants from cultured airway fibroblasts were collected at 48 hours after

Table 1. Subject Characteristics

	Subjects with Asthma (*n* = 25)	Control Subjects (*n* = 30)	*P* Value
Sex (female/male)	14/11	21/9	0.37
Age, yr	29 ± 2	28 ± 2	0.70
Ethnicity*	2A:7AA:16W	1A:13AA:17W	0.30
FEV_1, liters	3.3 ± 0.1	3.5 ± 0.1	0.38
FEV_1, % pred.	89 ± 3	102 ± 2	<0.01
FVC, liters	4.4 ± 0.2	4.3 ± 0.2	0.62
FVC, % pred.	101 ± 3	105 ± 2	0.28
FEV_1/FVC	82 ± 1	85 ± 1	0.06
Post-bronchodilator FEV_1/FVC	74 ± 2	85 ± 1	<0.01
PC_{20}, mg/ml	1.4 ± 0.44	>16	<0.01
F_{ENO}, ppb	59.2 ± 19.5	11.8 ± 1.3	<0.01
ACQ	1.6	—	—
Medications	SABA	None	

Definition of abbreviations: ACQ, Asthma Control Questionnaire; F_{ENO}, fraction of exhaled nitric oxide; PC_{20}, provocative concentration of methacholine resulting in a 20% fall in FEV_1; pred., predicted; SABA, short-acting β agonist.
*A, Asian; AA, African American; W, white.

treatment with IL-13 (50 ng/ml). Measurement of MMP-1 and tissue inhibitor of metalloproteinase (TIMP)-1 and -2 protein levels was performed using ELISA (R&D Systems, Minneapolis, MN). Endogenous MMP-1 activity levels were measured using a fluorimetric enzyme activity assay (R&D Systems). Active MMP-1 concentrations based on mean absorbance at 405 nm after 17-hour incubation were reported.

Statistical Analyses

Analyses were performed using JMP (SAS, Cary, NC) and Prism (GraphPad, La Jolla, CA) statistical software. Mean values from each condition were used for analyses. Baseline values were compared between groups, and differences are reported before using fold-change from baseline as the comparison variable. The fold change from baseline after exposure to mediators was used as the most relevant comparison to take baseline differences into account. Mediator-induced *ELN* expression relative to the unexposed control was compared within groups using a two-way ANOVA. Paired comparisons were evaluated using a two-tailed paired *t* test. If the overall *P* value for the comparisons was less than 0.05, individual contrasts determined significance within and between groups. Correlations were performed for PC_{20} and post-bronchodilator ratio of FEV_1/FVC data for each subject to determine the Pearson's correlation coefficient. For most analyses, data are expressed as means ± SEM because they were normally distributed; significance is denoted by $P < 0.05$. Data that were not normally distributed were expressed as medians (interquartile range [IQR]) and compared using a two-tailed Wilcoxon rank sum test; significance is denoted by $P < 0.05$.

Results

Subjects

Subject characteristics are shown in Table 1. The subjects with asthma were considered of mild severity because their mean percentage predicted FEV_1 was 89 ± 3%. The subjects with asthma exhibited AHR with a mean methacholine PC_{20} of 1.4 ± 0.4 mg/ml. The normal subjects exhibited normal airways responsiveness, with a methacholine PC_{20} of >16 mg/ml ($P < 0.01$). The subjects with asthma exhibited significantly elevated fraction of exhaled nitric oxide (F_{ENO}) as compared with the normal subjects (59.2 ± 19.5 ppb for asthma and 11.8 ± 1.3 ppb for normal; $P < 0.01$), suggesting significantly increased Th2-mediated airway inflammation in the subjects with asthma. The subjects with asthma used albuterol as needed and no

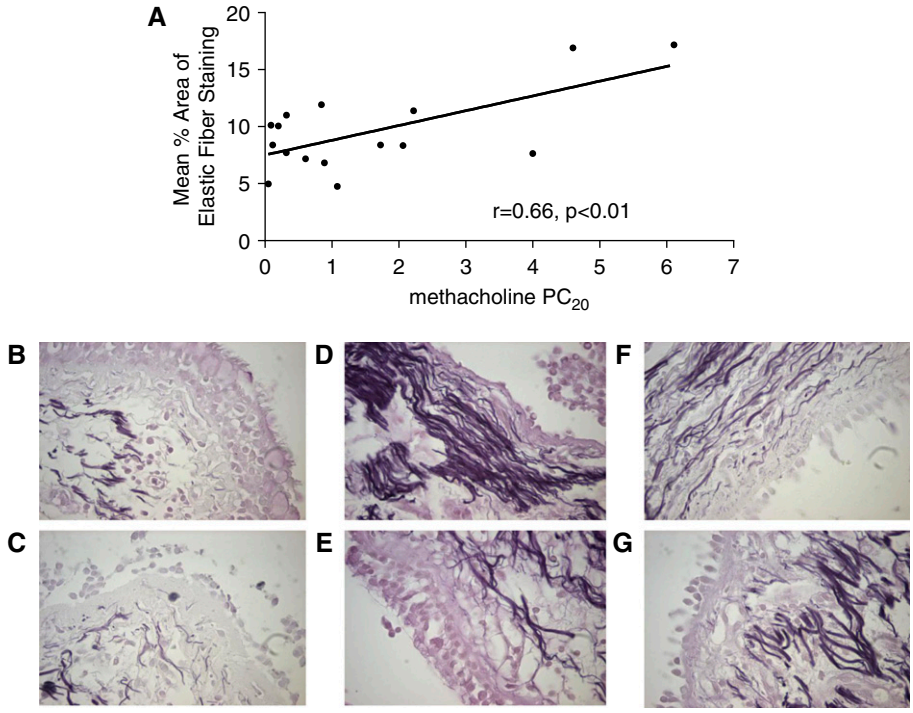

Figure 1. Elastic fiber staining was reduced with airway hyperresponsiveness in asthma. (*A*) Single linear regression of mean percent area of Weigert's resorcin-fuchsin–stained elastic fibers in airway tissue from subjects with asthma reveals significant positive correlation with methacholine PC_{20} values (i.e., the provocative concentration of methacholine resulting in a 20% fall in FEV_1 levels) ($r = 0.66$; $P < 0.01$). (*B* and *C*) Representative sections of airway biopsy tissue stained for elastic fibers in two subjects with asthma and relatively low methacholine PC_{20} measurements (1.08 mg/ml in *B*; 0.05 mg/ml in *C*). (*D* and *E*) Two subjects with asthma and relatively high methacholine PC_{20} measurements (4.6 mg/ml in *D*; 6.11 mg/ml in *E*). (*F* and *G*) Two healthy subjects (methacholine PC_{20} >16 mg/ml).

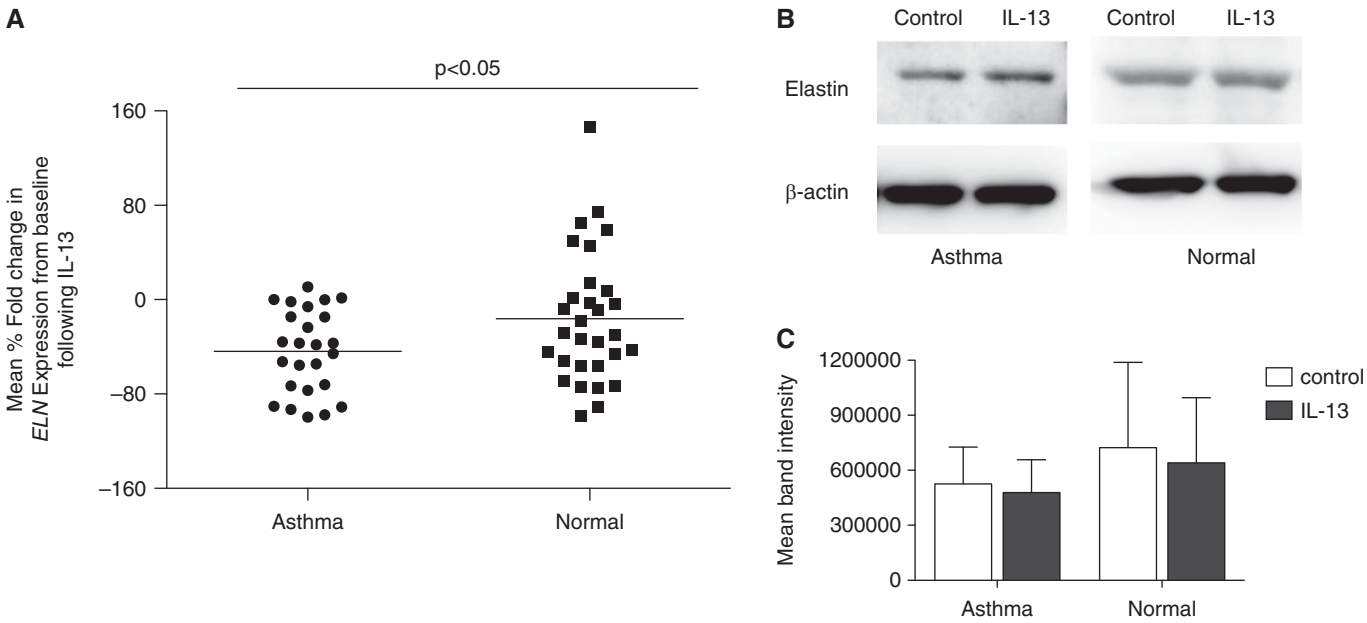

Figure 2. Elastin mRNA and protein expression by airway fibroblasts after IL-13 exposure. (*A*) Elastin mRNA expression, depicted as mean percent fold change from negative unexposed control subjects, was attenuated by IL-13 (50 ng/ml) in subjects with asthma as compared with normal control subjects ($P < 0.05$). (*B*) Representative immunoblot images indicating elastin and β-actin protein expression in whole cell lysates of airway fibroblasts from subjects with asthma or normal subjects after exposure to serum-free media (untreated control) or IL-13 (50 ng/ml) for 48 hours. (*C*) Mean ± SEM densitometry measurements of elastin protein signal in immunoblots from subjects with asthma ($n = 5$) and normal subjects ($n = 3$) ($P > 0.05$ comparing IL-13 with untreated control and asthma versus normal for either control or IL-13 treatments). *ELN*, elastin mRNA.

controller medications. The normal subjects took no chronic medications.

Elastic Fiber Staining Is Associated with AHR in Asthma

Sections of airway biopsy tissue from asthma and healthy control subjects were stained with Weigert's elastic fiber stain. Mean percentage area of elastic fiber staining was significantly and positively correlated with methacholine PC_{20} in asthma ($r = 0.66$; $P < 0.01$) (Figure 1A). Elastic fiber staining in airway biopsy tissue was reduced in patients with asthma who had low methacholine PC_{20} values (Figures 1B and 1C) as compared with patients with asthma who had relatively high methacholine PC_{20} values (Figures 1D and 1E) and healthy subjects (Figures 1F and 1G).

Effect of IL-13 on *ELN* mRNA and Elastin Protein Expression in Airway Fibroblasts

Baseline *ELN* mRNA expression in airway fibroblasts in asthma was not significantly different as compared with normal control

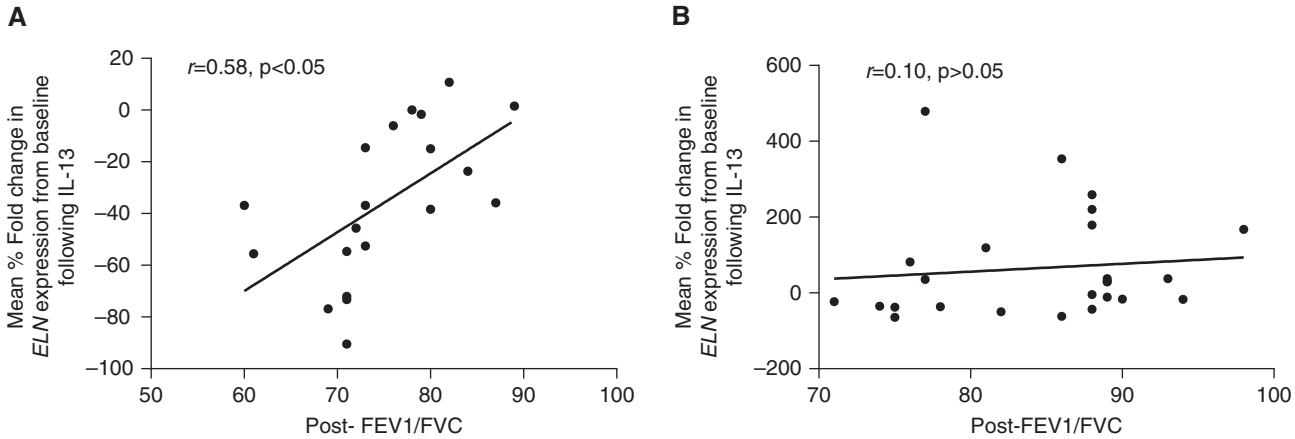

Figure 3. IL-13–induced *ELN* expression in airway fibroblasts from subjects with asthma was significantly associated with post-bronchodilator FEV_1/FVC. (*A*) Single linear regression of post-bronchodilator FEV_1/FVC data from subjects with asthma reveals significant correlation with mean percentage fold change in *ELN* expression after incubation with 50 ng/ml IL-13. (*B*) No significant association was observed between IL-13–induced elastin gene expression and post-bronchodilator FEV_1/FVC in normal subjects.

airway fibroblasts ($P > 0.05$) (*see* Figure E1 in the online supplement). IL-13 significantly suppressed *ELN* mRNA expression in asthmatic airway fibroblasts as compared with unexposed control fibroblasts (mean percentage fold change of *ELN* expression, $-44.0 \pm 7\%$; $P < 0.01$), and the magnitude of suppression in asthmatic airway fibroblasts was significantly greater than that observed in normal control airway fibroblasts (mean percentage fold change in *ELN* expression, $-44.0 \pm 7\%$ for subjects with asthma and $-16.1 \pm 10\%$ for normal control subjects; $P < 0.05$). In normal control subjects, IL-13 did not significantly alter *ELN* mRNA levels relative to untreated control ($P > 0.05$) (Figure 2A).

Elastin protein expression in airway fibroblasts, as detected by Western blot (Figure 2B), was not significantly changed with IL-13 relative to unexposed control in cells from subjects with asthma or from normal subjects ($P > 0.05$), although densitometry measurements indicated a slight reduction of elastin expression in immunoblots of both subjects with asthma and normal subjects (mean band intensity: 525,628.5 ± 201,243 for control subjects and 478,493.2 ± 179,217 for IL-13 in subjects with asthma; 723,506.5 ± 465,263 for control subjects and 640,562 ± 355,344 for IL-13 in normal subjects [$P > 0.05$]) (Figure 2C).

IL-13–Stimulated *ELN* Expression Correlates with Post-Bronchodilator FEV_1/FVC in Asthma

IL-13–induced *ELN* gene expression in airway fibroblasts was significantly and positively associated with post-bronchodilator FEV_1/FVC ($r = 0.58$; $P < 0.01$) (Figure 3A). No significant association between post-bronchodilator FEV_1/FVC and airway fibroblast IL-13–induced *ELN* gene expression was observed in normal subjects ($r = 0.10$; $P > 0.05$) (Figure 3B).

Inhibition of MMPs Reverses the Suppressive Effect of IL-13 on *ELN* Expression

As described above, baseline *ELN* mRNA expression in airway fibroblasts in asthma did not differ significantly from that of normal control airway fibroblasts (Figure E1). Similarly, the addition of a pan-MMP inhibitor (GM6001) alone did not significantly stimulate *ELN* mRNA expression in airway fibroblasts from subjects with asthma as compared with airway fibroblasts from normal control subjects (median percentage fold change in *ELN* expression, 0.0% [IQR, −44.5 to 79.8] for subjects with asthma and −41.0% [IQR, −54.9 to −4.07] for normal subjects; $P > 0.05$) (Figure 4A). Within asthma, treatment with the pan-MMP inhibitor significantly stimulated *ELN* gene expression in airway fibroblasts as compared with IL-13 treatment alone (mean percentage fold change in *ELN* expression, $-34.4 \pm 7\%$ for IL-13 and $1.0 \pm 15\%$ for pan-MMP inhibitor; $P < 0.05$) (Figure 4B). No significant difference was observed between IL-13 and pan-MMP inhibitor treatment in airway fibroblasts from normal control subjects ($P > 0.05$) (Figure 4C).

The effect of the pan-MMP inhibitor was also tested in the presence of IL-13. The combination of pan-MMP inhibitor and IL-13 resulted in airway fibroblast *ELN* mRNA levels that were not significantly different from untreated control subjects for both asthma and normal control groups (mean percentage fold change in *ELN*

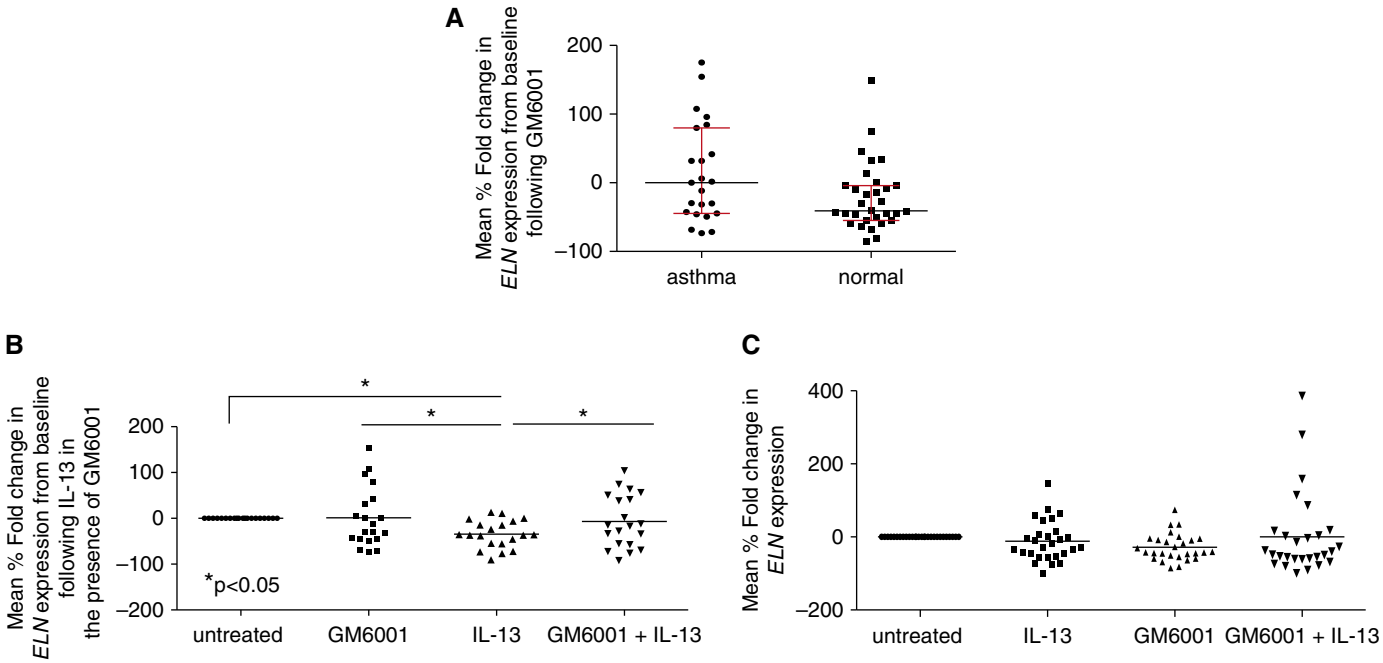

Figure 4. Elastin mRNA expression by airway fibroblasts after exposure to GM6001 alone and combined with IL-13. (*A*) Addition of GM6001 (10 μM) alone had no significant effect on elastin mRNA expression in subjects with asthma as compared with normal control subjects ($P > 0.05$). (*B*) In paired comparisons within fibroblasts from subjects with asthma, IL-13 (50 ng/ml), in combination with GM6001 (10 μM), stimulated a significant increase of *ELN* expression, in contrast to IL-13 alone (*$P < 0.05$). (*C*) In paired comparisons of fibroblasts isolated from normal control patients, no significant effect on *ELN* expression was observed in response to combined IL-13 and pan-matrix metalloproteinase (pan-MMP) inhibitor treatment relative to pan-MMP inhibitor alone or IL-13 alone ($P > 0.05$).

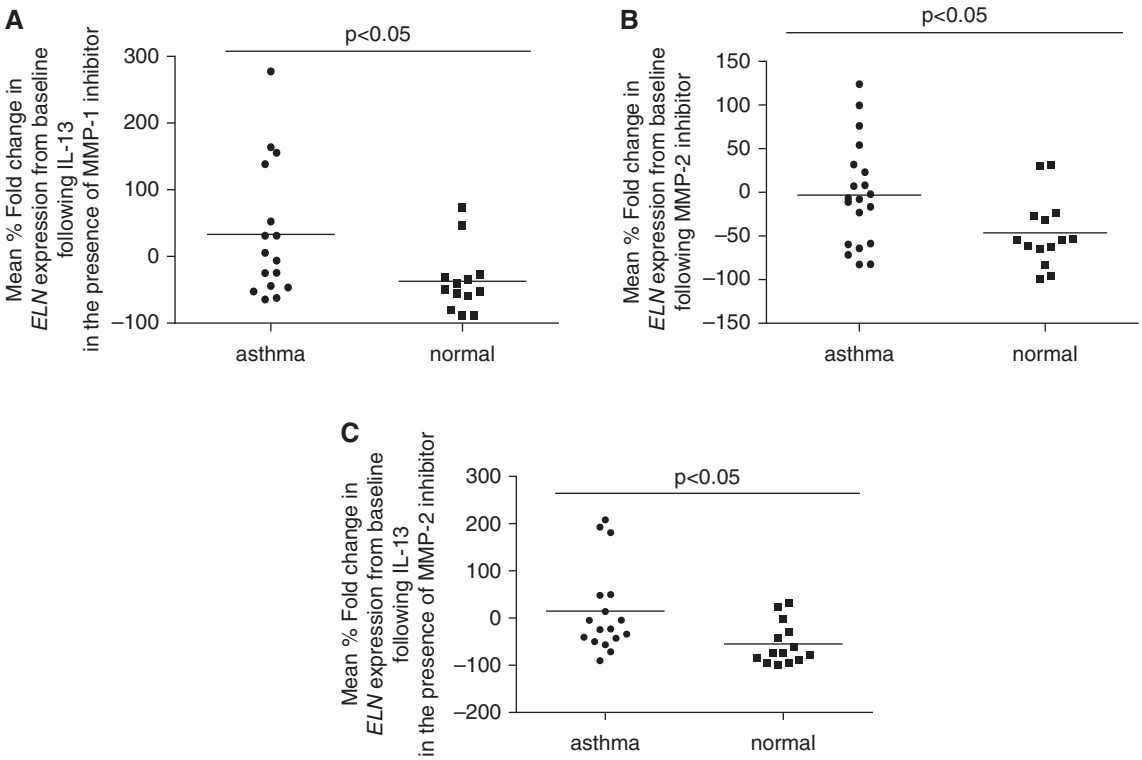

Figure 5. Inhibition of MMP-1 or MMP-2 stimulates increased *ELN* expression in airway fibroblasts from subjects with asthma as compared with fibroblasts from normal subjects. (*A*) MMP inhibitor I (10 μM) combined with IL-13 (50 ng/ml) significantly stimulated increased *ELN* expression in subjects with asthma as compared with normal subjects ($P < 0.05$). (*B*) The MMP-2 inhibitor alone (10 μM) stimulated significantly increased *ELN* expression in subjects with asthma as compared with normal subjects ($P < 0.05$). (*C*) MMP-2 inhibitor (10 μM) combined with IL-13 (50 ng/ml) significantly stimulated increased *ELN* expression in subjects with asthma as compared with normal subjects ($P < 0.05$).

expression, −6.7 ± 13% for subjects with asthma [$P > 0.05$] and 0.2 ± 21% for normal subjects [$P > 0.05$]) (Figures 4B and 4C). However, in asthma, a significant increase in *ELN* gene expression after treatment with the combined pan-MMP inhibitor and IL-13 was observed as compared with IL-13 alone (mean percentage fold change in *ELN* expression, −34.4 ± 7% for IL-13 alone; −6.7 ± 13% for combined MMP inhibitor and IL-13 [$P < 0.05$]) (Figure 4B). Thus, the combination of pan-MMP inhibitor with IL-13 reversed the suppressive effect of IL-13 alone.

MMP-1 and MMP-2 Mediate IL-13–Induced Suppression of *ELN* mRNA Expression

Because the pan-MMP inhibitor is a broad-spectrum inhibitor of several enzymes in the MMP family, these data prompted us to investigate the effects of inhibitors of specific MMPs on elastin gene expression in airway fibroblasts. We used MMP inhibitor I, which specifically inhibits MMP-1 and MMP-8 at low concentrations (half maximal inhibitory concentration [IC50] = 1 μM) and MMP-3 and MMP-9 at very high concentrations ($IC_{50} > 30$ μM). MMP-2 inhibitor-1 specifically inhibits MMP-2. MMP-3 inhibitor-1 specifically inhibits MMP-3. These specific inhibitors were tested alone and in combination with IL-13 (50 ng/ml) on airway fibroblasts from a group of patients. To discern whether effects of the MMP inhibitor I at the 10 μM concentration were due to inhibition of either MMP-1 or MMP-8, we also tested a specific inhibitor of MMP-8 (MMP-8 inhibitor I). No significant effects were observed after incubation of airway fibroblasts with the specific MMP-8 inhibitor alone or combined with IL-13 (Figure E2). Therefore, we conclude that any effects of the MMP inhibitor I in our experiments can be attributed to specific inhibition of MMP-1.

Inhibition of MMP-1 or MMP-2, but neither MMP-3 nor MMP-8, stimulated significantly increased *ELN* expression in airway fibroblasts in subjects with asthma as compared with normal subjects. Alone, inhibition of MMP-1 resulted in no significant change in *ELN* expression in subjects with asthma as compared with normal subjects ($P > 0.05$) (Figure E3). However, when combined with IL-13, the MMP-1 inhibitor resulted in a significant increase in *ELN* expression in subjects with asthma as compared with normal control subjects (median percentage fold change in *ELN* expression, −0.44% [IQR, −45.9 to 116.9] for subjects with asthma and −49.07% [IQR, −69.7 to −29.4] for normal subjects; $P < 0.05$) (Figure 5A).

Similarly, the MMP-2 inhibitor alone significantly increased *ELN* expression in subjects with asthma as compared with normal subjects (mean percentage fold change in *ELN* expression, −3.1 ± 13% for subjects with asthma and −46.5 ± 11% for normal subjects; $P < 0.05$ for inhibitor alone) (Figure 5B). MMP-2 inhibitor combined with IL-13 also resulted in significantly increased *ELN* expression in subjects with asthma (median percentage fold change in *ELN*

expression, −23.2% [IQR, −46.3 to 48.9] for subjects with asthma and −73.8% [IQR, −90 to −22.9] for normal subjects; $P < 0.05$) (Figure 5C).

Within asthma, the combination of IL-13 and the inhibitors of either MMP-1 or MMP-2 reversed the effect of IL-13 in that *ELN* mRNA levels significantly increased relative to the effect of IL-13 alone ($P < 0.05$) (mean percentage fold change in *ELN* expression, −31.7 ± 9% for IL-13 alone, 10.9 ± 24% for MMP-1 inhibitor alone, 39.4 ± 30% for combined MMP-1 inhibitor and IL-13, −36.4 ± 9% for IL-13 alone, 4.0 ± 16% for MMP-2 inhibitor alone, and 16.3 ± 25% for combined MMP-2 inhibitor and IL-13) (Figures 6A and 6B). No significant effect of MMP-1 or MMP-2 inhibitors as compared with IL-13 alone was observed in airway fibroblasts from normal patients (Figure E4). No significant effect on *ELN* mRNA levels in airway fibroblasts in subjects with asthma as compared with normal control subjects or within subjects with asthma was appreciated with specific inhibition of MMP-3 alone or combined with IL-13 ($P > 0.05$) (Figure E5).

IL-13 Stimulates Increased MMP-1 Secretion and Activity in Airway Fibroblasts in Asthma

We have previously shown that IL-13 stimulates significantly increased MMP-2 production (12) and activity (13) in asthmatic airway fibroblasts as compared with normal airway fibroblasts and as compared with untreated control cells. Because inhibition of MMP-1 alters IL-13–induced *ELN* expression in asthmatic airway fibroblasts (Figure 6A), we measured production and activity of MMP-1 in airway fibroblasts from subjects with asthma and normal subjects after exposure to IL-13. IL-13 significantly stimulated total MMP-1 secretion from airway fibroblasts from subjects with asthma as compared with cells from normal control subjects (mean MMP-1 production, 73.89 ± 21.8 ng/ml with IL-13 in airway fibroblasts from subjects with asthma compared with 19.85 ± 4.1 ng/ml with IL-13 in airway fibroblasts from normal control subjects; $P < 0.05$) (Figure 7A). Similarly, the effect of IL-13 on MMP-1 activity was significantly increased in airway fibroblasts from subjects with asthma as compared with cells derived from normal healthy control subjects ($P < 0.05$) and as compared with fibroblasts from untreated control subjects with asthma ($P < 0.05$) (mean active MMP-1, 1.27 ± 0.6 ng/ml for untreated control cells from subjects with asthma; 3.26 ± 1.1 ng/ml for IL-13–treated cells from subjects with asthma compared with 2.46 ± 0.9 ng/ml for normal airway fibroblasts from untreated control subjects; 1.48 ± 0.6 ng/ml for IL-13–treated airway fibroblasts from normal subjects) (Figure 7C). The effect of IL-13 to induce increased MMP-1 secretion or activity was elevated in subjects with asthma as compared with normal control subjects; however, this effect was not significant in the small numbers of patients that we analyzed for this study (Figures 7B and 7D). IL-13 exerted no significant effect on the production of TIMP-1 or -2, endogenous inhibitors of MMP-1 and MMP-2, respectively, by airway fibroblasts ($P > 0.05$, comparing IL-13 with fibroblasts for untreated control subjects with asthma or comparing subjects with asthma with normal subjects for TIMP-1 or TIMP-2) (Figures E6A and E6B).

Discussion

Airway remodeling can lead to permanent biomechanical and pathologic alterations of airways. Despite extended courses of steroids, some patients manifest persistent airway obstruction. Previously, we have shown that elastin fiber degradation in the airways was associated with loss of elastic recoil in asthma (3). We demonstrate in this study that elastin loss occurs in the airways of patients with allergic asthma along with airways obstruction and AHR. These data suggest that elastin synthesis is impaired and/or elastin degradation is augmented with increased AHR and remodeling in asthma.

The mechanisms directing elastin loss in asthma are unclear. In this study, we show that IL-13, a key cytokine that directs airway remodeling in allergic asthma (15, 16), suppresses elastin mRNA expression in airway fibroblasts isolated from subjects with mild allergic asthma. Our data suggest that IL-13 is a critical modulator of elastin mRNA, and this process occurs through effects of MMPs on elastin mRNA levels. The MMPs have emerged as key mediators

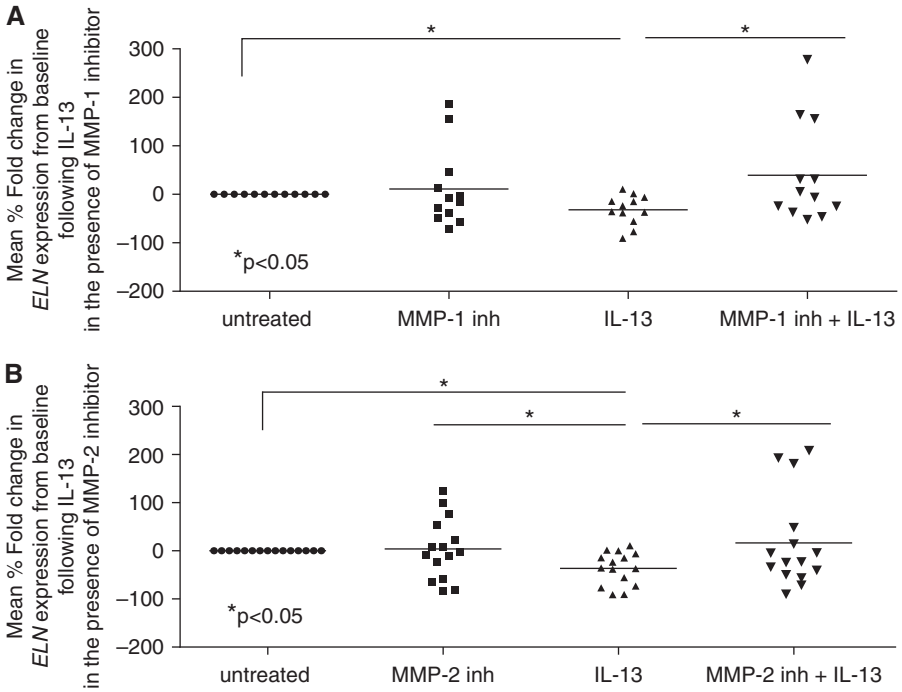

Figure 6. In asthma, inhibition of MMP-1 and MMP-2 reverses the suppressive effect of IL-13 on *ELN* expression in airway fibroblasts. (*A*) In paired comparisons, the combination of MMP-1 inhibitor with IL-13 significantly stimulated increased *ELN* expression as compared with IL-13 alone (*$P < 0.05$). (*B*) In paired comparisons, the combination of MMP-2 inhibitor with IL-13 significantly stimulated increased *ELN* expression as compared with IL-13 alone (*$P < 0.05$).

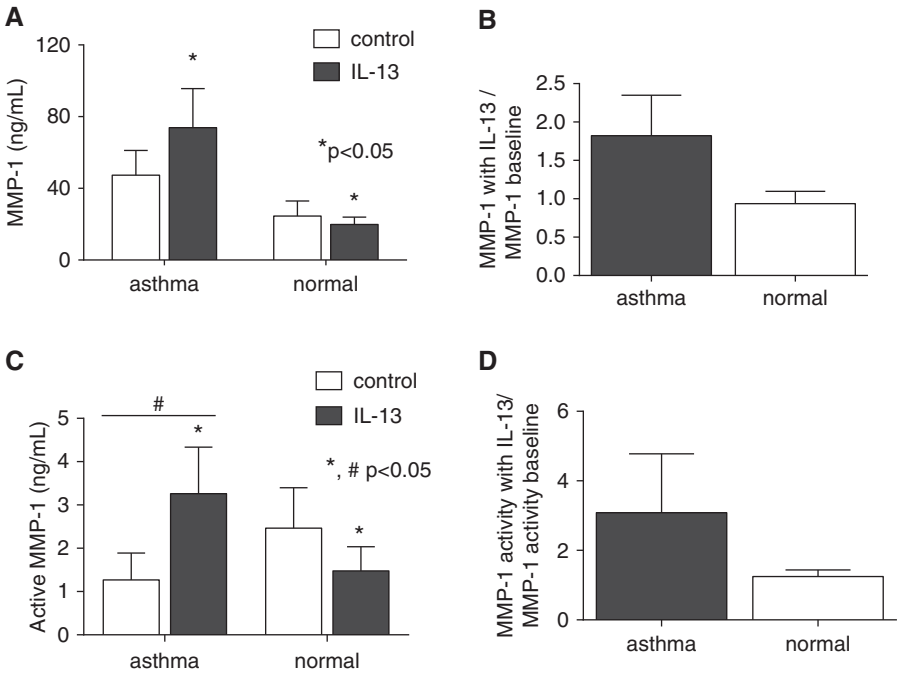

Figure 7. IL-13 stimulated increased MMP-1 secretion and activity in airway fibroblasts from subjects with asthma as compared with fibroblasts from normal control subjects. (*A*) MMP-1 protein production by airway fibroblasts, as measured by ELISA, was significantly increased after exposure to IL-13 (50 ng/ml) in asthmatic fibroblasts ($n = 10$) as compared with normal control ($n = 4$) fibroblasts ($^*P < 0.05$). (*B*) IL-13–induced MMP-1 protein production by airway fibroblasts normalized to baseline MMP-1 protein production indicates a trend toward increased effect of IL-13 on MMP-1 protein levels in asthmatic fibroblasts as compared with normal control fibroblasts ($P > 0.05$). (*C*) Endogenously active MMP-1 levels in asthmatic airway fibroblasts ($n = 12$) were increased after exposure to IL-13 (50 ng/ml) as compared with normal control fibroblasts ($n = 14$) ($^*P < 0.05$) and untreated control fibroblasts ($^\#P < 0.05$). (*D*) IL-13–induced active MMP-1 levels in airway fibroblasts normalized to baseline active MMP-1 levels indicates a trend toward increased effect of IL-13 on active MMP-1 levels in asthmatic fibroblasts as compared with normal control fibroblasts ($P > 0.05$).

of extracellular matrix remodeling in asthma and are an important link between IL-13 and airway elastin, but most reported effects are manifested in protein degradation, not mRNA expression (21). Our study shows that MMPs may also directly or indirectly modulate mRNA expression. Although we have not fully characterized the cellular signaling that accounts for IL-13–directed *ELN* mRNA suppression via MMP activity, one possible mechanism may be through the heparin-binding epidermal growth factor (HB-EGF)/EGF receptor (EGFR) pathway. It is plausible that MMP-1 and MMP-2, induced by IL-13 in airway fibroblasts, mediate proteolytic cleavage of membrane-tethered HB-EGF at the cell surface (22–24). The shed HB-EGF binds to EGFR and, through autocrine signaling, suppresses *ELN* mRNA expression, as previously demonstrated by DiCamillo and colleagues (25) and Liu and colleagues (26) in lung fibroblasts (25, 26). Levels of HB-EGF are increased in bronchoalveolar lavage fluid after allergen stimulation (27). Also, IL-13 stimulates proteolytic release of HB-EGF in airway epithelial cells (28). Thus, we postulate that in patients with allergic asthma, elevated responsiveness to IL-13 by airway fibroblasts stimulates MMP1 and MMP2 secretion, which acts indirectly to initiate negative regulation of *ELN* transcription through shedding of HB-EGF (or other mediators) rather than acting directly to degrade positive regulators of elastin gene expression. Future experiments will determine the role of HB-EGF and EGFR in IL-13–directed *ELN* suppression in airway fibroblasts in asthma.

Murine models of tobacco-induced emphysema show that IL-13 overexpression induces MMP-2, MMP-9, MMP-12, MMP-13, and MMP-14 (21). In this model, the effects of IL-13–related emphysema were attenuated by MMP blockade. Although matrix destruction by MMPs is a potential cause of elastin loss in patients with asthma, we present evidence for the ability of MMPs to mediate suppression of elastin mRNA levels in the airway fibroblast. Thus, IL-13, through its influence on MMP-1 and -2, is linked not only to elastin protein integrity but also to its production. Furthermore, we have previously shown that IL-13 stimulates increased *COL1A2* expression and cellular invasion by airway fibroblasts in asthma through a mechanism requiring MMP-2 activity (12, 13). Therefore, MMP-2 has emerged as an important signaling mediator of several different features of IL-13–induced airway remodeling in asthma, including subepithelial fibrosis and loss of elastin in the submucosa.

We propose that loss of elastin in the asthmatic airway is a facet of remodeling that can lead to significant physiologic changes, including airflow obstruction, increased airways collapsibility, and loss of elastic recoil. Indeed, IL-13–induced suppression of elastin gene expression in airway fibroblasts was significantly correlated with post-bronchodilator FEV_1/FVC, an established measure of airways obstruction and possibly remodeling (29, 30). Our data suggest that IL-13–induced suppression of elastin gene expression in airway fibroblasts could be associated with airways obstruction in asthma.

These data are in agreement with another study that demonstrated significant associations between elastic fiber assembly patterns and post-bronchodilator FEV_1% predicted (31). Our data suggest that increased elastin gene expression is associated with increased post-bronchodilator FEV_1/FVC, or less airway obstruction, and that elastic fiber staining in airway biopsy tissue is directly correlated with methacholine PC_{20}, or inversely correlated with AHR in asthma. We hypothesize that airways collapsibility associated with reduced airway elastin increases susceptibility to not only methacholine but also to other mediators known to cause bronchoconstriction, such as allergens and airway irritants. Furthermore, we have previously shown that IL-13–induced airway fibroblast invasion in asthma was inversely associated with methacholine PC_{20} (12). Taken together, these data suggest that

airway fibroblasts isolated from subjects with mild asthma exhibiting the most severe AHR are more susceptible to IL-13–directed signaling than those with less severe AHR, associating AHR with airway remodeling as manifested by airway wall thickening and stiffening due to increased subepithelial fibrosis and reduced elastin gene expression. Airway collapsibility and loss of elastic recoil may, in part, represent the spectrum of changes caused by IL-13 in asthma; the physiologic manifestation of increased airway collapsibility due to loss of elastin versus increased airway wall stiffness due to increased collagen production, which can each modulate FEV_1/FVC and AHR, will be determined by which process is dominant. This latter issue may be the most important in understanding the phenotypic change seen in asthmatic airway fibroblasts.

A previous study has shown an inverse relationship between methacholine PC_{20} and elastic fiber staining in the large airways in mild asthma (32). Although our study was similar to this previous study in the methods of investigating extracellular matrix in airway biopsy tissue in a small group of patients with mild asthma on no controller medications, we observed an opposite association between elastic fiber staining and methacholine PC_{20}. Because asthma is a heterogeneous disease characterized by several different clinical, inflammatory, and molecular phenotypes (33, 34), many different factors, such as T helper 2–driven inflammation or obesity, may affect the association of elastin fiber staining and AHR. Larger cohorts of patients are required to discern the exact relationship between elastic fiber expression and AHR in specific phenotypes of patients with asthma.

In summary, we have demonstrated that a reduction of elastic fiber staining is associated with increasing AHR in a cohort of subjects with mild allergic asthma. One possible mechanism for this observed elastin loss in asthma might be directed through IL-13–induced suppression of *ELN* mRNA expression in airway fibroblasts. Inhibition of MMP-1 and MMP-2 significantly reverses the IL-13–induced elastin suppression, pointing to an important role for MMPs beyond protein destruction, at the level of elastin gene regulation. Finally, IL-13–induced suppression of *ELN* mRNA expression was correlated with increased airways obstruction, suggesting that loss of elastin contributes to IL-13–induced airway wall stiffening and remodeling in asthma and may partially account for the observed process of airways collapsibility in asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgements: The authors thank Denise Beaver, R.R.T., R.C.P., of Duke University Medical Center for excellence in recruiting patients and coordinating this study, and Joseph Thomas, M.S., M.B.A., of Duke University Medical Center and Juno Pak, B.S., of National Jewish Health for technical assistance with fibroblast cultures and other experiments during the preparation of this manuscript.

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ONLINE DATA SUPPLEMENT

Matrix metalloproteinases-1 and -2 Mediate IL-13-induced Suppression of Elastin in Airway Fibroblasts in Asthma

Jennifer L. Ingram, David Slade, Tony D. Church, Dave Francisco, Karissa Heck, R. Wesley Sigmon, Michael Ghio, Anays Murillo, Rafael Firszt, Njira L. Lugogo, Loretta Que, Mary E. Sunday and Monica Kraft

METHODS

Subjects

All asthmatics were atopic, as demonstrated by at least one positive skin test to common Colorado or North Carolina allergens associated with a correlative clinical history. 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 beta-2 agonists only were recruited. Exclusion criteria included post-bronchodilator FEV_1 < 50% predicted, inpatient status, upper or lower respiratory tract infection within one month of study, use of inhaled corticosteroids, leukotriene modifiers and/or theophylline preparations within four weeks of study, long acting beta-2 agonists within two weeks of study, smoking history greater than 5 pack-years or any cigarette use within the previous two years, significant non-asthma pulmonary disease or other medical problems.

Bronchoscopy

Prior to the bronchoscopy, spirometry was performed and asthmatics received 0.18 mg of inhaled albuterol. Lidocaine (2%) was used to anesthetize the upper airway and lidocaine (1%) was applied to the laryngeal area, trachea and orifice of the right lower or left lower lobe bronchi via the bronchoscope. Biopsy sites were randomized to the right or left lower lobe. Four to five endobronchial tissue samples were taken from third and fourth generation airways by direct visualization. Supplemental oxygen was administered throughout the procedure along with monitoring of heart rate, blood pressure and oxygen saturation. Upon completion of the procedure, subjects were

carefully monitored and then discharged once their FEV_1 reached 90% of their pre-bronchoscopy, post-bronchodilator value.

Airway tissue staining

Rehydrated airway tissue sections were stained for 3 hours using Weigert's resorcin-fuchsin; no counterstain was applied. After being stained, slides were washed in 0.4 mL glacial acetic acid in ~200 mL tap water for 10 min. They were then reprocessed through 1X PBS, alcohol, and xylene before being cover-slipped. 4X images were taken of each slide to prevent sampling error with the small specimens. In the event the entire specimen did not fit on the screen, additional non-overlapping 4X images were taken and analyses of those images were averaged. Before analysis, all images were edited through ImageJ to have uniform white backgrounds and to exclude cartilage and glands, which were present in only a few biopsies.

Fibroblast Culture

Endobronchial tissue specimens were rinsed in Dulbecco's Modified Eagle Medium (DMEM) and manually sectioned into 5 to 6 smaller pieces. Equal numbers of tissue fragments were plated onto three sterile, 60mm collagen-coated culture dishes (BioCoat) with 4 ml of growth medium (DMEM with 10% Fetal Bovine Serum (FBS), gentamicin, penicillin/streptomycin mixture, and nystatin). The tissue pieces were incubated at 37°C/5% CO_2, with fresh media added every 2 to 3 days, until they reached >80% confluency. At confluency, the tissue pieces were removed and the cells passaged, via trypsinization, onto sterile, collagen-coated tissue culture flasks (either $75cm^2$ or $150cm^2$, depending on the number of cells harvested).

Mediator Exposure

GM6001 is a pan-inhibitor of MMPs and has been shown to inhibit: MMP-1 (fibroblast collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-8 (neutrophil collagenase), MMP-9 (gelatinase B), MMP-12 (metalloelastase), MMP-14, MMP-26 (endometase), MMP-10, MMP-13, MMP-15, MMP-17, and MMP-21 (18).

Quantitative Real-time PCR

Total RNA from airway fibroblasts was extracted using TRIzol reagent (Gibco BRL, Rockville, MD) according to manufacturer's protocol. Briefly, cells in each well of a 12-well plate were lysed in 1 mL Trizol reagent. Total RNA was extracted in the aqueous phase following addition of chloroform. After precipitation with isopropanol, the RNA was washed several times with 75% ethanol, then resuspended in 30 µL RNAse-free deionized water. Total RNA was quantified by determining absorbance at 260nm using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE). First-strand cDNA was prepared using 1µg of total RNA and random hexamers in a 50 µl reaction according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The 50 µl PCR reaction contains 50 ng cDNA, 100 nM fluorogenic probe and 200 nM primers and other components contributed from Applied Biosystems 2x RT-PCR Master Mix. Elastin (*ELN*) primers (Genbank, accession # NM_000501) were purchased from Applied Biosystems; product number for primer: Hs0355783_m1. Expression of the housekeeping gene *GAPDH* (Applied Biosystems, Hs9999995_m1) was also evaluated. The specificity of PCR was verified by lack of a signal in no-template controls or RT (–) RNA samples. The threshold cycle (C_T) was recorded for each sample, in each

condition. Fold changes in *ELN* mRNA expression was calculated by normalization of all expression levels to *GAPDH* mRNA levels and then compared to those of untreated cells by the $\Delta\Delta C_T$ method. Baseline fold-change in ELN mRNA expression in both asthma and normal control airway fibroblasts was calculated by normalizing to the mean ΔC_T value of the normal control subjects.

Western Blot Analyses

Whole cell lysates from cultured airway fibroblasts were collected at 48 hours after IL-13 (50 ng/ml) treatment. Proteins in cell lysates (15 µg/lane) were resolved on 4-12% SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were probed with 1:100 dilution of mouse monoclonal anti-human elastin antibody (Santa Cruz Biotechnology, Dallas, TX) and elastin protein signal was detected using enhanced chemiluminescence. The membranes were then stripped using western blot stripping buffer (Thermo Scientific, Rockford, IL) and re-probed using 1:1000 dilution of mouse anti-human β-actin antibody (Santa Cruz Biotechnology, Dallas, TX). The blots were documented with the Kodak Image Station 4000 MM PRO and elastin protein band intensity was quantified using Carestream Molecular Image software (version 5.0).

Figure E1: Elastin mRNA expression by airway fibroblasts at baseline. *ELN* mRNA expression, depicted as mean percent fold change in airway fibroblasts from asthma and normal control subjects, normalized to the mean cycle threshold (cT) of the normal controls and to *GAPDH* expression ($p>0.05$).

Figure E2: **Elastin mRNA expression by airway fibroblasts following exposure to MMP-8 inhibitor alone or combined with IL-13.** Addition of MMP-8 inhibitor (10 μM), alone (**A**) or in combination with IL-13 (**B**) had no significant effect on elastin mRNA expression in asthma as compared to normal controls ($p>0.05$). In fibroblasts isolated from asthma patients, (**C**) no significant effect on ELN expression was observed in response to combined IL-13 and MMP-8 inhibitor relative to MMP-8 inhibitor alone or IL-13 alone ($p>0.05$, paired comparisons).

Figure E3: **Elastin mRNA expression by airway fibroblasts following exposure to MMP-1 inhibitor alone.** Addition of MMP-1 inhibitor (10 μM), alone had no significant effect on elastin mRNA expression in asthma as compared to normal controls ($p>0.05$).

Figure E4: Elastin mRNA expression by airway fibroblasts following exposure to MMP-1 inhibitor or MMP-2 inhibitor combined with IL-13. In paired comparisons, in fibroblasts isolated from normal control patients, no significant effect on ELN expression was observed in response to combined IL-13 and MMP-1 (**A**) or MMP-2 (**B**) inhibitors relative to MMP-1 or -2 inhibitor alone or IL-13 alone ($p>0.05$).

Figure E5: Elastin mRNA expression by airway fibroblasts following exposure to MMP-3 inhibitor alone or combined with IL-13. Addition of MMP-3 inhibitor (10 μM), alone (**A**) or in combination with IL-13 (**B**) had no significant effect on elastin mRNA

expression in asthma as compared to normal controls ($p>0.05$). In fibroblasts isolated from asthma patients, (**C**) no significant effect on ELN expression was observed in response to combined IL-13 and MMP-3 inhibitor relative to MMP-3 inhibitor alone or IL-13 alone ($p>0.05$, paired comparisons).

Figure E6: IL-13 stimulated no significant change in TIMP-1 and TIMP-2 levels with IL-13 in asthma as compared to normal controls or untreated control. A) TIMP-1 or **B**) TIMP-2 protein production in airway fibroblasts, as measured by ELISA, was not significantly altered in asthma (n=15) following exposure to IL-13 (50 ng/mL) as compared to normal control fibroblasts (n=16) or untreated control fibroblasts ($p>0.05$).

Figure E1

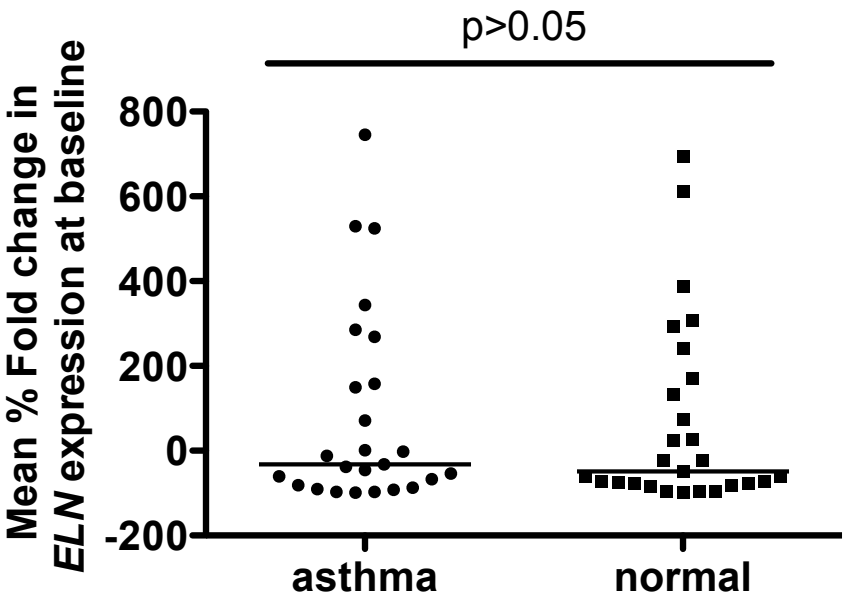

p>0.05
Mean % Fold change in
ELN expression at baseline
800
600
400
200
0
-200
asthma
normal

Figure E2

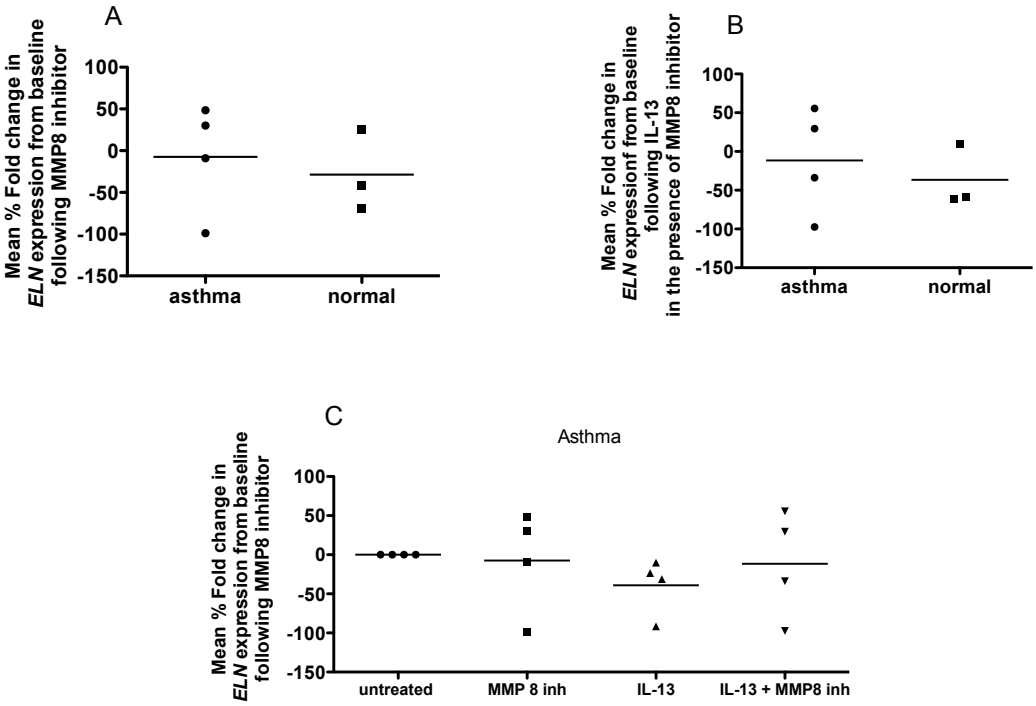

A
Mean % Fold change in
ELN expression from baseline
following MMP8 inhibitor
100
50
0
-50
-100
-150
asthma
normal
B
Mean % Fold change in
ELN expressionf from baseline
following IL-13
in the presence of MMP8 inhibitor
100
50
0
-50
-100
-150
asthma
normal
C
Asthma
Mean % Fold change in
ELN expression from baseline
following MMP8 inhibitor
100
50
0
-50
-100
-150
untreated
MMP 8 inh
IL-13
IL-13 + MMP8 inh

Figure E3

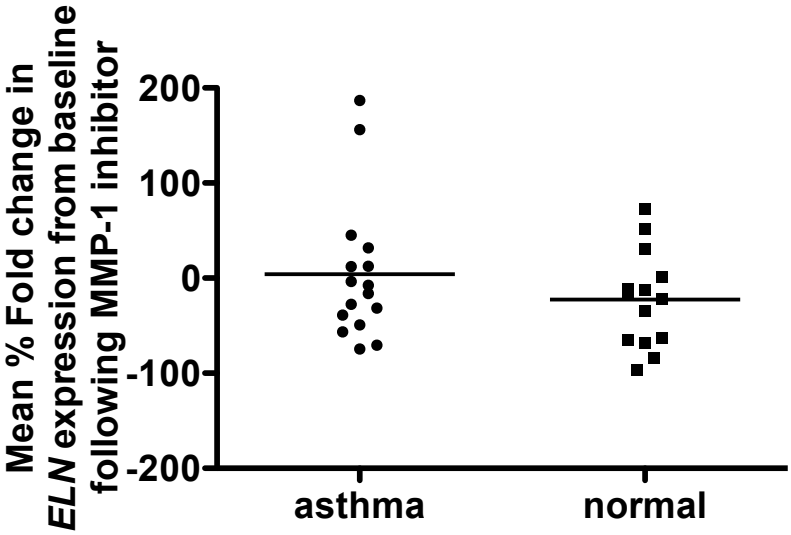
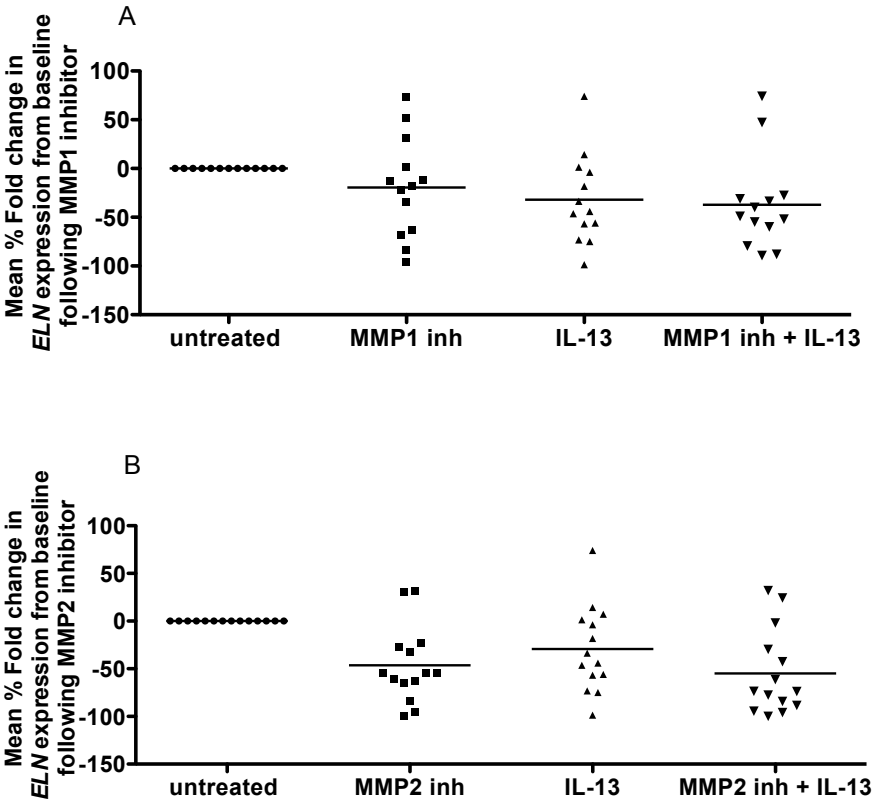

Mean % Fold change in
ELN expression from baseline
following MMP-1 inhibitor
200
100
0
-100
-200
asthma
normal

Figure E4


A
Mean % Fold change in
ELN expression from baseline
following MMP1 inhibitor
100
50
0
-50
-100
-150
untreated
MMP1 inh
IL-13
MMP1 inh + IL-13
B
Mean % Fold change in
ELN expression from baseline
following MMP2 inhibitor
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50
0
-50
-100
-150
untreated
MMP2 inh
IL-13
MMP2 inh + IL-13

Figure E5

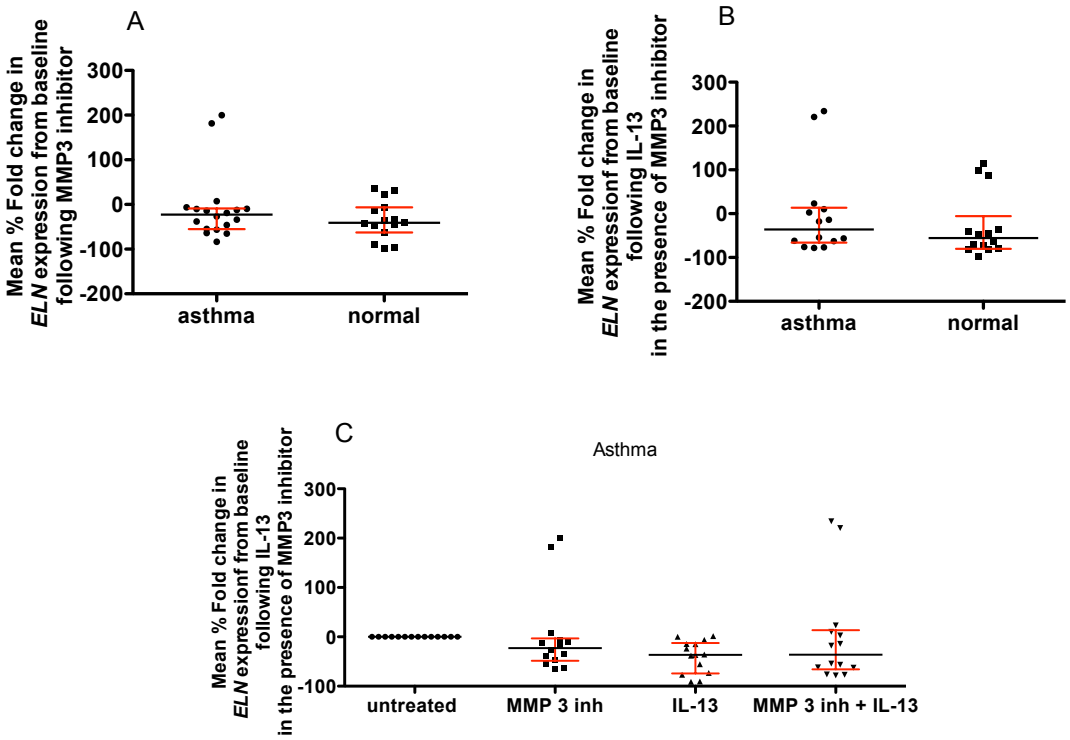
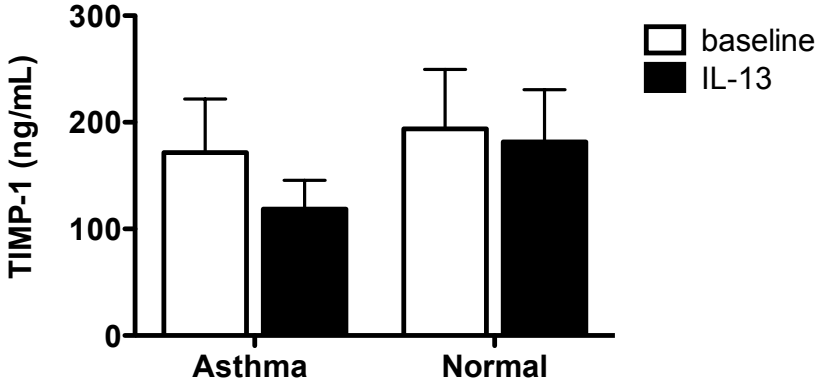
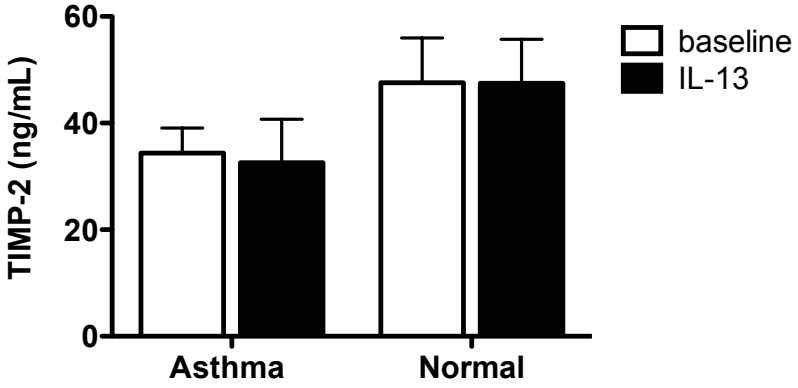

A
Mean % Fold change in
ELN expression from baseline
following MMP3 inhibitor
300
200
100
0
-100
-200
asthma
normal
B
Mean % Fold change in
ELN expressionf from baseline
following IL-13
in the presence of MMP3 inhibitor
300
200
100
0
-100
-200
asthma
normal
C
Asthma
Mean % Fold change in
ELN expressionf from baseline
following IL-13
in the presence of MMP3 inhibitor
300
200
100
0
-100
untreated
MMP 3 inh
IL-13
MMP 3 inh + IL-13

Figure E6

A


300
200
100
0
TIMP-1 (ng/mL)
Asthma
Normal
baseline
IL-13

B


60
40
20
0
TIMP-2 (ng/mL)
Asthma
Normal
baseline
IL-13