

Eotaxin-2/CCL24 and eotaxin-3/CCL26 exert differential profibrogenic effects on human lung fibroblasts

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Background: Eotaxin-2/CCL24 and eotaxin-3/CCL26 play an important role in eosinophil chemotaxis and activation in asthma. We previously demonstrated that eotaxin/CCL11 is profibrogenic for human lung fibroblasts. The effect of eotaxin-2/CCL24 and eotaxin-3/CCL26 on lung fibroblasts has not yet been investigated.

Objective: To evaluate whether eotaxin-2/CCL24 and eotaxin-3/CCL26 modulate fibrotic properties of lung fibroblasts.

Methods: Fibroblast proliferation was evaluated by means of 3-hydroxythymidine incorporation. Collagen production was assessed by means of 3-hydroxyproline incorporation and biochemical staining. Chemotaxis was determined using Boyden chambers. Expression of α -smooth muscle actin was evaluated by means of immunostaining. Transforming growth factor β 1 release was assessed using enzyme-linked immunosorbent assay. Parametric analysis of variance, followed by the Tukey-Kramer multiple comparisons test, was used to calculate statistical significance.

Results: Eotaxin-2/CCL24 but not eotaxin-3/CCL26 stimulated human lung fibroblast proliferation and collagen synthesis. In contrast, eotaxin-3/CCL26 but not eotaxin-2/CCL24 promoted fibroblast migration. Neither eotaxin-2/CCL24 nor eotaxin-3/CCL26 induced the expression of α -smooth muscle actin or transforming growth factor β 1 from lung fibroblasts.

Conclusions: Eotaxin-2/CCL24 and eotaxin-3/CCL26 have differential profibrogenic effects on human lung fibroblasts. These CC chemokines may, therefore, contribute to airway remodeling in asthma.

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INTRODUCTION

ptk;4Asthma is a complex chronic disease characterized by airway obstruction, airway hyperresponsiveness, inflammation, and remodeling.^{1,2} Airway remodeling is defined as the qualitative and quantitative changes observed in structural components of the airways, including hyperplasia of mucous-secreting cells, increased subepithelial deposition of extracellular matrix, increased smooth muscle, and angiogenesis.³ The molecular mechanisms that underlie the remodeling changes in chronic asthma are not yet fully understood.

Fibroblast activation and differentiation to a myofibroblastic phenotype is a feature of airway remodeling^{1–3} and has been reported in asthmatic patients.⁴ Fibroblasts/myofibroblasts are a major source of collagen production, and after

activation, they account for the increased extracellular matrix deposition and subsequent subepithelial fibrosis seen in chronic asthma.⁵

Eotaxin-1/CCL11,⁶ eotaxin-2/CCL24,⁷ and eotaxin-3/CCL26⁸ are members of the CC family of chemokines. Several studies provide evidence of a role for CC chemokines in human asthma, where they play an important role in airway inflammation, particularly via induction of eosinophil chemotaxis and activation.^{9,10} Increased levels of eotaxin/CCL11 and its receptor, CC chemokine receptor 3 (CCR-3), were observed in bronchial biopsy samples from asthmatic patients¹¹ and in lungs and sputum after allergen challenge.¹² Eotaxin-2/CCL24 expression is also increased in endobronchial biopsy samples and bronchial epithelium of asthmatic patients.^{13,14} Eotaxin-3/CCL26 is upregulated in bronchial epithelium and airways of asthmatic individuals 24 hours after allergen challenge.^{13,14}

We have previously shown that human lung fibroblasts express CCR-3 and that eotaxin/CCL11 has selective profibrogenic properties on human lung fibroblasts.¹⁵ Although eotaxin-2/CCL24 and eotaxin-3/CCL26 are expressed by human lung and dermal fibroblasts,^{16,17} there are no data, to our knowledge, that explore whether these chemokines can modulate fibroblast behavior. In this study, we demonstrate that eotaxin-2/CCL24 and eotaxin-3/CCL26 have differential profibrogenic activity on lung fibroblasts.

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METHODS

Cell Culture

The human fetal lung fibroblast line MRC-5 (American Type Culture Collection, Manassas, Virginia) was cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2-mmol/L L-glutamine, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin (all from Biological Industries, Beit Haemek, Israel) at 37°C in 5% carbon dioxide. Human lung fibroblasts were obtained from lung biopsy samples as previously described.¹⁵

Fibroblast Proliferation

MRC-5 fibroblasts and primary lung fibroblasts (5×10^3 per 0.2 mL) were seeded in 96-well plates. When cells reached semiconfluence (approximately 80%), they were incubated for 24 hours at 37°C in 5% carbon dioxide with DMEM (0.5% FCS) alone or containing increasing concentrations of eotaxin/CCL11, eotaxin-2/CCL24, or eotaxin-3/CCL26 (5–500 ng/mL) (all from PreproTech Asia, Rehovot, Israel). Transforming growth factor β 1 (TGF- β 1) (10 ng/mL) (R&D Systems, Minneapolis, Minnesota) was used as a positive control. 3-Hydroxythymidine ($[^3\text{H}]$ -thymidine) (1 μ Ci per well) (NEN Life Science Products Inc, Boston, Massachusetts) was added as a final 24-hour pulse, and samples were processed as previously described.¹⁸

Collagen Production and Deposition

Collagen production was determined based on 3-hydroxyproline ($[^3\text{H}]$ -proline) incorporation into collagen proteins.¹⁸ MRC-5 cells (5×10^4 per well) were seeded in 24-well plates. When cells reached confluence, they were exposed to DMEM containing 5% FCS, 50 μ g/mL of β -aminopropionitrile, and 50 μ g/mL of ascorbic acid in the presence or absence of increasing concentrations of eotaxin/CCL11, eotaxin-2/CCL24, or eotaxin-3/CCL26 (5–500 ng/mL) at 37°C in 5% carbon dioxide. TGF- β 1 (10 ng/mL) was used as a positive control. Cells were incubated in these conditions for 24 hours, and then $[^3\text{H}]$ -proline (10 μ Ci/mL) (NEN Life Science Products Inc) was added for an additional 24 hours. Samples were subsequently processed as previously described.¹⁸

Collagen deposition was determined using the Sircol Collagen Assay Kit (Biocolor Ltd, Belfast, Northern Ireland) as previously described.¹⁹ Briefly, MRC-5 cells or primary lung fibroblasts seeded in 24-well plates were incubated in DMEM (10% FCS) at 37°C in 5% carbon dioxide. When cells reached semiconfluence (approximately 80%), they were exposed to increasing concentrations of eotaxin/CCL11, eotaxin-2/CCL24, or eotaxin-3/CCL26 (5–500 ng/mL) for 48 hours. TGF- β 1 (10 ng/mL) was used as a positive control. Collagen deposited in each well was then stained. The amount of collagen was evaluated by means of spectrophotometry at 540 nm. Results are expressed as fold increase over untreated cells.

Fibroblast Migration

Chemotaxis of MRC-5 fibroblasts (10^4 cells per well) was evaluated in Boyden chambers.¹⁵ Increasing concentrations of

eotaxin-2/CCL24 and eotaxin-3/CCL26 (5–500 ng/mL) were used as chemoattractants for the cells resting in the lower chambers. Cells were incubated for 6 hours at 37°C in 5% carbon dioxide (short-term treatment). In a different set of experiments, MRC-5 fibroblasts were exposed overnight to increasing concentrations of eotaxin-2/CCL24 and eotaxin-3/CCL26 (5–500 ng/mL), and their migration in response to the conditioned medium of 3T3 fibroblasts (nonspecific chemoattractants) was evaluated (long-term treatment). Eotaxin/CCL11 (200 ng/mL) was used as a positive control.¹⁵

Immunofluorescence for α -Smooth Muscle Actin

Confluent MRC-5 fibroblasts were cultured on round coverslips and were prepared as previously described.¹⁹ Cells were incubated with monoclonal anti-mouse α -smooth muscle actin (α -SMA) (Sigma-Aldrich Corp, St Louis, Missouri) or irrelevant isotype-matched IgG antibodies (60 minutes at 4°C) in the presence or absence of increasing concentrations of eotaxin-2/CCL24 or eotaxin-3/CCL26 (5–500 ng/mL). TGF- β 1 (10 ng/mL) was used as a positive control. Binding was detected using Cy5-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc, West Grove, Pennsylvania). Nuclei were counterstained with propidium iodide (Sigma-Aldrich Corp). Tissue slides were examined using a FluoView FV 300 confocal laser scanning biological microscope (Olympus Corp, Center Valley, Pennsylvania). Quantification was evaluated using Image-Pro Plus (Media Cybernetics Inc, Silver Spring, Maryland) on photographs taken at a magnification of 600 $\times$ and was expressed as integrated optical density.

TGF- β 1 Release

Confluent MRC-5 fibroblasts were cultured in 24-well plates at 37°C in 5% carbon dioxide and were exposed to increasing concentrations of eotaxin/CCL11, eotaxin-2/CCL24, and eotaxin-3/CCL26 (48 hours, 5–500 ng/mL). Active TGF- β 1 was measured in culture supernatants by means of enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems).

Statistical Analysis

Data are expressed as the mean (SEM) of at least 3 independent experiments. Parametric analysis of variance, followed by the Tukey-Kramer multiple comparisons test, was used to compare groups. $P < .05$ was considered statistically significant.

RESULTS

Effects of Eotaxin-2/CCL24 and Eotaxin-3/CCL26 on Fibroblast Proliferation

To investigate whether eotaxin-2/CCL24 and eotaxin-3/CCL26 induce fibroblast proliferation, the MRC-5 line and primary human lung fibroblasts were exposed to increasing concentrations of eotaxin-2/CCL24 and eotaxin-3/CCL26 (5–500 ng/mL), and proliferation was then determined by means of $[^3\text{H}]$ -thymidine incorporation. As shown in Figure 1A, 500 ng/mL of eotaxin-2/CCL24 increased MRC-5 fibroblast proliferation by 1.8-fold ($P < .01$), whereas eotaxin-3/CCL26 had no effect. The effect observed for eotaxin-2/

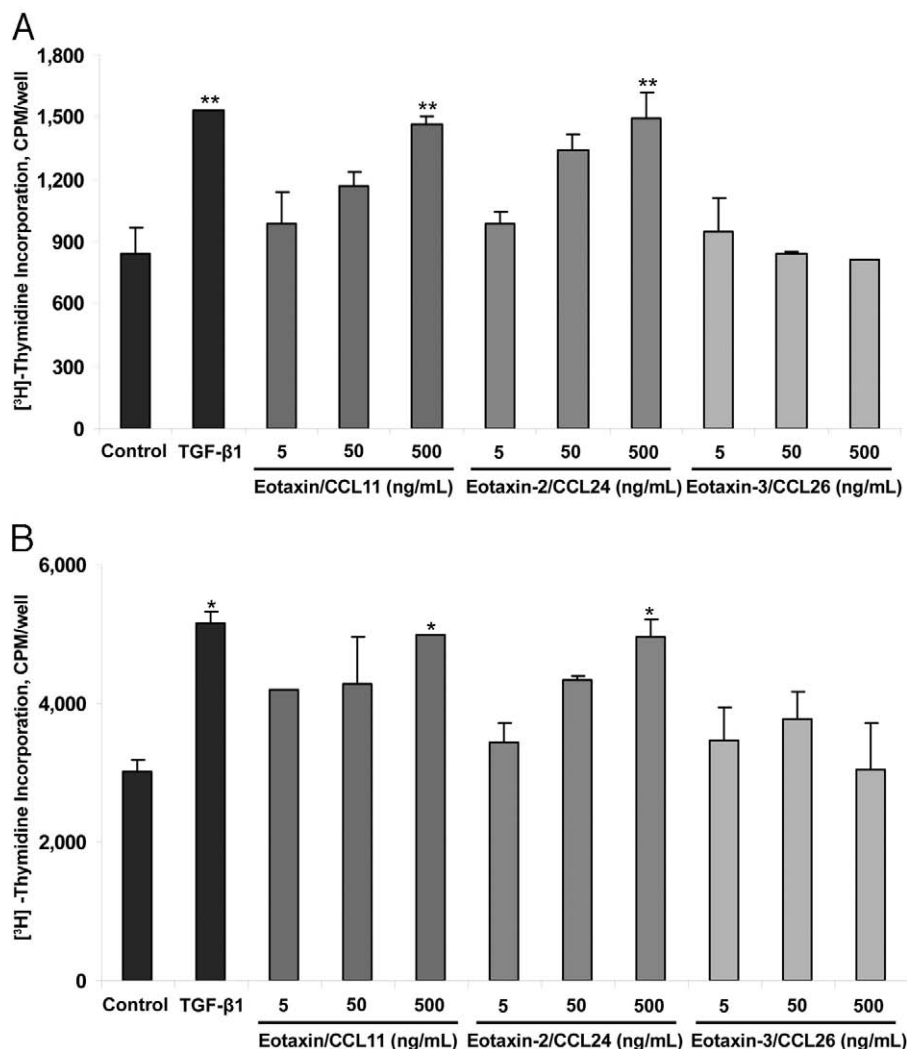


Figure 1. Eotaxin-2/CCL24 increases human lung fibroblast proliferation. MRC-5 (A) and primary human lung fibroblasts (B) were treated with increasing concentrations of eotaxin/CCL11, eotaxin-2/CCL24, or eotaxin-3/CCL26 for 24 hours, and proliferation was then evaluated. Data are given as the mean of 3 experiments performed in triplicate. Error bars represent SEM. * $P < .05$ and ** $P < .01$ vs control. [³H]-thymidine indicates 3-hydroxythymidine; TGF-β1, transforming growth factor β1.

CCL24 was similar to that of TGF-β1, a potent inducer of fibroblast proliferation used as a positive control. Exposure of primary cultured human fibroblasts to 500 ng/mL of eotaxin-2/CCL24 also increased the proliferation rate of these cells by 1.6-fold ($P < .05$) (Fig 1B).

Effect of Eotaxin-2/CCL24 and Eotaxin-3/CCL26 on Collagen Synthesis

We examined whether eotaxin-2/CCL24 and eotaxin-3/CCL26 have an effect on collagen production and deposition. We observed that eotaxin-2/CCL24 (5 ng/mL) but not eotaxin-3/CCL26 significantly enhanced collagen production by MRC-5 cells, as determined by [³H]-proline incorporation ($P < .05$) (Fig 2A). Increased doses of eotaxin-2/CCL24 (50 and 500 ng/mL) did not lead to a further increase in collagen production in these cells. Consistent

with the findings for collagen production, exposure to eotaxin-2/CCL24 (5 ng/mL) also increased collagen deposition by MRC-5 fibroblasts ($P < .01$) (Fig 2B) and by primary lung fibroblasts ($P < .01$) (Fig 2C).

Effects of Eotaxin-2/CCL24 and Eotaxin-3/CCL26 on Fibroblast Chemotaxis

We investigated whether eotaxin-2/CCL24 and eotaxin-3/CCL26 promote fibroblast chemotaxis. Stimulation for 6 hours with increasing concentrations of eotaxin-2/CCL24 or eotaxin-3/CCL26 (5–500 ng/mL) did not induce any chemotactic effect on MRC-5 cells (data not shown). Conversely, after overnight treatment (18 hours of stimulation) with eotaxin-3/CCL26, the migration of MRC-5 fibroblasts in response to conditioned medium of 3T3 fibroblasts increased 2.0-, 2.0-, and 2.83-fold ($P < .05$).

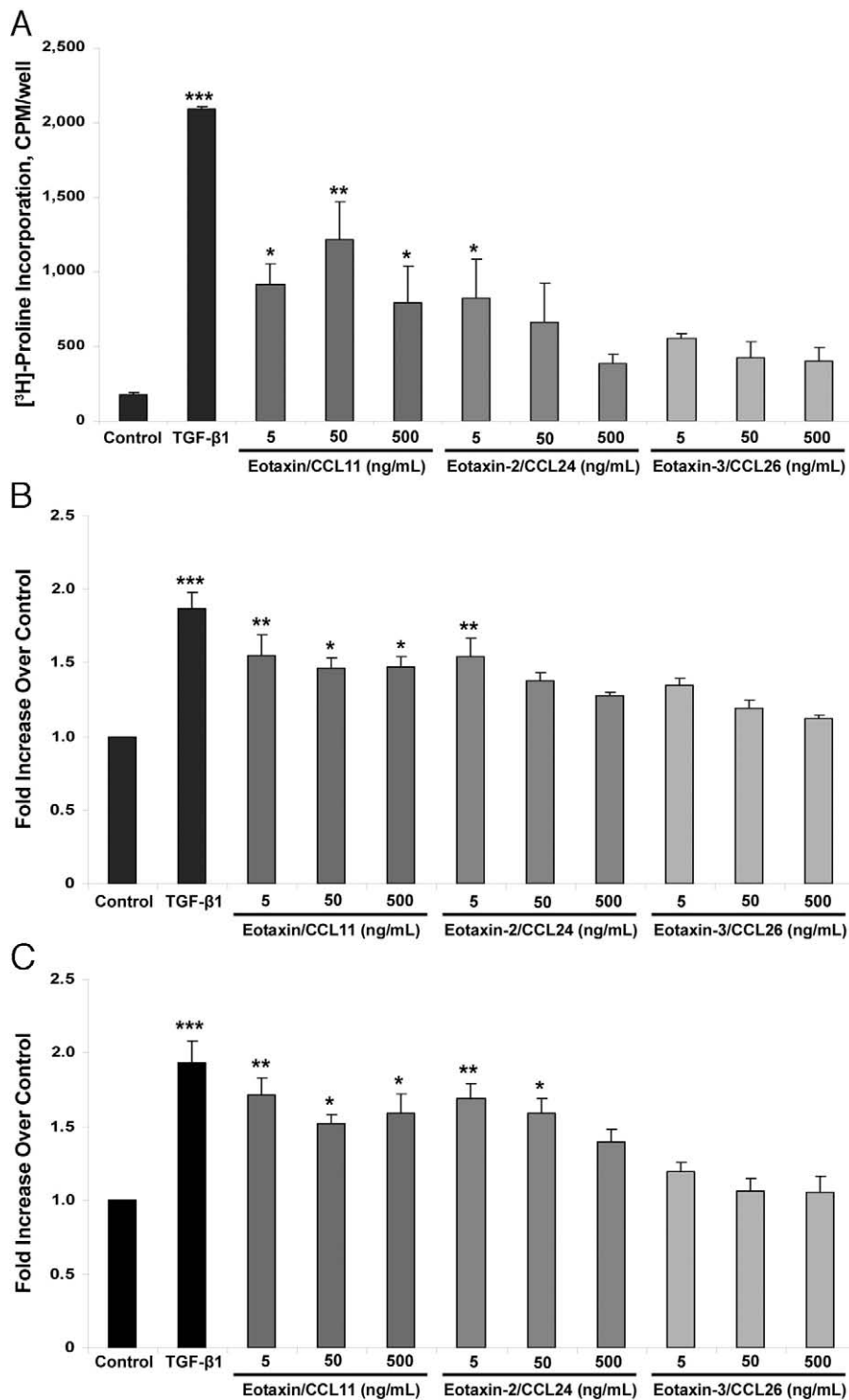


Figure 2. Eotaxin-2/CCL24 increases human lung fibroblast collagen production and deposition. MRC-5 fibroblasts and primary human lung fibroblasts were incubated with increasing concentrations of eotaxin/CCL11, eotaxin-2/CCL24, or eotaxin-3/CCL26. Collagen production and deposition were evaluated by means of 3-hydroxyproline (³H]-proline) incorporation and the Sircol assay, respectively. Eotaxin-2/CCL24, 5 ng/mL, increased collagen production (A) and deposition (B) in MRC-5 fibroblasts and collagen deposition in primary lung fibroblasts (C). Data are given as the mean of 4 experiments. Error bars represent SEM. **P* < .05, ***P* < .01, and ****P* < .001 vs control. TGF-β1 indicates transforming growth factor β1.

.001) at 5, 50, and 500 ng/mL, respectively, compared with cells cultured in the absence of eotaxin-3/CCL26 (control) (Fig 3). In contrast, no chemotactic effect was observed with eotaxin-2/CCL24. Eotaxin/CCL11 (200 ng/mL), used as a positive control, increased fibroblast chemotaxis by 5-fold compared with untreated cells.

Effect of Eotaxin-2/CCL24 and Eotaxin-3/CCL26 on Fibroblast Differentiation and TGF- β 1 Release

To investigate fibroblast differentiation, we evaluated the expression of α -SMA by means of immunostaining. After incubation with increasing concentrations of eotaxin-2/CCL24 and eotaxin-3/CCL26 (5–500 ng/mL) for 48 hours, we did not observe any effect on α -SMA expression in MRC-5 fibroblasts. TGF- β 1 (10 ng/mL), used as a positive control, increased α -SMA expression (Fig 4). In addition, neither eotaxin-2/CCL24 nor eotaxin-3/CCL26 stimulated TGF- β 1 release after exposure of MRC-5 cells to 500 ng/mL of each eotaxin (Table 1).

DISCUSSION

Fibroblasts and myofibroblasts are key contributors to the development of airway remodeling in chronic asthma.^{4,20} We previously showed that eotaxin/CCL11 has profibrotic effects on human fibroblasts.¹⁵ In the present study, we show that eotaxin-2/CCL24 and eotaxin-3/CCL26 function as differential profibrotic factors for human lung fibroblasts. Eotaxin-2/CCL24 promotes fibroblast proliferation and collagen production, whereas eotaxin-3/CCL26 stimulates fibroblast motility. Neither chemokine induced lung fibroblast α -SMA expression or TGF- β 1 release.

Although a role for eotaxin-2/CCL24 and eotaxin-3/CCL26 on eosinophil chemotaxis and activation is well established,^{21,22} these chemokines also demonstrate activity on structural cells.^{15,23} Eotaxin-2/CCL24 and eotaxin-3/CCL26

accelerate wound closure on cell monolayers of human epithelial cells.²³ While fibroblasts express these chemokines,^{16,17} to date, no data have been reported regarding the effect of these chemokines on lung fibroblast behavior.

In the present study, we found that eotaxin-2/CCL24 increases fibroblast proliferation. The proliferative effect induced by eotaxin-2/CCL24 (65%–70% over nonstimulated cells) is comparable with that previously reported for TGF- β 1 and eotaxin/CCL11.¹⁵ The human fetal lung fibroblast line and primary human lung fibroblasts showed similar proliferative responses to the group of eotaxins. Other CC chemokines, such as pulmonary and activation-regulated chemokine/CCL18, stimulate lung fibroblast proliferation.²⁴ Although with a less pronounced effect than that observed for lung fibroblasts in this study, similar concentrations of eotaxin-2/CCL24 stimulate primary bronchial epithelial cell proliferation via CCR-3 in a dose-dependent manner.²³ We also observed that eotaxin-2/CCL24 increases collagen production by MRC-5 cells and primary human lung fibroblasts. Other studies^{24,25} have reported increased collagen production from lung fibroblasts by pulmonary and activation-regulated chemokine/CCL18 and by monocyte chemoattractant protein 1 (MCP-1)/CCL2 in a dose- and time-dependent manner.

We found that eotaxin-3/CCL26 increases the ability of human lung fibroblasts to migrate in response to nonspecific chemoattractants. The effect observed for eotaxin-3/CCL26 (2- to 3-fold increase) is not as efficient as that observed for eotaxin/CCL11 (5- to 6-fold increase). Albeit these observations are consistent with previous studies that showed less potency for eotaxin-3/CCL26 compared with eotaxin/CCL11 on eosinophil chemotaxis,¹⁰ others have reported similar efficacy for both chemokines on eosinophil migration.¹⁶ Exposure to eotaxin-2/CCL24 did not show any effect on the chemotactic properties of cultured human lung fibroblasts.

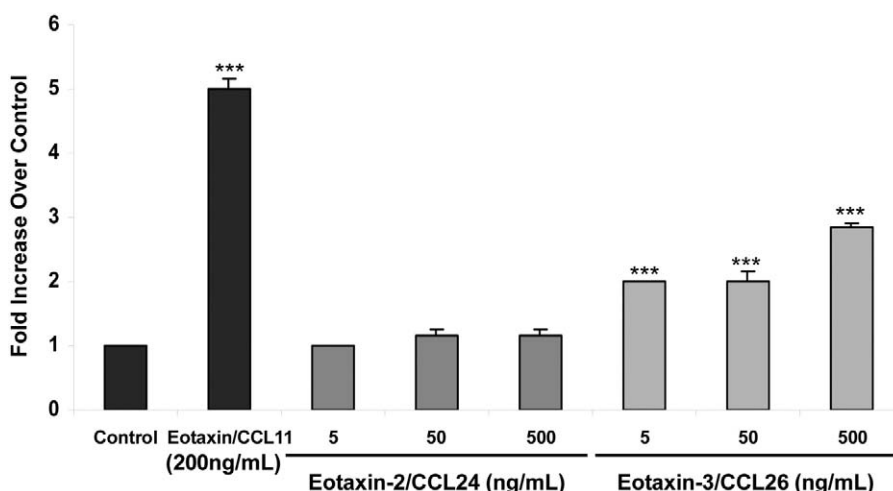


Figure 3. Eotaxin-3/CCL26 stimulates human lung fibroblast migration. MRC-5 fibroblasts were exposed to eotaxin-2/CCL24 or eotaxin-3/CCL26 (for 18 hours), and their chemotactic activity in response to conditioned medium of 3T3 fibroblasts was evaluated. Data are expressed as the mean fold increase over untreated cells (control) of 3 experiments. Error bars represent SEM. *** P < .001 vs control.

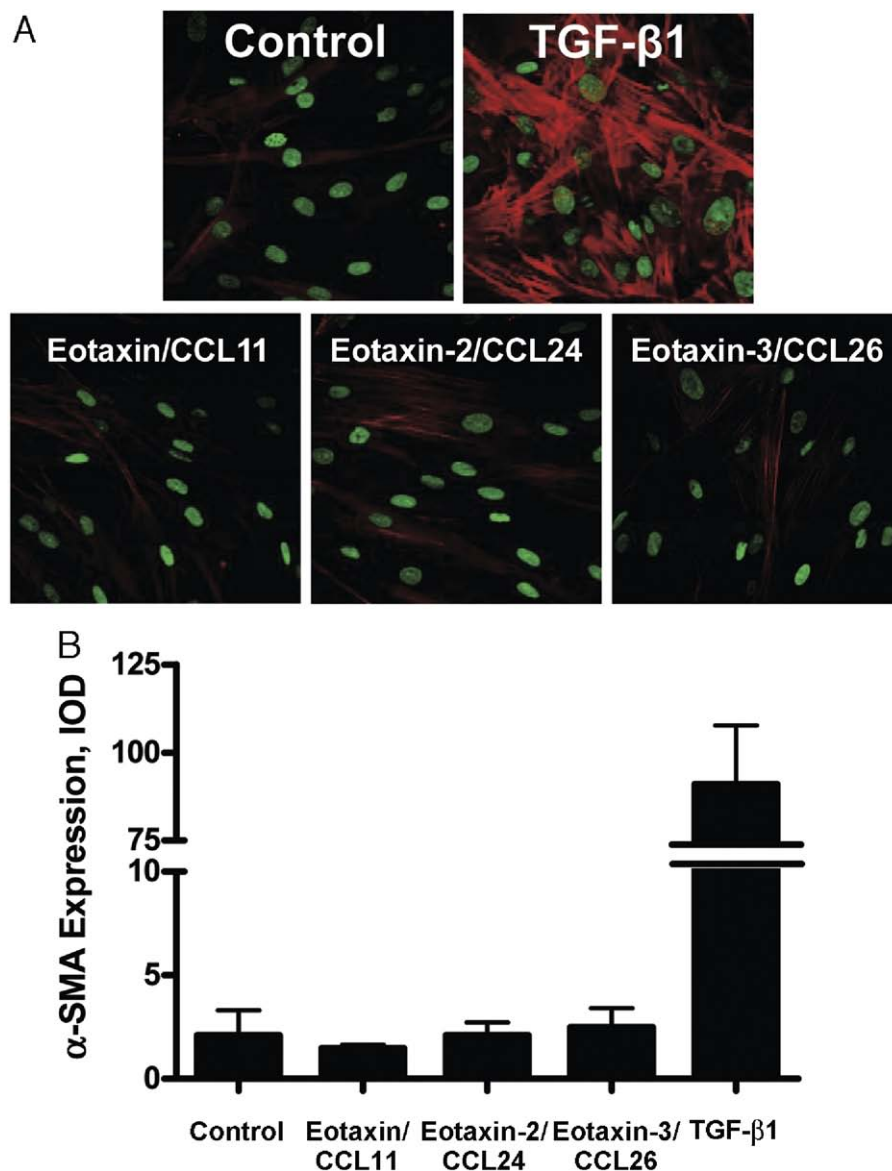


Figure 4. Eotaxins do not promote human lung fibroblast differentiation. MRC-5 fibroblasts were exposed to eotaxin/CCL11, eotaxin-2/CCL24, or eotaxin-3/CCL26 (5–500 ng/mL) or to transforming growth factor β 1 (TGF- β 1) (10 ng/mL) for 48 hours, and fibroblast differentiation was evaluated by means of immunostaining for α -smooth muscle actin (α -SMA). Representative images (A) and quantification (B) of α -SMA expression on fibroblasts exposed to 500 ng/mL of each eotaxin are shown. IOD indicates integrated optical density.

This is in contrast to the direct chemotactic effect previously reported on airway epithelial cells.²³

An important finding of this study is the differential effect exhibited by eotaxin-2/CCL24 and eotaxin-3/CCL26 on human lung fibroblast behavior. Even though both chemokines demonstrate similar chemotactic activity toward eosinophils and bind exclusively to CCR-3, they differ in their expression kinetics and biological activity. *CCL24* and *CCL26* genes are located on the q arm of chromosome 7 but share only 33% homology.^{8,10} While eotaxin-2/CCL24 is constitutively ex-

pressed in asthmatic patients, only eotaxin-3/CCL26 is increased in these patients 24 hours after allergen challenge.¹³

Differentiation of fibroblasts into myofibroblasts is a critical step in fibrogenesis. The most important marker of the fibroblast/myofibroblast transition is the de novo expression of α -SMA.²⁶ We observed that neither eotaxin-2/CCL24 nor eotaxin-3/CCL26 induces α -SMA expression in lung fibroblasts, demonstrating that the profibrogenic effect of the eotaxins is a selective one. MCP-1/CCL2 also has chemotactic and proliferative effects on lung fibroblasts but does not induce α -SMA expression.^{27,28} These observations, there-

Table 1. TGF- β 1 Release From Human Lung Fibroblasts^a

Treatment	TGF- β 1, ng/mL
Untreated cells	0.96 (0.03)
Eotaxin/CCL11	0.98 (0.07)
Eotaxin-2/CCL24	0.92 (0.05)
Eotaxin-3/CCL26	0.93 (0.06)

Abbreviation: TGF- β 1, transforming growth factor β 1.

^a Concentrations of TGF- β 1 were determined by means of enzyme-linked immunosorbent assay as described in the "Methods" section. Treatment with 500 ng/mL of each eotaxin is shown. Data are expressed as the mean (SEM) of 3 experiments.

fore, suggest that the eotaxins are implicated in early events of remodeling, where fibroblasts migrate and proliferate but do not express markers of differentiation. TGF- β 1, which directly induces an α -SMA-positive myofibroblastic phenotype,^{19,26} may be required, in turn, for later events during the progression of remodeling. The fact that chemokines involved in the early stages of remodeling, such as MCP-1/CCL2, can modulate TGF- β 1 expression^{21,25} supports this notion.

In summary, we show that eotaxin-2/CCL24 and eotaxin-3/CCL26 have differential profibrogenic effects on lung fibroblasts. Whereas eotaxin-2/CCL24 promotes fibroblast proliferation and collagen production, eotaxin-3/CCL26 induces fibroblast migration. These data demonstrate a role for eotaxin molecules in fibrogenesis and, therefore, raise the possibility that they may contribute to the process of airway remodeling observed in asthma. Thus, selective blocking of eotaxin/CCL11 may not be sufficient to prevent fibroblast activation, and "pan-inhibition" of eotaxin molecules, such as CCR-3 blockade, may be a more pharmacologically sound approach to attenuate fibrosis in asthma.

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