

The CC chemokine eotaxin/CCL11 has a selective profibrogenic effect on human lung fibroblasts

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Background: Eotaxin/CCL11 plays an important role in asthma. It acts through the chemokine receptor CCR3 expressed on hematopoietic and nonhematopoietic cells in the lung.

Objective: To determine whether eotaxin/CCL11 modulates lung and bronchial fibroblast properties and thereby might contribute to airway remodeling.

Methods: CCR3 expression was characterized on a lung fibroblast line (MRC-5; flow cytometry, fluorescent microscopy, RT-PCR, and Northern blotting), on primary bronchial fibroblasts (flow cytometry), and on fibroblasts in human lung tissue (confocal laser microscopy). The effects of eotaxin/CCL11 on lung fibroblast migration (Boyden chamber), proliferation (tritiated thymidine incorporation), α -smooth muscle actin expression (ELISA), 3-dimensional collagen gel contraction (floating gel), pro- α 1(I) collagen mRNA (Northern blotting), total collagen synthesis (tritiated proline incorporation), matrix metalloproteinase activity (gelatin zymography), and TGF- β ₁ release (ELISA) were evaluated. The contribution of eotaxin/CCL11/CCR3 binding on lung fibroblasts was also investigated by neutralizing experiments. **Results:** CCR3 is constitutively expressed in cultured lung and primary bronchial fibroblasts and colocalizes with specific surface markers for human fibroblasts in lung tissue. Eotaxin/CCL11 selectively modulates fibroblast activities by increasing their proliferation, matrix metalloproteinase 2 activity, and collagen synthesis but not their differentiation into myofibroblasts, contractility in collagen gel, or TGF- β ₁ release.

Eotaxin/CCL11 enhances migration of lung fibroblasts in response to nonspecific chemoattractants, and this effect is completely inhibited by anti-CCR3-neutralizing antibodies. **Conclusion:** These data demonstrate that eotaxin/CCL11 has a direct and selective profibrogenic effect on lung and bronchial fibroblasts, providing a novel mechanism whereby eotaxin/CCL11 can participate in airway remodeling in asthma. (J Allergy Clin Immunol 2006;117:103-10.)

Key words: CC chemokine, eotaxin/CCL11, CCR3, fibroblast, myofibroblast, airway remodeling, asthma, α -smooth muscle actin

Airway remodeling is a prominent pathophysiologic feature of asthma. It is characterized by structural changes in the airway wall, such as increased deposition of extracellular matrix (ECM), smooth muscle hypertrophy, hyperplasia of mucus-secreting cells, and angiogenesis.¹⁻³ A link between airway inflammation and airway remodeling in asthma has recently been proposed. Several CC chemokines responsible for initiating and perpetuating airway allergic inflammation in asthma might also contribute to the pathogenesis of airway remodeling.⁴⁻⁷ Among them, monocyte chemoattractant protein 1 (MCP-1)/CCL2, which is increased in asthma^{8,9} and in pulmonary fibrotic diseases,^{10,11} is a well-established profibrogenic mediator *in vitro* and *in vivo*. MCP-1/CCL2 promotes tissue remodeling and fibrosis in the airways by inducing TGF- β ₁ release and collagen deposition from lung fibroblasts^{12,13} and by recruiting T_H2 cells in the lung.¹⁴ Other CC chemokines, such as MCP-3/CCL7, thymus and activation-regulated chemokine/CCL17, and monocyte-derived chemokine/CCL22, also contribute to the development of pulmonary fibrosis.^{15,16}

Eotaxin/CCL11, a potent chemoattractant for eosinophils,¹⁷⁻²⁰ is a critical mediator for the development and perpetuation of allergen-induced eosinophilic airway inflammation.²¹ Eotaxin/CCL11 is upregulated in the bronchial mucosa of patients with asthma, and its expression correlates with airway hyperresponsiveness after allergen exposure.²² Eotaxin/CCL11 induces eosinophil migration,¹⁷⁻²⁰ degranulation,^{23,24} and differentiation from CD34⁺ progenitor cells²⁵ by acting through its specific receptor, CCR3, expressed on inflammatory and structural cells of the lung.²⁶ It also regulates migration of mast cell progenitors into inflamed tissue and mast cell activation.²⁷

Eotaxin/CCL11 is likely to play an indirect role in airway remodeling through recruitment of eosinophils and mast cells, which have profibrogenic activity.²⁸ However, no data regarding a direct involvement of eotaxin/CCL11 in airway remodeling have been reported. Salcedo et al²⁹ have demonstrated that eotaxin/CCL11 has a proangiogenic effect by acting on endothelial cells through CCR3, even in the absence of eosinophil infiltration. Eotaxin/CCL11 also induces cytokine release from bronchial epithelial cells³⁰ and increases smooth muscle cell migration.³¹ Taken together, these findings prompted us

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Abbreviations used

α -SMA: α -Smooth muscle actin
 DMEM: Dulbecco's modified Eagle's medium
 ECM: Extracellular matrix
 HBF: Human bronchial fibroblast
 HLF: Human lung fibroblast
 MCP-1: Monocyte chemoattractant protein 1
 MMP: Matrix metalloproteinase
 PARC: Pulmonary and activation-regulated chemokine

to investigate whether eotaxin/CCL11 might also act directly on lung fibroblasts, which are important target cells in airway remodeling. In this study we characterize the expression of CCR3 in human lung fibroblasts (HLFs) and human bronchial fibroblasts (HBFs) and demonstrate a direct effect of eotaxin/CCL11 on their migration, proliferation, and differentiation and on ECM metabolism.

METHODS

Cell culture

Human bronchial fibroblasts were obtained from endobronchial biopsy specimens of normal mucosa from 3 subjects (age, 54–64 years; 1 female and 2 male subjects not receiving any medications) undergoing fiberoptic bronchoscopy for assessment of contralateral localized lung cancer, as previously described.³² Bronchoscopy was performed according to published guidelines,³³ and informed consent was obtained. Fibroblasts were expanded in T21/75-cm² flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (DMEM⁺; 37°C in a 5% CO₂ atmosphere; all from Biological Industries, Beit Haemek, Israel). HBFs were then passed weekly by means of Trypsin-EDTA (Biological Industries) and used for assays between the second and third passages. The human fetal lung fibroblast line MRC-5 (American Type Culture Collection, Manassas, Va) was cultured in DMEM⁺ (37°C in a 5% CO₂ atmosphere) and used for assays between the second and seventh passages. In all the experiments, the HBFs and HLFs were incubated overnight with serum-free DMEM (DMEM⁻) before stimulation. Eosinophils and neutrophils were purified from peripheral blood of mildly atopic volunteers not receiving any pharmacologic treatment by using the magnetic cell separation system, as previously described.³⁴

Fluorescence microscopy

Costaining and confocal microscopy were performed on normal human lung tissue obtained after lobectomy for stage I lung cancer. The 4% paraformaldehyde-fixed 2- μ m tissue sections were incubated with rat anti-human CCR3 (15 μ g/mL; R&D System, Minneapolis, Minn) and mouse anti-human CD90 (Ab-1; Calbiochem, San Diego, Calif) antibodies (1 hour at room temperature). Binding was detected by using Cy2 goat anti-rat and Cy5 goat anti-mouse fluorescent secondary antibodies (1:200; Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa; 40 minutes at 37°C). Cells were examined with a Zeiss 410 laser scanning confocal microscope attached to the Zeiss Axiovert 135 M inverted microscope with 63 $\times$ /1.2 C-Apochromat water immersion lens (Carl Zeiss, Thornwood, NY). HLFs cultured until confluence on round cover slips placed in

24-well plates were fixed in 2% buffered paraformaldehyde (10 minutes at 4°C), permeabilized in 0.2% Triton 100X (5 minutes at room temperature), blocked in 1% BSA (20 minutes at room temperature), and incubated with rat anti-human CCR3 or mouse anti-human CCR5 (Cymbus Biotechnology Ltd, Southampton, United Kingdom) or with irrelevant isotype-matched IgG antibodies (1 hour at 4°C). Binding was detected by using fluorescein isothiocyanate-conjugated F(ab')₂ fragments of rabbit anti-rat or goat anti-mouse antibodies (1:200; Jackson; 40 minutes at 37°C). The nuclei were counterstained with propidium iodide (Sigma Chemicals, St Louis, Mo).

Flow cytometry

Single-cell suspensions were washed in PBS, fixed in 2% paraformaldehyde (15 minutes at room temperature), and incubated with rat anti-human CCR3 (5 μ g/mL), goat anti-human CCR5 (1:50; Cymbus), or irrelevant isotype-matched IgG (Chemicon, San Diego, Calif) antibodies (1 hour at 4°C). Binding was detected by using phycoerythrin-conjugated rabbit anti-rat antibodies or fluorescein isothiocyanate-conjugated rabbit anti-rat or anti-goat antibodies (1:200; Jackson; 40 minutes at 4°C). Fluorescence was quantified by using a FACSCalibur flow cytometer (Becton Dickinson, Cowley, Oxfordshire, United Kingdom), and data were processed with the CellQuest software program (BD Biosciences, San Jose, Calif). Human eosinophils and neutrophils were used as positive and negative controls, respectively.

RNA analysis

Total RNA (15 μ g) from HLFs cultured in DMEM (0.1% BSA) alone or with eotaxin/CCL11 (5–500 ng/mL) or TGF- β ₁ (10 ng/mL; 24 hours at 37°C in a 5% CO₂ atmosphere) was extracted, electrophoresed, and transferred by using standard procedures. The cDNA probe for pro- α 1(I) collagen was generated by means of PCR, and the sequence was confirmed, radiolabeled with phosphorous 32, and hybridized by using standard procedures. A glyceraldehyde-3-phosphate dehydrogenase cDNA probe was used as a control. The intensity of the bands on autoradiography was quantified on a Fluor-S MultiImager analyzer (Bio-Rad, Hercules, Calif), and the ratio between the different bands and glyceraldehyde-3-phosphate dehydrogenase was calculated for each lane and expressed as a fold increase over medium alone. mRNA expression for CCR3 was determined by using RT-PCR. Total RNA (1 μ g) from HLFs and from eosinophils (positive control) was reverse transcribed, and cDNA was amplified by using primers for human CCR3 (forward 5'-CCACACTCTGAGAATGACCATC-3', reverse 5'-GCCAGGTGCATGAGCAAGTG CC-3', 381 bp). PCR products were visualized by means of ethidium bromide-stained gel electrophoresis. Northern blot analysis for CCR3 was also performed (as above) to confirm the findings obtained with RT-PCR.

Cell migration assay

Migration of HLFs (10⁴ cells per well) was evaluated in Boyden well chambers.³⁵ Different concentrations of eotaxin/CCL11 (0–500 ng/mL) were used as chemoattractants for resting HLFs in the lower chambers (6 hours at 37°C in a 5% CO₂ atmosphere, acute treatment). In another set of experiments, the cells were incubated overnight with different concentrations of eotaxin/CCL11 (0–500 ng/mL), and their migration in response to the conditioned medium of 3T3 fibroblasts, as nonspecific chemoattractants, was evaluated (chronic treatment). Goat anti-human eotaxin/CCL11, irrelevant IgG antibodies (R&D Systems), or both were used as controls.

To elucidate whether eotaxin/CCL11 acts through CCR3, CCR5, or both, the cells were treated (30 minutes at 37°C in a 5% CO₂ atmosphere) with rat anti-human CCR3 (10 μ g/mL), mouse anti-

human CCR5 (10 μ g/mL), or irrelevant isotype-matched IgG (10 μ g/mL) antibodies before addition of eotaxin/CCL11 (200 ng/mL), and their chemotaxis in response to 3T3 fibroblast-conditioned medium was evaluated as described above.

Cell proliferation assay

Subconfluent HLFs and HBFs (5×10^3 /0.2 mL) were incubated (24 hours at 37°C in a 5% CO₂ atmosphere) in DMEM (0.5% FCS) alone or with eotaxin/CCL11 (5-500 ng/mL) or TGF- β_1 (10 ng/mL) (R&D). Tritiated thymidine (1 μ Ci/well; NEN Life Science Products, Inc, Boston, Mass) was added as a final 24-hour pulse, and samples were processed as previously described.³⁴ Blocking experiments were performed by means of preincubation of HLFs with anti-CCR3 antibody, anti-CCR5 antibody, or both (30 minutes at 37°C in a 5% CO₂ atmosphere) before adding eotaxin/CCL11.

Collagen production

Collagen production of confluent HLF monolayers was assessed on the basis of tritiated proline incorporation into collagenous proteins.³⁴ HLFs were seeded in 24-well tissue-culture plates (10⁴/well) in 1 mL of DMEM (10% FCS) until confluence. Eotaxin/CCL11 (0-500 ng/mL) or TGF- β_1 (10 ng/mL; R&D) in 0.5 mL of DMEM containing 5% FCS, 50 μ g/mL β -aminopropionitrile, and 50 μ g/mL ascorbic acid (modified DMEM) was added, and the cultures were incubated for 24 hours. Then tritiated proline (10 μ Ci/well) was added for an additional 24 hours, and samples were processed as described previously.³⁴

Metalloproteinase activity

Subconfluent HLFs (5×10^4 /2 mL) were cultured (6 or 18 hours at 37°C in a 5% CO₂ atmosphere) in DMEM (0.1% BSA) alone or with different concentrations of eotaxin/CCL11 (5-500 ng/mL). Metalloproteinase activity in the supernatants of cultured HLFs was evaluated by means of gelatin zymography, as previously described.³⁶

ELISA surface for α -smooth muscle actin expression and 3-dimensional gel contraction

Confluent HLFs were cultured (5 days at 37°C in a 5% CO₂ atmosphere) in 96-well plates in DMEM⁺ alone or with eotaxin/CCL11 (5-500 ng/mL) or TGF- β_1 (10 ng/mL). α -Smooth muscle actin (α -SMA) expression was assessed by means of surface ELISA, as previously described.³⁷ Three-dimensional lattice cultures were performed in collagen matrices, as described previously.³⁷ Briefly, type I collagen (2 mg/mL from rat-tail tendon in 18 mM acetic acid) was added to bacteriologic 35-mm dishes containing supplemented DMEM⁺ and 0.1 M NaOH. HLFs (10⁵ per lattice) were added to the gel with or without eotaxin/CCL11 (5-500 ng/mL) or TGF- β_1 (10 ng/mL) before gel fibrillation and lattice formation. Lattice diameters were measured daily in a blinded manner for 7 days.

TGF- β_1 release

Confluent HLFs were cultured (6, 12, or 24 hours at 37°C in a 5% CO₂ atmosphere) in 24-well plates with different concentrations of eotaxin/CCL11 (5-500 ng/mL), and active TGF- β_1 was measured in the culture supernatants by means of ELISA, according to the manufacturer's instructions (R&D).

Statistical analysis

Data are expressed as means $\pm$ SEM of at least 3 independent experiments. Parametric ANOVA, followed by the Tukey-Kramer

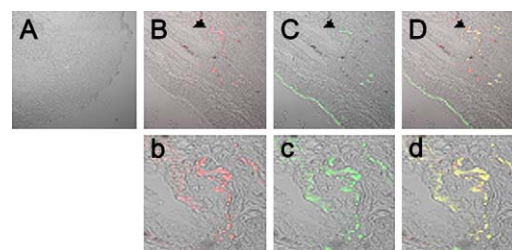


FIG 1. Fibroblasts express CCR3 in human lung tissue: double-immunofluorescent staining for fibroblasts and CCR3 in human lung tissue biopsy specimens. **A**, irrelevant isotype-matched IgG antibody. **B**, Fibroblast (CD90, red). **C**, CCR3 (green). **D**, Overlay of **B** and **C** (yellow). Panels **b**, **c**, and **d** show higher magnifications of selected areas of **Fig 1**, **B**, **C**, and **D**, respectively.

multiple comparisons test, was used to compare groups. A *P* value of less than .05 was considered statistically significant.

RESULTS

CCR3 is expressed on fibroblasts in human lung tissue

Human lung sections were analyzed by means of confocal laser microscopy for the presence of CCR3⁺ fibroblasts. As shown in **Fig 1**, **D** and **d**, the tissue sections contained cells that expressed both Ab-1 (fibroblast marker) and CCR3, demonstrating that human lung tissue fibroblasts express CCR3 *in vivo*.

Cultured HLFs express CCR3 mRNA and protein

Cultured HLFs constitutively expressed CCR3 mRNA and protein (**Fig 2**). The intensity of the CCR3 mRNA band in HLFs was weaker than that observed in eosinophils used as a positive control (**Fig 2**, **A**). PCR controls (specific primers with no cDNA and cDNA without primers) were negative (not shown). Northern blot analysis confirmed the specificity of CCR3 mRNA. More than one band was observed, with the major one observed at 1.6 kb (**Fig 2**, **B**), indicating the presence of more than one CCR3 RNA species. By means of fluorescence microscopy, we characterized the expression of CCR3 in cultured HLFs. As shown in **Fig 2**, **C**, HLFs expressed CCR3 protein with a pattern compatible with surface distribution. We also investigated the expression of the chemokine receptor CCR5 that primarily binds RANTES/CCL5 but can also bind eotaxin/CCL11. The expression and distribution of CCR5 was similar to that observed for CCR3 (not shown). After quantification with FACS analysis (**Fig 2**, **D**), we found that the surface expression of CCR3 on HLFs was similar to that detected on HBFs (mean fluorescence intensity fold control = 7.9 ± 0.9 and 8.3 ± 1.1 , respectively). Peripheral blood eosinophils and neutrophils from mildly atopic donors were used as positive and negative controls, respectively, to confirm the specificity of CCR3 staining on HLFs and HBFs. The levels of CCR3 on HLFs and HBFs were less than those detected on

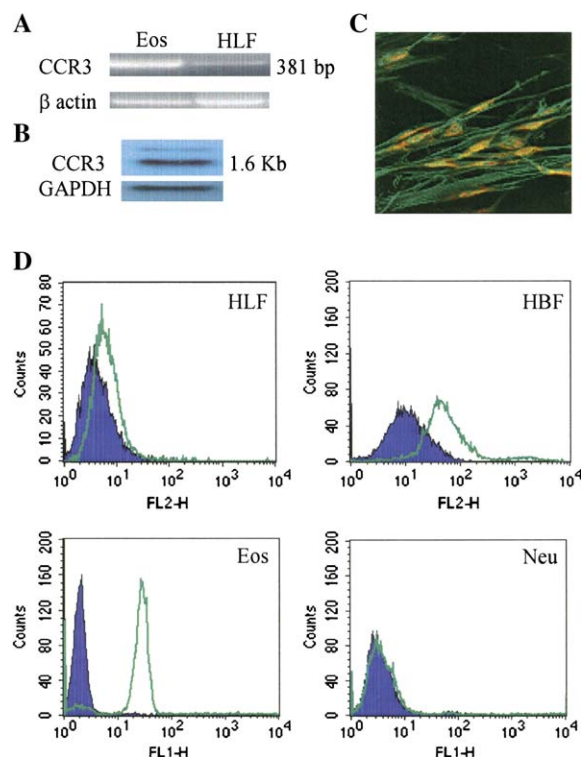


FIG 2. CCR3 expression in HLFs. **A**, RT-PCR: fragment (381 bp) amplified from mRNA of human eosinophils (*Eos*) and HLFs. **B**, Northern blotting. RNA from HLFs shows more than one band, with the major band at 1.6 Kb. **C**, Fluorescence microscopy: distribution of CCR3 (green) in cultured HLFs. Fibroblast nuclei are stained with propidium iodide. **D**, Flow cytometry: CCR3 staining of HLFs, HBFs, eosinophils (*Eos*), and neutrophils (*Neu*; green line histograms) and the IgG2a isotype control (blue histograms).

eosinophils (mean fluorescence intensity fold control = 13.4 ± 1.5).

Eotaxin/CCL11 enhances HLF chemotaxis

After characterization of the eotaxin/CCL11-specific receptor CCR3 on cultured HLFs, we investigated whether this CC chemokine might exert a direct effect on HLFs. Because eotaxin/CCL11 is a potent chemoattractant for several inflammatory cells, we first examined its activity on HLF chemotaxis in a Boyden chamber. Acute stimulation with different concentrations of eotaxin/CCL11 did not induce any effect. However, after overnight treatment with eotaxin/CCL11, the number of migrating cells in response to the conditioned medium of 3T3 fibroblasts increased by 3.0-fold ($P < .05$), 4.5-fold ($P < .01$), 5.9-fold ($P < .001$), and 6.0-fold ($P < .001$) at 5, 50, 200, and 500 ng/mL, respectively, versus medium alone (Fig 3, A). The addition of anti-human eotaxin/CCL11 antibodies completely abrogated this effect (47.5 ± 3.1 vs 16.25 ± 2.5 migrated cells $\times 10^4$). Pretreatment of HLFs with anti-CCR3 antibodies resulted in a complete inhibition of this effect (Fig 3, B), providing strong evidence that eotaxin/CCL11 acts on HLFs through CCR3 binding.

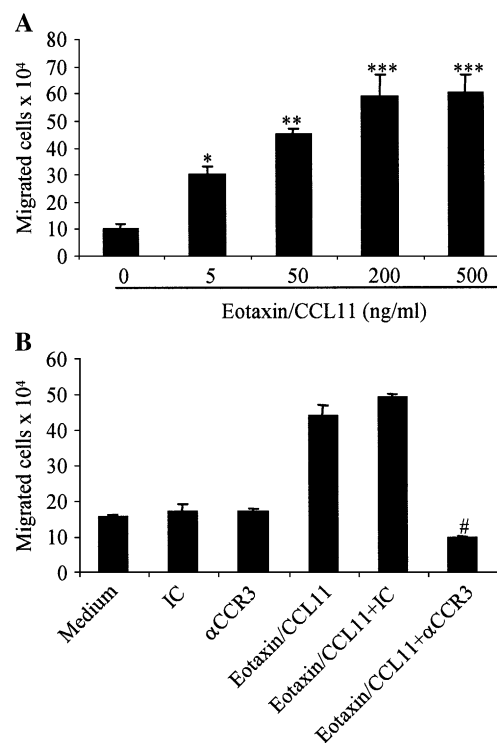


FIG 3. Effect of eotaxin/CCL11 on HLF migration. **A**, HLFs were treated with eotaxin/CCL11 (18 hours), and their migration in response to conditioned medium of 3T3 fibroblasts was evaluated (Boyden chambers). **B**, Neutralizing experiments with anti-CCR3 or with isotype control (*IC*) antibodies. Data represent means $\pm$ SEM of 3 experiments performed in triplicate. * $P < .05$, ** $P < .01$, and *** $P < .001$ versus medium alone. # $P < .001$ versus eotaxin/CCL11 plus isotype control.

Preincubation of HLFs with anti-CCR5 antibody did not exert any inhibitory effect (not shown).

Eotaxin/CCL11 increases HLF and HBF proliferation

Because fibroblast proliferation is an important step during airway remodeling, we determined whether eotaxin/CCL11 modulates HLF and HBF proliferation. As shown in Fig 4, A, eotaxin/CCL11 increased HLF proliferation by 1.3- and 1.4-fold at 200 and 500 ng/mL, respectively ($P < .01$). At 500 ng/mL, the effect was comparable with that of TGF- β_1 used as a positive control. In HBFs (Fig 4, B) eotaxin/CCL11 increased proliferation already at 50 ng/mL (1.6-fold, $P < .05$), with a maximal effect at 200 and 500 ng/mL (1.7-fold and 1.8-fold, $P < .01$). Blocking experiments with anti-CCR3 antibody, anti-CCR5 antibody, or both resulted in a similar inhibitory effect to that observed in the chemotaxis experiments (not shown).

Eotaxin/CCL11 enhances matrix metalloproteinase 2 activity in HLFs

Increased matrix metalloproteinase (MMP) activity in the lung is a common feature in asthma. In particular,

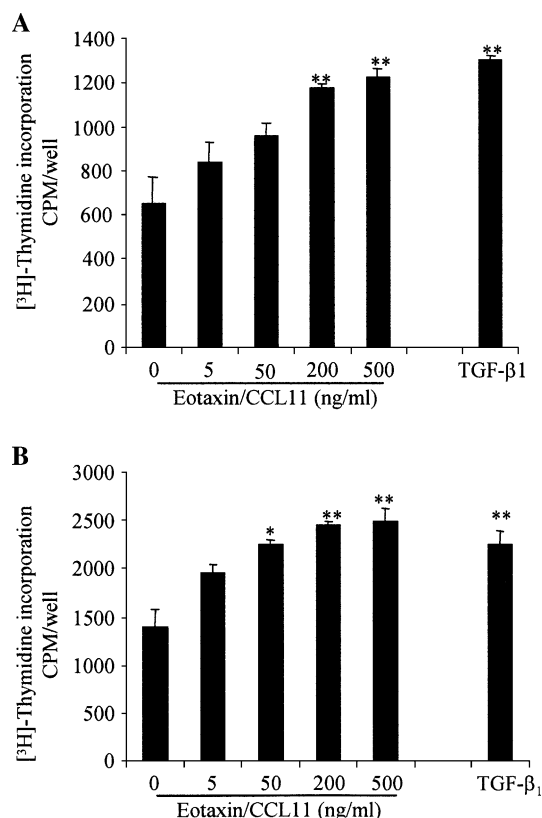


FIG 4. Effect of eotaxin/CCL11 on HLF and HBF proliferation. HLFs (A) and HBFs (B) were treated with eotaxin/CCL11 or TGF-β₁ for 24 hours, and their proliferation was evaluated (tritiated thymidine incorporation). Data represent means ± SEM of 3 experiments performed in triplicate. **P* < .05 and ***P* < .01 versus medium alone.

MMP-2 regulates the chemotactic gradients in the lung in experimental asthma, promoting inflammatory cell egression from the lung parenchyma.³⁸ Deficiency in MMP-2 results in marked reduction in eotaxin/CCL11 in the bronchoalveolar lavage fluid of these mice. Interestingly, we found that increased concentrations of eotaxin/CCL11 induced activation of MMP-2 in HLFs by 1.2-fold, 1.6-fold, and 2.2-fold (*P* < .01) at 50, 200, and 500 ng/mL, respectively (Fig 5). In contrast, we did not observe any effect on MMP-9 activity (data not shown). This finding introduces a new mechanism whereby MMP-2 can be activated and therefore can contribute to the inflammation and remodeling of the lung.

Eotaxin/CCL11 increases pro-α1(I) collagen mRNA and collagen synthesis

The fibroblast is the main cellular source of collagen type I in the airways. By means of Northern blot analysis, we showed that eotaxin/CCL11 increased pro-α1(I) collagen mRNA in HLFs at all the concentrations tested, with a maximal effect at 50 ng/mL (2.5-fold, *P* < .01; Fig 6, A, B). However, the effect induced by eotaxin/CCL11 was lower than that induced by TGF-β₁ at 10 ng/mL

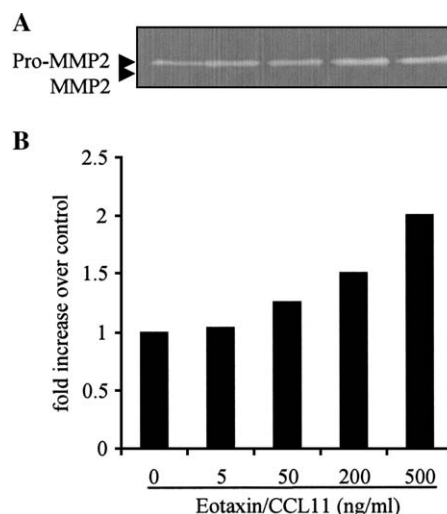


FIG 5. Effect of eotaxin/CCL11 on MMP-2 activity. A, HLFs were treated with eotaxin/CCL11 for 18 hours, and MMP-2 activity in the supernatants of HLFs was analyzed (gel zymography, representative of 3 separate experiments). B, Densitometric analysis of Fig 5, A.

(3.0-fold, *P* < .001 vs control). As shown in Fig 6, C, eotaxin/CCL11 also enhanced collagen protein production in HLFs in a dose-dependent manner, with a maximal effect at 200 ng/mL (1.5-fold, *P* < .01).

Effect of eotaxin/CCL11 on TGF-β₁ release

TGF-β₁ is the major profibrogenic cytokine in the pathogenesis of asthma and pulmonary fibrosis. The CC chemokines MCP-1/CCL2 and MCP-3/CCL7 exert their profibrogenic effects on fibroblasts through TGF-β₁. At all time points and different concentrations used, eotaxin/CCL11 failed to induce HLFs to release the active form of TGF-β₁ (data not shown). Thus, unlike other CC chemokines, we found that the profibrogenic effect of eotaxin/CCL11 is independent on TGF-β₁.

Eotaxin/CCL11 did not induce myofibroblast differentiation

Differentiation of fibroblasts into myofibroblasts is a crucial event in airway remodeling. We used α-SMA actin expression and collagen gel contraction as markers of fibroblast differentiation. After incubation with increasing concentrations of eotaxin/CCL11 for 5 days, we did not detect any effect on α-SMA expression or on contractility in HLF. TGF-β₁ (10 ng/mL) used as a positive control increased α-SMA expression and collagen gel contraction by 2-fold (*P* < .01) and 4.7-fold (*P* < .01), respectively.

DISCUSSION

In this study we demonstrated that primary HBFs and a human fetal lung fibroblast line constitutively express the CCR3 receptor and that eotaxin/CCL11 promotes fibroblast migration and proliferation and increases MMP-2

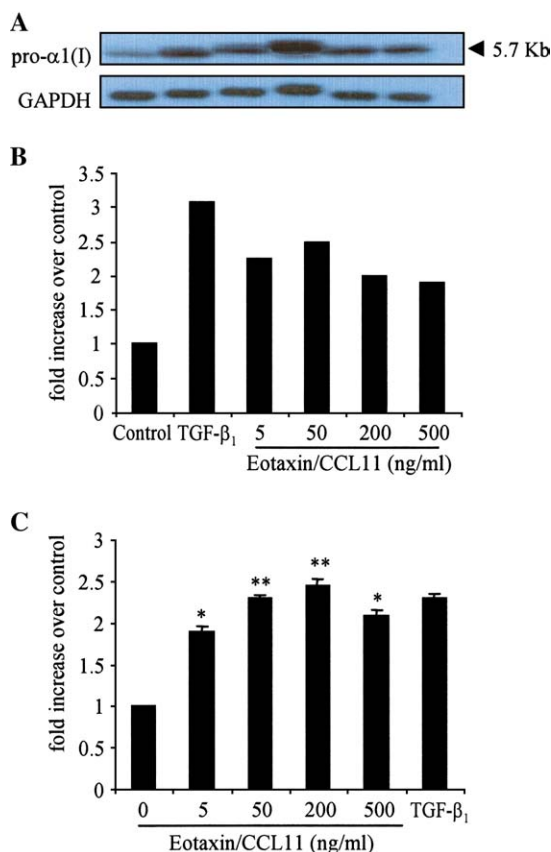


FIG 6. Effect of eotaxin/CCL11 on pro- α 1(I) collagen mRNA and collagen production. **A**, Total RNA was extracted from HLFs treated with medium alone (control), TGF- β ₁, or eotaxin/CCL11 for 18 hours and mRNA for pro- α 1(I) collagen, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was analyzed (Northern blotting, representative of 3 separate experiments). **B**, Densitometric analysis of Fig 6, A. **C**, HLFs were incubated with eotaxin/CCL11 or TGF- β ₁ for 24 hours, and collagen production was evaluated (tritiated proline incorporation). Data represent means $\pm$ SEM of 3 experiments performed in triplicate. * P < .05 and ** P < .01 versus medium alone.

activity, pro- α 1(I) collagen mRNA expression and collagen protein production but has no effect on differentiation into myofibroblasts or TGF- β ₁ release.

Airway remodeling is a central pathophysiologic feature of chronic asthma.¹⁻³ Recent evidence suggests that infiltrating inflammatory cells, such as eosinophils, play an active role in airway remodeling by promoting the activation of fibroblasts^{34,37,39,40} and of endothelial cells.⁴¹ Eotaxin/CCL11 is a potent chemoattractant for eosinophils¹⁷⁻²⁰ and is a critical mediator for the development and perpetuation of allergen-induced eosinophilic airway inflammation.²¹

We hypothesized that eotaxin/CCL11, in addition to its well-established proinflammatory role, might also contribute to tissue remodeling by acting directly on lung fibroblasts. This hypothesis is strengthened by the recent finding that eotaxin/CCL11 modulates the activity of several nonhematopoietic cells. For example, it induces

angiogenesis by acting on endothelial cells through CCR3,²⁹ enhances IL-8 and GM-CSF production from bronchial epithelial cells,³⁰ and induces migration of mouse aortic vascular smooth muscle cells.³¹

In our study we found that eotaxin/CCL11 increased fibroblast proliferation, and this effect was comparable with that induced by TGF- β ₁. We found no significant difference between the human fetal lung fibroblast line and primary HBFs regarding their proliferative response to recombinant human eotaxin/CCL11. In addition, as shown in Fig 2, C, the surface expression of CCR3 on the human fetal lung fibroblast line was similar to that detected on primary HBFs. In previous studies we and others have also observed similar behavior in fetal and adult fibroblasts. We therefore limited subsequent *in vitro* experiments to the HLF line. A proliferative effect of eotaxin/CCL11 has been reported for keratinocytes,⁴² and the intensity of the effect was comparable with that observed in our system. Pulmonary and activation-regulated chemokine (PARC)/CCL18, another CC chemokine with a profibrogenic effect, has been previously demonstrated to induce lung fibroblast proliferation⁴³; however, the effect of PARC/CCL18 was less pronounced than that of eotaxin/CCL11.

In a similar fashion to that of other CC chemokines, such as MCP-1/CCL2^{12,13} and PARC/CCL18,⁴³ we showed that eotaxin/CCL11 upregulates procollagen type I gene expression and, more importantly, collagen protein production in lung fibroblasts, suggesting a potential contribution of this CC chemokine to the deposition of ECM in the lung.

Collagen type I is increased in asthmatic airways and is strongly induced by TGF- β ₁.⁴⁴ The effect of MCP-1,¹³ but not of PARC/CCL18,⁴³ on collagen synthesis in lung fibroblasts is mediated through induction of autocrine TGF- β ₁. These observations led us to investigate whether the effect of eotaxin/CCL11 on collagen synthesis is TGF- β ₁ dependent. After treatment of the fibroblasts with eotaxin/CCL11 at different concentrations and time points, we did not detect any increase in TGF- β ₁ release, indicating that the effect of eotaxin/CCL11 on lung fibroblasts is likely to be a direct one.

Proteases and their inhibitors play important roles in tissue remodeling. MMP-2, MMP-3, MMP-9, and MMP-12 are upregulated in asthmatic airways and contribute to the ECM degradation.³ We found that eotaxin/CCL11 induced MMP-2, but not MMP-9, activity in lung fibroblasts, suggesting a role of eotaxin/CCL11 in the degradation of the ECM.

The results we observed for fibroblast chemotaxis with Boyden chambers introduces another important point. Eotaxin/CCL11 does not induce fibroblast migration directly, but by means of a priming effect, it increases the susceptibility of the cells to nonspecific chemoattractants present in the conditioned medium of 3T3 fibroblasts. This observation is in contrast to the direct chemoattractant effect observed on endothelial and smooth muscle cells,^{29,31} indicating again that eotaxin/CCL11 has variable effects on different cell types.

Eotaxin/CCL11-induced lung fibroblast activation requires binding to the CCR3 receptor, as we demonstrated in neutralizing experiments. The importance of CCR3 for eotaxin/CCL11 activity is well documented in other nonhematopoietic cells, such as endothelial cells,²⁹ epithelial cells,³⁰ and smooth muscle cells.³¹

It is well accepted that eotaxin/CCL11 can also bind and signal through CCR5, an important receptor for RANTES/CCL5 signaling. We confirmed the expression of CCR5 on both the fetal lung fibroblast line and adult bronchial fibroblasts by means of FACS analysis and cytoimmunofluorescent staining. However, blocking experiments with a monoclonal anti-CCR5 antibody had no effect on fibroblast migration or proliferation, demonstrating that CCR3 is the predominant functional receptor for eotaxin/CCL11 activity in HLFs.

An important finding in our study is that the effect of eotaxin/CCL11 on lung fibroblasts is selective. Although this chemokine enhances cell migration and proliferation, it does not induce myofibroblast differentiation. On the basis of these observations, it is possible that eotaxin/CCL11 acts in the early phase of remodeling, and additional profibrogenic mediators that induce fibroblast differentiation, such as TGF- β ,⁴⁵ are required for progression of this process.

The role of eotaxin/CCL11 in airway remodeling might also be due to its ability to recruit eosinophils and mast cells, important sources of profibrogenic and proangiogenic mediators, into the site of inflammation.²⁸ Similarly thymus and activation-regulated chemokine/CCL17, a ligand of CCR4, plays an indirect role in the pathogenesis of pulmonary fibrosis through the recruitment of lymphocytes and macrophages.¹⁶ These inflammatory cells, through the release of TGF- β , IL-4, and IL-13, are also able to enhance the release of eotaxin/CCL11 from lung fibroblasts,⁴⁶ resulting in an amplification loop of airway remodeling.

In conclusion, we show that eotaxin/CCL11 has direct and selective profibrogenic activity on lung fibroblasts, and these effects are mediated through the CCR3 receptor. Further studies with knockout animals or *in vivo* administration of anti-eotaxin/CCL11 antibody will help to clarify the contribution of eotaxin/CCR3 to airway remodeling and to establish whether eotaxin/CCL11, CCR3, or both can be specifically targeted for the treatment or prevention of airway remodeling in asthma.

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