TGF-β differentially regulates T_H2 cytokine-induced eotaxin and eotaxin-3 release by human airway smooth muscle cells

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Background: Human airway smooth muscle cells (HASMs) are involved in the pathogenesis of asthma. By producing chemokines, HASMs play a role in the inflammatory processes observed in this disease. Eotaxin, eotaxin-2, and eotaxin-3 are important chemoattractants for eosinophils, and these chemokines are expressed during different phases of the allergic reaction. T_H2 cytokines and TGF-β can be found in increased levels in patients with asthma, and these cytokines may be involved in the regulation of chemokine expression.
Objective: The aim of this study was to determine the effect of T_H2 cytokines and TGF-β on the regulation of expression of eotaxin, eotaxin-2, and eotaxin-3 by HASMs.
Methods: HASMs were incubated for 24 hours with IL-4, IL-13, TGF-β1, or combinations of these cytokines. Protein and mRNA levels of eotaxin and eotaxin-3 were evaluated by sandwich ELISA and reverse transcriptase-PCR.
Results: IL-4 and IL-13 induced mRNA and protein for both eotaxin and eotaxin-3. Eotaxin-2 mRNA and protein were not detected in HASMs. TGF-β alone did not induce expression of the eotaxins. However, in combination with IL-4 or IL-13, TGF-β enhanced eotaxin production and inhibited T_H2 cytokine-induced eotaxin-3 production.
Conclusion: TGF-β differentially regulates T_H2 cytokine-induced eotaxin and eotaxin-3 release. (J Allergy Clin Immunol 2004;114:791-8.)

Key words: *Human airway smooth muscle cells, T_H2 cytokines, TGF-β, chemokines, allergy, asthma*

Human airway smooth muscle cells (HASMs) are involved in the pathogenesis of asthma, because these cells contribute to airway hyperresponsiveness and airway obstruction. Furthermore, HASMs may play a role in the inflammatory processes of this disease by producing chemokines. This more recently recognized function of HASMs, together with their involvement in the previously mentioned features, implicates these cells as key players in asthma. Several chemokines are produced by HASMs *in vitro* upon stimulation with cytokines, including eotaxin, RANTES, monocyte chemoattractant protein (MCP)–3, and MCP-4.[1,2] These chemokines are involved in the recruitment of inflammatory cells into the lung. Eosinophilic inflammation is characteristic for atopic asthma, and eosinophils are recruited into the lung by eosinophil-selective chemokines such as the eotaxins.[3]

Abbreviations used
BAL: Bronchoalveolar lavage
CCL: CC chemokine ligand
HASM: Human airway smooth muscle cell
MCP: Monocyte chemoattractant protein
MMP: Matrix metalloproteinase

Eotaxin (CC chemokine ligand [CCL]11), an eosinophil-selective chemoattractant, was first identified in bronchoalveolar lavage (BAL) fluid from guinea pigs and was shown to be involved in eosinophil recruitment to the lung.[4] Later, 2 other CC chemokines with preferential eosinophil-attracting activity were identified and named eotaxin-2 (CCL24) and eotaxin-3 (CCL26), because they share their function and receptor (CCR3) with eotaxin.[5,6] Studies in human subjects have shown that allergen challenge induces eotaxin expression in bronchial tissue and BAL from atopic patients with asthma and that the production of eotaxin is associated with the early phase of allergen-induced recruitment of eosinophils.[7] Other studies in atopic subjects have shown that eotaxin-2 and eotaxin-3 may be more involved in late-phase eosinophil recruitment.[8,9] Injection of allergen into the skin of sensitized atopic subjects showed early induction of eotaxin and late induction of eotaxin-2 mRNA and protein.[8] These data suggest that eotaxin-2 is involved in late infiltration of eosinophils. In addition, another study in atopic patients showed that eotaxin-3 mRNA levels were increased after allergen challenge in patients with asthma, whereas allergen challenge did not affect eotaxin or eotaxin-2 levels, thus suggesting the involvement of eotaxin-3 in late-phase eosinophil recruitment.[9]

A variety of factors known to be involved in asthma have been shown to increase eotaxin production *in vitro* in various cell types. These include growth factors, allergens, proinflammatory cytokines, T_H2 cytokines, and rhinovirus.[1,7,10-13] In epithelial cells and macrophage cell lines,

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eotaxin is induced by proinflammatory cytokines, whereas mast cells produce eotaxin upon stimulation with stem cell factor.[10] In fibroblasts and airway smooth muscle cells, both proinflammatory and T_H2 cytokines can induce eotaxin release. The regulation of the expression of eotaxin-2 and eotaxin-3 in the lungs is largely unknown.

The T_H2 cytokines IL-4 and IL-13 are increased in BAL fluid and lung tissue from patients with asthma when compared with nonatopic control subjects and have been associated with eosinophilia.[14] In addition to T_H2 cytokines, TGF-β is also associated with increased numbers of airway eosinophils, and TGF-β levels are higher in patients with asthma.[15,16] This cytokine has been shown to have multiple properties: it is a modulator of inflammation because it has both proinflammatory and anti-inflammatory activities, and it can act as a growth factor. Persistent TGF-β activity may lead to tissue fibrosis and airway remodelling.[17] Recently, it has been shown that TGF-β and IL-13 synergistically increase eotaxin expression in human airway fibroblasts.[15] Although HASMs have been reported to produce eotaxin, not much is known about the regulation of eotaxin, eotaxin-2, and eotaxin-3 in HASMs. Because *in vivo* studies have indicated that the different eotaxins are involved in different phases of the allergic reaction, it is likely that the expression of these chemokines is differentially regulated. Therefore, we hypothesized that TGF-β is involved in differential regulation of eotaxins by HASMs. To this end, we have explored the effects of TGF-β and T_H2 cytokines on the expression of eotaxins by cultured HASMs.

METHODS

HASM culture

HASMs from 2 donors were purchased from Stratagene (La Jolla, Calif). Cells were characterized and stained positive for α-smooth muscle actin. Cells were cultured in Dulbecco modified Eagle medium/nutrient mixture F12 (1:1; Invitrogen, Carlsbad, Calif) containing 25 mmol/L HEPES (Invitrogen), 10% (vol/vol) FCS (Invitrogen) supplemented with 2.5 mmol/L L-glutamine (Cambrex, East Rutherford, NJ), 1% (vol/vol) nonessential amino acids (Invitrogen), 50 U/mL penicillin, and 50 μg/mL streptomycin (Cambrex). Cells were seeded at 5000/cm^2, and medium was replaced every 72 hours. Confluent cells from passages 5 to 7 were used for experiments.

Stimulation of HASMs

Confluent cells were washed with PBS and cultured in serum-free Dulbecco modified Eagle medium/nutrient mixture F12 containing 1% (vol/vol) insulin, transferrin, and sodium selenite liquid media supplement (stock: insulin 5 mg/mL, transferrin 0.5 mg/mL, and sodium selenite 0.5 μg/mL; Sigma, St. Louis, Mo) for 24 hours. After starvation, cells were stimulated with IL-4, IL-9, IL-13 (Peprotech, Rocky Hill, NJ), or TGF-β1 (R&D Systems, Abingdon, United Kingdom) in the same medium.

Measurement of chemokine release

Eotaxin, eotaxin-2, and eotaxin-3 protein levels were measured in supernatants from HASMs stimulated with IL-4, IL-9, IL-13, or TGF-β by using sandwich ELISAs from R&D Systems. The detection limit of the eotaxin and eotaxin-2 ELISA was 20 pg/mL, whereas the detection limit for eotaxin-3 was 50 pg/mL.

Flow cytometric analysis

The expression of eotaxin in HASMs was evaluated by flow cytometric analysis. HASMs were cultured until confluence and serum-deprived for 24 hours before stimulation. Cells were stimulated with IL-4, and 1 μg/mL brefeldin A (Sigma) was added in the last 4 hours of the stimulation period to accumulate proteins in the Golgi apparatus. Cells were harvested and fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany). After fixation, cells were permeabilized for 10 minutes with 0.1% (wt/vol) saponin (Riedel de Haën, Seelze, Germany) to allow detection of intracellular antigens. All subsequent staining steps were performed in the presence of 0.1% saponin. After permeabilization, cells were incubated for 30 minutes at 4°C with 50 μL of monoclonal anti-eotaxin antibody (clone 43911.11; R&D Systems) or a monoclonal anti–eotaxin-3 antibody (clone 115002; R&D Systems). After incubation, cells were washed with wash buffer (PBS/0.5% [wt/vol] BSA/0.2% [wt/vol] sodium azide/0.1% [wt/vol] saponin) and incubated with fluorescein isothiocyanate–conjugated goat anti-mouse IgG antibody (Southern Biotechnology Associates, Birmingham, Ala) for 30 minutes at 4°C. Cells were washed and resuspended in wash buffer. In each experiment, parallel stainings with an isotype-matched control antibody (mouse IgG1, Dako, Glostrup, Denmark) were performed as a control for nonspecific Ig binding. Cells were analyzed with a FACScalibur (Becton Dickinson, Franklin Lakes, NJ) by using Cellquest Pro software.

Isolation of RNA and PCR

After stimulation, RNA was isolated by using the Qiagen RNeasy Minikit (Qiagen, Valencia, Calif). The RNA concentration and purity were assessed with optical density measurements. Reverse transcriptase-PCR was performed to detect eotaxin or eotaxin-3 mRNA expression in cultured HASMs. Reverse transcription of 2 μg of total RNA was performed by using 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen); 1.25 mmol/L of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate (Invitrogen); 0.5 μg of oligo$(dT)_{12\text{-}18}$ primer (Invitrogen); 10 mmol/L dithiothreitol; 50 mmol/L Tris-HCl (pH 8.3); 75 mmol/L KCl; and 3.0 mmol/L $MgCl_2$ in a total volume of 20 μL. The mix was incubated at 37°C for 1 hour. PCR was performed with 2 pmol of forward and reverse primers (Invitrogen); deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate at a final concentration of 0.2 mmol/L; 1 U of Taq polymerase (Promega, Madison, Wis); 50 mmol/L KCl; 10 mmol/L Tris-HCl (pH 9.0 at 25°C); 0.1% (vol/vol) Triton X-100; and 2 or 2.5 mmol/L $MgCl_2$ in a final volume of 25 μL. Primers for eotaxin, eotaxin-3, and β-actin are specified in Table I. Amplification of β-actin was used as a reference.

PCR was performed at 95°C for an initial 10 minutes followed by 30 (eotaxin) or 25 (eotaxin-3 and β-actin) cycles of denaturation at 95°C for 45 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 60 seconds. Final extension was performed at 72°C for 10 minutes. PCR products were visualized by electrophoresis on a 1.5% agarose gel containing ethidium bromide as a fluorescent dye and subsequent ultraviolet luminescence.

Quantitative real-time PCR

Quantitative real-time PCR was performed on an iCycler PCR machine (Bio-Rad, Hercules, Calif) by using SYBR Green I

TABLE I. PCR primer sequences

Name	Sequence	Product size (base pairs)	$MgCl_2$ (mmol/L)
Eotaxin	F: 5′-CCTTCAGCGACTAGAGAGC-3′ R: 5′-ACACTCAGGCTCTGGTTTGG-3′	209	2
Eotaxin-3	F: 5′-GCCTGATTTGCAGCATCATGATGG-3′ R: 5′-CGGATGACAATTCAGCTGAGTCAC-3′	290	2.5
β-Actin	F: 5′-CTACAATGAGCTGCGTGTGG-3′ R: 5′-AAGGAAGGCTGGAAGAGTGC-3′	528	2

F, Forward primer; *R*, reverse primer

TABLE II. Primer sequences for quantitative real-time PCR

Name	Sequence	Product size (base pairs)	$MgCl_2$ (mmol/L)
Eotaxin	F: 5′-GAAACCACCACCTCTCACG-3′ R: 5′-GCTCTCTAGTCGCTGAAGGG-3′	190	3
Eotaxin-3	F: 5′-ATATCCAAGACCTGCTGCTTC-3′ R: 5′-TTTTTCCTTGGATGGGTACAG-3′	152	3
β-Actin	F: 5′-TGCGTGACATTAAGGAGAAG-3′ R: 5′-TGAAGGTAGTTTCGTGGATG-3′	213	3

F, Forward primer; *R*, reverse primer.

chemistry and the primers specified in Table II. Samples were analyzed in triplicate, and threshold cycles were calculated by using iCycler version 3.0a analysis software (Bio-Rad). Threshold cycles were used to calculate arbitrary mRNA concentrations by using the relative standard curve method. The standard curve was constructed by using serial dilutions of cDNA containing message for eotaxin and eotaxin-3. Relative mRNA concentrations for eotaxin, eotaxin-3, β-actin, and glyceraldehyde phosphate dehydrogenase (GAPDH) were determined and used to calculate the expression ratios.

Statistical analysis

Statistical analysis was performed with a paired-samples *t* test. Differences were considered significant when *P* values were smaller than .05.

RESULTS

Cytokine-induced eotaxin and eotaxin-3 release

To study the effect of T_H2 cytokines on the release of eotaxins by HASMs, serum-starved confluent monolayers were incubated with IL-4, IL-13, and IL-9 in serum-free medium. The T_H2 cytokines IL-4 and IL-13 induced eotaxin and eotaxin-3 release by HASMs in a dose-dependent manner (Fig 1, *A* and *B*). IL-4 induced eotaxin release at low concentrations, whereas IL-13 was less potent in inducing eotaxin release. Eotaxin-3 release, however, was induced at low concentrations of both IL-4 and IL-13. Control-treated cells did not release eotaxin or eotaxin-3. In contrast to IL-4 and IL-13, the other T_H2 cytokine, IL-9, did not induce eotaxin or eotaxin-3 release from HASMs. Eotaxin-2 was not released by HASMs. Flow cytometric analysis revealed one population of cells expressing eotaxin and eotaxin-3, indicating that the production of eotaxin and eotaxin-3 was not the result of contaminating fibroblasts in the HASM culture (Fig 1, *C*).

Cytokine-induced eotaxin and eotaxin-3 mRNA

IL-4 (20 ng/mL) and IL-13 (20 ng/mL) induced eotaxin and eotaxin-3 mRNA expression in HASMs (Fig 2). In line with the protein data, IL-9 did not induce eotaxin or eotaxin-3 mRNA expression.

TGF-β and T_H2 cytokines

To study the effect of TGF-β on IL-4–induced and IL-13–induced eotaxin and eotaxin-3 release, cells were incubated with the T_H2 cytokines in combination with TGF-β. A recent study by Wenzel et al[15] showed that a low concentration of TGF-β (0.5 ng/mL) synergistically enhanced IL-13–induced eotaxin release by primary human airway fibroblasts, whereas TGF-β alone did not induce release of eotaxin. In our study, HASMs were stimulated with 0.5 ng/mL TGF-β1, 10 ng/mL IL-4, and 30 ng/mL IL-13 or combinations thereof for 24 hours. Stimulation with the combination of TGF-β and a T_H2 cytokine resulted in an enhanced release of eotaxin by HASMs (Fig 3, *A*). Both IL-4–induced and IL-13–induced eotaxin-3 release, however, was inhibited by TGF-β (Fig 3, *B*).

Eotaxin and eotaxin-3 mRNA expression by HASMs from 2 donors was assessed by quantitative real-time PCR, and results were normalized to β-actin expression (Fig 4). For comparison, mRNA expression normalized to GAPDH showed the same results (data not shown). In line with the protein data, IL-4–induced eotaxin mRNA expression was enhanced by TGF-β, whereas eotaxin-3 mRNA expression was inhibited. These effects were seen only at 20 hours; at the earlier time points, mRNA expression for eotaxin and eotaxin-3 was low.

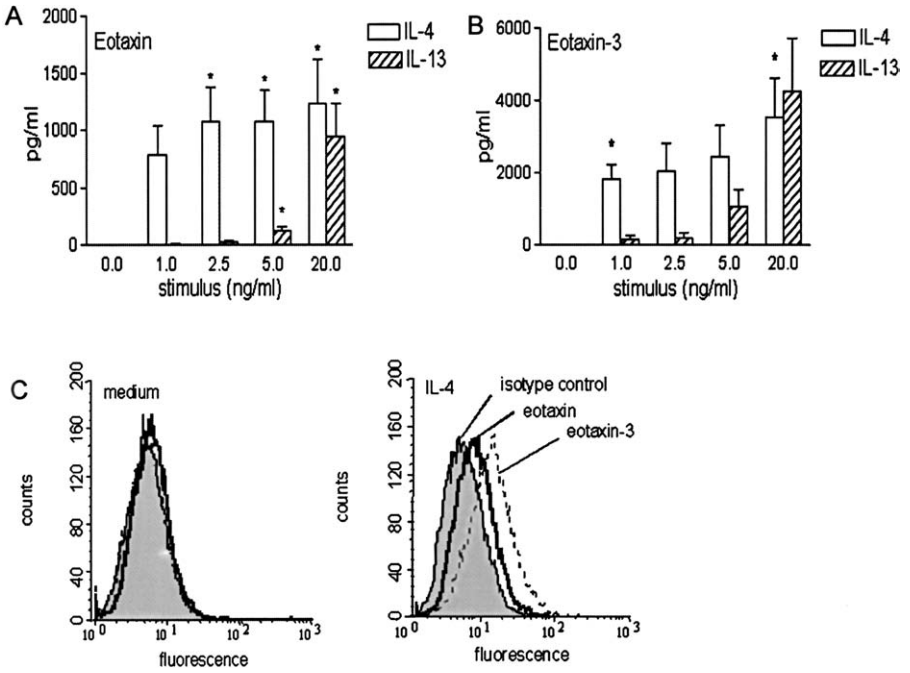

FIG 1. Effect of T_H2 cytokines on eotaxin **(A)** and eotaxin-3 **(B)** release by HASMs. HASMs were stimulated with IL-4 or IL-13 for 24 hours. Results are mean ± SEM of 4 separate experiments, each performed in triplicate, with cells from 2 donors. $*P < .05$ compared with medium-treated cells. Flow cytometric analysis of HASMs stimulated for 24 hours with medium or 20 ng/mL IL-4 in the presence of brefeldin A **(C)**.

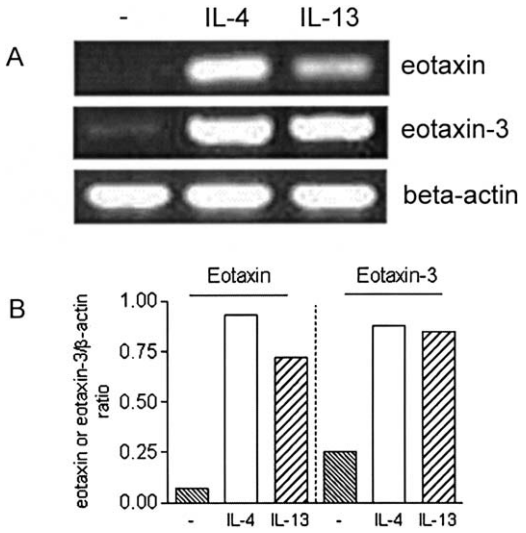

FIG 2. A, Representative eotaxin and eotaxin-3 PCR from 1 donor of HASMs. HASMs were stimulated for 6 hours with medium alone or with 20 ng/mL of IL-4 or IL-13. **B,** Eotaxin and eotaxin-3 mRNA levels were quantified by scanning densitometry and corrected for β-actin in the same samples. Similar results were obtained in another experiment with cells from a different donor.

DISCUSSION

In this study we have shown that HASMs produce eotaxin and eotaxin-3 upon stimulation with the T_H2 cytokines IL-4 and IL-13. HASMs did not release eotaxin-2 protein upon stimulation with the T_H2 cytokines IL-4 and IL-13. A low concentration (0.5 ng/mL) of TGF-β increased IL-4–induced and IL-13–induced eotaxin release, whereas it decreased the release of eotaxin-3. At the same concentration, TGF-β alone altered neither eotaxin nor eotaxin-3 release.

We have shown for the first time that IL-4 and IL-13 induce both mRNA expression and release of eotaxin-3 in HASMs. Eotaxin-3 mRNA has been shown to be in-

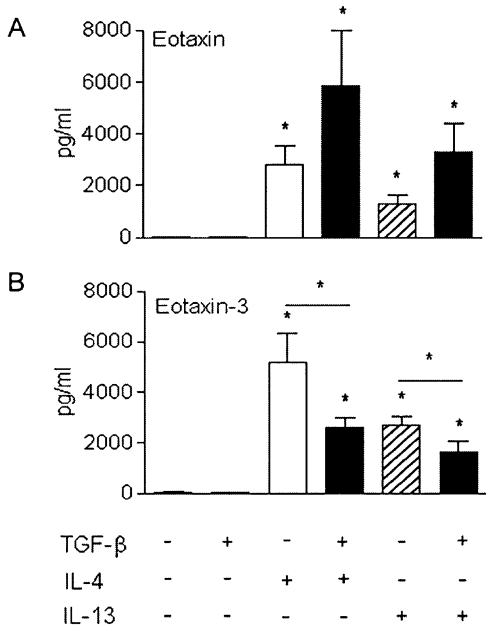

FIG 3. Modulation of T_H2 cytokine-induced eotaxin **(A)** and eotaxin-3 **(B)** release by TGF-β. HASMs from 2 donors were stimulated with IL-4 (10 ng/mL), IL-13 (30 ng/mL), TGF-β1 (0.5 ng/mL), or combinations of these cytokines for 24 hours. Data are mean ± SEM of 6 separate experiments, each performed in triplicate, with cells from 2 donors. $*P < .05$.

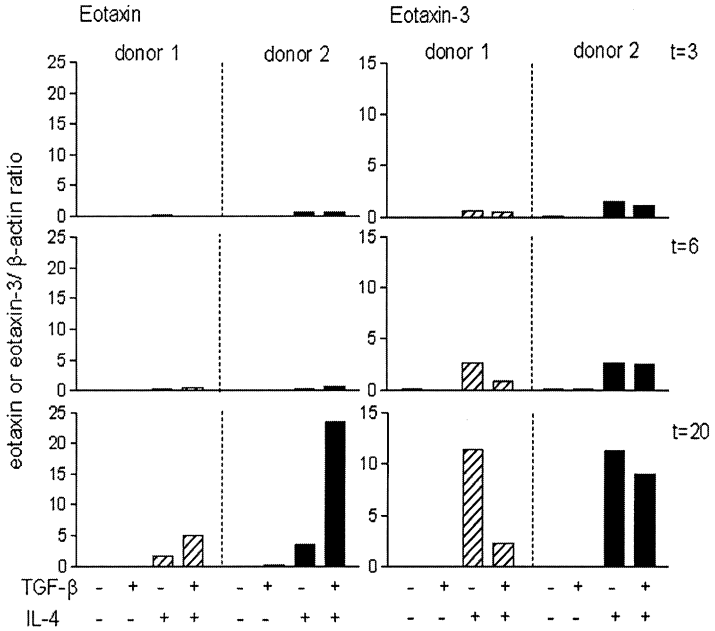

FIG 4. Effect of TGF-β on IL-4–induced and IL-13–induced eotaxin and eotaxin-3 mRNA expression. HASMs from 2 donors were stimulated for 3, 6, or 20 hours with medium alone, IL-4 (10 ng/mL), TGF-β1 (0.5 ng/mL), or the combination of both cytokines, and quantitative real-time PCR was performed.

creased by IL-4 and IL-13 in *in vitro* studies with cell types other than HASMs, such as human dermal fibroblasts, airway epithelial cells, and vascular endothelial cells.[6,18,19] The ability of IL-4 and IL-13 to increase production of eotaxin by HASMs has been reported previously.[20,21] Eotaxin-2 protein was not detected in supernatants of HASMs. Studies with bronchial biopsies have shown that patients with asthma have more eotaxin-2 mRNA–positive cells in their mucosa and that epithelial cells and $CD68^+$ macrophages are the main source of

eotaxin-2.[22] To our knowledge, no reports on eotaxin-2 expression by HASMs have been published.

The effect of TGF-β on eotaxin release has been studied in primary human airway fibroblasts from the third- to fifth-order subcarinas by Wenzel et al.[15] In these cells, IL-13–induced eotaxin release was synergistically enhanced by TGF-β at both the protein and mRNA levels. The authors suggest that the synergistic increase in mRNA resulted from increased transcription or from increased stabilization of the mRNA. Although we did not find a synergistic increase in T_H2 cytokine-induced eotaxin release, our findings on the modulation of T_H2 cytokine-induced eotaxin release by TGF-β are in concordance with this study.

We have used well-characterized airway smooth muscle cell cultures from a commercial source (Stratagene), which stained positive for α-smooth muscle actin. Studies with airway smooth muscle cells from other sources have shown that these cells respond to IL-4 and IL-13 in the same manner.[20,21] In addition, we believe that we excluded the possibility that our cell culture was contaminated with fibroblasts that would contribute to the observed eotaxin release, because flow cytometry showed that a single cell population was responsible for the production of eotaxin and eotaxin-3.

The mechanism by which TGF-β enhances eotaxin expression and inhibits eotaxin-3 expression is unknown. Eotaxin and eotaxin-3 are structurally different, and their genes are located on different chromosomes. The gene encoding for eotaxin lies on chromosome 17q11.2, whereas the genes for eotaxin-2 and eotaxin-3 are located on chromosome 7q11.2. The promotors for these genes have been partially characterized. The eotaxin promotor contains binding sites for many transcription factors, including Sp1, NF-κB, AP-1, CEBP, and STAT6.[23-25] A binding site for STAT6 was also found in the eotaxin-3 promotor.[19] Binding of IL-4 or IL-13 to the IL-4 receptor leads to activation of STAT6, which can bind to specific sequences in promotor regions of the eotaxin and eotaxin-3 gene, thus explaining the observed stimulatory effect of these T_H2 cytokines on expression of both eotaxin and eotaxin-3. The differential effect of TGF-β on T_H2 cytokine-mediated eotaxin and eotaxin-3 expression may be explained by a differential effect on the activity of the eotaxin and eotaxin-3 promotor. In most promotors, binding sites for coactivators and repressors can be found. One possible explanation for our findings is that TGF-β may induce a repressor that binds to the eotaxin-3 promotor and inhibits STAT6-induced transcription, whereas this repressor does not bind to the eotaxin promotor. We have found that both IL-4–induced eotaxin protein and mRNA levels are enhanced by TGF-β, whereas eotaxin-3 protein and mRNA levels are inhibited. Therefore, TGF-β probably exerts at least part of its modulating effect at the transcriptional level.

Posttranscriptional mechanisms may also play a role in the differential regulation of eotaxin and eotaxin-3. TGF-β has been shown to increase the stability of elastin mRNA by pathways dependent on SMAD, p38, and protein kinase C.[26] TGF-β was found to induce the transcription of a product that binds to elastin mRNA and stabilizes it. Studies on eotaxin gene expression have shown that RNA-binding proteins, such as HuR, may regulate the increased stabilization of eotaxin mRNA, which contributes to the increase in eotaxin expression seen in cytokine-treated airway epithelial cells.[27] An alternative posttranscriptional mechanism involved in the observed TGF-β–mediated suppression of eotaxin-3 release is proteolytic degradation of eotaxin-3. Matrix metalloproteinases (MMPs) have been shown to cleave a variety of chemokines. MMP-2 (gelatinase A) has been shown to cleave MCP-3, whereas MCP-1, MCP-2, and MCP-4 are cleaved by MMP-1 (collagenase-1) and MMP-3 (stromelysin).[28] As eotaxin and eotaxin-3 differ structurally, they may be susceptible to cleavage by different MMPs. Because eotaxin is structurally more closely related to the MCPs than to eotaxin-3, it may be cleaved by the same enzymes that cleave MCPs: MMP-3 and MMP-1. MMP-9 (gelatinase B) has been shown to be present in the extracellular matrix of bronchial biopsy samples from patients with asthma. This MMP can cleave different matrix proteins, including collagens, fibronectin, and laminin. In addition, MMP-9 can cleave the CXC chemokines stromal cell–derived factor (SDF) and growth-regulated oncogene α. Eotaxin-3 may be more sensitive to MMP-9 than eotaxin, which would account for the reduction in eotaxin-3 protein after stimulation with T_H2 cytokines and TGF-β. Although both TGF-β and IL-13 have been shown to induce MMP-9 production in keratinocytes, it is unknown how the production of MMPs by HASMs is regulated by these cytokines.[29,30] The reduction of eotaxin-3 levels after stimulation with the combination of these cytokines may be caused by the induction of MMP-9 or other MMPs and may lead to the resolution of the eotaxin-3 gradient. This in turn may result in less recruitment of new eosinophils into the lung tissue and could result in resolution of chronic eosinophilia. In addition, the reduction of the concentration of stromal chemoattractant may lead to a gradient of chemokine toward the epithelium, resulting in clearance of inflammatory cells into the lumen.[31-33]

The differential regulation of eotaxin and eotaxin-3 by TGF-β implies that the eotaxins may differ in function. The receptor for the eotaxins, CCR3, is present on inflammatory cells such as eosinophils, basophils, mast cells, T cells, and dendritic cells.[34-37] In addition, CCR3 can be found on airway resident cells such as epithelial cells.[38,39] We have found expression of CCR3 on HASMs.[40] The finding of CCR3 on airway resident cells suggests that the ligands for CCR3 may not only function as chemokines for inflammatory cells. Stimulation of airway epithelial cells with eotaxin induced IL-8 and GM-CSF production, which was inhibited by a CCR3 antagonist.[41] We speculate that eotaxin may affect HASMs in an autocrine manner by binding to CCR3. The increase in T_H2 cytokine-induced eotaxin by TGF-β may result in direct effects on HASMs instead of leading to a new influx of eosinophils.

Although it has been shown that all eotaxins can induce chemotaxis of eosinophils, contradictory results have been found on the relative potency of the selected eotaxins.[6,42,43] Their location on different chromosomes and low sequence homology, together with the differential regulation of these chemokines in HASMs, suggests that the eotaxins have different functions and are not redundant.

The inflammatory process in asthma is an intricate network of cytokines, growth factors, chemokines, and inflammatory cells. T_H2 cytokines induce recruitment of eosinophils by production of chemokines. It is interesting to note that eosinophils themselves may regulate their recruitment by altering the production of eotaxin and eotaxin-3 as they release TGF-β. Regulatory T cells are another important source of TGF-β. These cells have been shown to reduce airway inflammation and airway hyperresponsiveness.[44] Our findings implicate TGF-β as an important cytokine for fine-tuning the inflammatory process.

In summary, this study shows that HASMs produce eotaxin and eotaxin-3 upon stimulation, whereas eotaxin-2 is not induced. Furthermore, our study demonstrates that T_H2 cytokine-induced eotaxin and eotaxin-3 expression by HASMs is differentially regulated by TGF-β. TGF-β may dampen the inflammatory response by reducing eotaxin-3 levels, thereby resolving chronic eosinophilia. The increase of T_H2 cytokine-induced eotaxin expression by TGF-β may lead to an increased influx of eosinophils but may also lead to effects on airway smooth muscle itself. Our data support the dual function of TGF-β in inflammation, because we have shown that TGF-β has both anti-inflammatory and proinflammatory functions. The finding of production of different eotaxins by HASMs further implicates these cells as important regulators in the pathogenesis of asthma.

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REFERENCES

1. Ghaffar O, Hamid Q, Renzi PM, Allakhverdi Z, Molet S, Hogg JC, et al. Constitutive and cytokine-stimulated expression of eotaxin by human airway smooth muscle cells. Am J Respir Crit Care Med 1999;159: 1933-42.
2. Chung KF, Patel HJ, Fadlon EJ, Rousell J, Haddad EB, Jose PJ, et al. Induction of eotaxin expression and release from human airway smooth muscle cells by IL-1beta and TNFalpha: effects of IL-10 and corticosteroids. Br J Pharmacol 1999;127:1145-50.
3. Bandeira-Melo C, Herbst A, Weller PF. Eotaxins. Contributing to the diversity of eosinophil recruitment and activation. Am J Respir Cell Mol Biol 2001;24:653-7.
4. Jose PJ, Griffiths-Johnson DA, Collins PD, Walsh DT, Moqbel R, Totty NF, et al. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. J Exp Med 1994; 179:881-7.
5. Forssmann U, Uguccioni M, Loetscher P, Dahinden CA, Langen H, Thelen M, et al. Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes. J Exp Med 1997;185:2171-6.
6. Shinkai A, Yoshisue H, Koike M, Shoji E, Nakagawa S, Saito A, et al. A novel human CC chemokine, eotaxin-3, which is expressed in IL-4-stimulated vascular endothelial cells, exhibits potent activity toward eosinophils. J Immunol 1999;163:1602-10.
7. Brown JR, Kleimberg J, Marini M, Sun G, Bellini A, Mattoli S. Kinetics of eotaxin expression and its relationship to eosinophil accumulation and activation in bronchial biopsies and bronchoalveolar lavage (BAL) of asthmatic patients after allergen inhalation. Clin Exp Immunol 1998;114: 137-46.
8. Ying S, Robinson DS, Meng Q, Barata LT, McEuen AR, Buckley MG, et al. C-C chemokines in allergen-induced late-phase cutaneous responses in atopic subjects: association of eotaxin with early 6-hour eosinophils, and of eotaxin-2 and monocyte chemoattractant protein-4 with the later 24-hour tissue eosinophilia, and relationship to basophils and other C-C chemokines (monocyte chemoattractant protein-3 and RANTES). J Immunol 1999;163:3976-84.
9. Berkman N, Ohnona S, Chung FK, Breuer R. Eotaxin-3 but not eotaxin gene expression is upregulated in asthmatics 24 hours after allergen challenge. Am J Respir Cell Mol Biol 2001;24:682-7.
10. Rothenberg ME. Eotaxin. An essential mediator of eosinophil trafficking into mucosal tissues. Am J Respir Cell Mol Biol 1999;21:291-5.
11. Teran LM, Mochizuki M, Bartels J, Valencia EL, Nakajima T, Hirai K, et al. Th1- and Th2-type cytokines regulate the expression and production of eotaxin and RANTES by human lung fibroblasts. Am J Respir Cell Mol Biol 1999;20:777-86.
12. Mochizuki M, Bartels J, Mallet AI, Christophers E, Schroder JM. IL-4 induces eotaxin: a possible mechanism of selective eosinophil recruitment in helminth infection and atopy. J Immunol 1998;160:60-8.
13. Papadopoulos NG, Papi A, Meyer J, Stanciu LA, Salvi S, Holgate ST, et al. Rhinovirus infection up-regulates eotaxin and eotaxin-2 expression in bronchial epithelial cells. Clin Exp Allergy 2001;31:1060-6.
14. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. Science 1998; 282:2258-61.
15. Wenzel SE, Trudeau JB, Barnes S, Zhou X, Cundall M, Westcott JY, et al. TGF-beta and IL-13 synergistically increase eotaxin-1 production in human airway fibroblasts. J Immunol 2002;169:4613-9.
16. Redington AE, Madden J, Frew AJ, Djukanovic R, Roche WR, Holgate ST, et al. Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. Am J Respir Crit Care Med 1997; 156(2 pt 1):642-7.
17. Nakao A. Is TGF-beta1 the key to suppression of human asthma? Trends Immunol 2001;22:115-8.
18. Banwell ME, Tolley NS, Williams TJ, Mitchell TJ. Regulation of human eotaxin-3/CCL26 expression: modulation by cytokines and glucocorticoids. Cytokine 2002;17:317-23.
19. Hoeck J, Woisetschlager M. Activation of eotaxin-3/CCLl26 gene expression in human dermal fibroblasts is mediated by STAT6. J Immunol 2001;167:3216-22.
20. Hirst SJ, Hallsworth MP, Peng Q, Lee TH. Selective induction of eotaxin release by interleukin-13 or interleukin-4 in human airway smooth muscle cells is synergistic with interleukin-1beta and is mediated by the interleukin-4 receptor alpha-chain. Am J Respir Crit Care Med 2002;165: 1161-71.
21. Moore PE, Church TL, Chism DD, Panettieri RA Jr, Shore SA. IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK. Am J Physiol Lung Cell Mol Physiol 2002;282: L847-53.
22. Ying S, Meng Q, Zeibecoglou K, Robinson DS, Macfarlane A, Humbert M, et al. Eosinophil chemotactic chemokines (eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein-3 (MCP-3), and MCP-4), and C-C chemokine receptor 3 expression in bronchial biopsies from atopic and nonatopic (intrinsic) asthmatics. J Immunol 1999;163: 6321-9.
23. Garcia-Zepeda EA, Rothenberg ME, Weremowicz S, Sarafi MN, Morton CC, Luster AD. Genomic organization, complete sequence, and chromosomal location of the gene for human eotaxin (SCYA11), an eosinophil-specific CC chemokine. Genomics 1997;41:471-6.
24. Matsukura S, Stellato C, Plitt JR, Bickel C, Miura K, Georas SN, et al. Activation of eotaxin gene transcription by NF-kappa B and STAT6 in human airway epithelial cells. J Immunol 1999;163:6876-83.
25. Hoeck J, Woisetschlager M. STAT6 mediates eotaxin-1 expression in IL-4 or TNF-alpha-induced fibroblasts. J Immunol 2001;166: 4507-15.

26. Kucich U, Rosenbloom JC, Abrams WR, Rosenbloom J. Transforming growth factor-beta stabilizes elastin mRNA by a pathway requiring active Smads, protein kinase C-delta, and p38. Am J Respir Cell Mol Biol 2002;26:183-8.
27. Atasoy U, Curry SL, Lopez de Silanes I, Shyu AB, Casolaro V, Gorospe M, et al. Regulation of eotaxin gene expression by TNF-alpha and IL-4 through mRNA stabilization: involvement of the RNA-binding protein HuR. J Immunol 2003;171:4369-78.
28. McQuibban GA, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, Overall CM. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. Blood 2002;100:1160-7.
29. Santibanez JF, Guerrero J, Quintanilla M, Fabra A, Martinez J. Transforming growth factor-beta1 modulates matrix metalloproteinase-9 production through the Ras/MAPK signaling pathway in transformed keratinocytes. Biochem Biophys Res Commun 2002;296: 267-73.
30. Lanone S, Zheng T, Zhu Z, Liu W, Lee CG, Ma B, et al. Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling. J Clin Invest 2002;110: 463-74.
31. Corry DB, Rishi K, Kanellis J, Kiss A, Song LZ, Xu J, et al. Decreased allergic lung inflammatory cell egression and increased susceptibility to asphyxiation in MMP2-deficiency. Nat Immunol 2002;3:347-53.
32. Erjefalt JS, Korsgren M, Malm-Erjefalt M, Conroy DM, Williams TJ, Persson CG. Acute allergic responses induce a prompt luminal entry of airway tissue eosinophils. Am J Respir Cell Mol Biol 2003;29: 439-48.
33. Sheppard D. Killed by leukocytes that don't know when to leave. Nat Immunol 2002;3:337-8.
34. Dulkys Y, Kluthe C, Buschermohle T, Barg I, Knoss S, Kapp A, et al. IL-3 induces down-regulation of CCR3 protein and mRNA in human eosinophils. J Immunol 2001;167:3443-53.
35. Cosmi L, Annunziato F, Maggi E, Romagnani S, Manetti R. Chemoattractant receptors expressed on type 2 T cells and their role in disease. Int Arch Allergy Immunol 2001;125:273-9.
36. Scotet E, Schroeder S, Lanzavecchia A. Molecular regulation of CC-chemokine receptor 3 expression in human T helper 2 cells. Blood 2001;98:2568-70.
37. Beaulieu S, Robbiani DF, Du X, Rodrigues E, Ignatius R, Wei Y, et al. Expression of a functional eotaxin (CC chemokine ligand 11) receptor CCR3 by human dendritic cells. J Immunol 2002;169:2925-36.
38. Stellato C, Brummet ME, Plitt JR, Shahabuddin S, Baroody FM, Liu MC, et al. Expression of the C-C chemokine receptor CCR3 in human airway epithelial cells. J Immunol 2001;166:1457-61.
39. Petering H, Kluthe C, Dulkys Y, Kiehl P, Ponath PD, Kapp A, et al. Characterization of the CC chemokine receptor 3 on human keratinocytes. J Invest Dermatol 2001;116:549-55.
40. Zuyderduyn S, Ravensberg AJJ, van Schadewijk A, Mauad T, Hiemstra PS, Rabe KF. Eotaxin and CCR3 expression in cultured human airway smooth muscle cells. Eur Respir J 2002;20(suppl 38):2S.
41. Cui CH, Adachi T, Oyamada H, Kamada Y, Kuwasaki T, Yamada Y, et al. The role of mitogen-activated protein kinases in eotaxin-induced cytokine production from bronchial epithelial cells. Am J Respir Cell Mol Biol 2002;27:329-35.
42. Kitaura M, Suzuki N, Imai T, Takagi S, Suzuki R, Nakajima T, et al. Molecular cloning of a novel human CC chemokine (eotaxin-3) that is a functional ligand of CC chemokine receptor 3. J Biol Chem 1999;274: 27975-80.
43. Dulkys Y, Schramm G, Kimmig D, Knoss S, Weyergraf A, Kapp A, et al. Detection of mRNA for eotaxin-2 and eotaxin-3 in human dermal fibroblasts and their distinct activation profile on human eosinophils. J Invest Dermatol 2001;116:498-505.
44. Umetsu DT, Akbari O, Dekruyff RH. Regulatory T cells control the development of allergic disease and asthma. J Allergy Clin Immunol 2003;112:480-7.