CCR3 Expression and Function in Asthmatic Airway Smooth Muscle Cells

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Asthma is a common, chronic, inflammatory condition of the respiratory system, associated with paroxysmal bronchospasm, bronchial hyperresponsiveness, and airway remodeling (1, 2). Many factors, such as inflammatory mediators, have been implicated in the initiation and the perpetuation of airway remodeling (2, 3), with increases in airway smooth muscle mass being one of the major structural changes described in the airways of asthmatics (4–6). Mechanisms responsible for this phenomenon are thought to be an increase in cell proliferation (hyperplasia) and/or an increase in the size of individual cells (hypertrrophy) (7). Recently, it has been proposed that migration of the airway smooth muscle cells (ASM) toward the epithelium might also contribute to this phenomenon (8, 9). These migrating cells could originate from deep smooth muscle bundles or from bone marrow cells, in a fashion similar to the migration of vascular smooth muscle cells in vascular diseases (10).

Airway smooth muscle was traditionally considered to be a structural cell involved primarily in bronchoconstriction. However, it has been recently shown that ASM could play an important part in regulating local immune response. ASM produce inflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8, eotaxin, and RANTES that may act in both an autocrine and paracrine manner (11). ASM also express mRNA and immunoreactivity for a number of cytokine receptors (12). To our knowledge, however, there has been no report on the expression of chemokine receptors in ASM. CCR3 is a receptor for eotaxin-1 (CCL11), eotaxin-2 (CCL24), eotaxin-3 (CCL26), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13), and RANTES (CCL5). Expression of CCR3 and expression of eotaxin have been shown to be increased in asthmatic lungs and have been linked to the pathogenesis of asthma (13, 14). Therefore, we hypothesized that ASM express CCR3 and that eotaxin promotes the migration of ASM through the activation of CCR3.

The aims of this study were to demonstrate the expression of CCR3 by ASM and to compare this expression between asthmatic and control subjects. To determine the presence and the functional relevance of this receptor on ASM, we assessed the effect of eotaxin on ASM function. Our results provide evidence for the expression of a functional CCR3 by ASM. Furthermore, we have shown that ASM migrate toward a gradient of eotaxin. These data suggest that the up-regulation of CCR3 by ASM in asthmatic subjects might be partly responsible for the increased smooth muscle mass and the airway hyperresponsiveness observed in such patients.

Materials and Methods

Cell culture

Human ASM were obtained from two sources. Bronchial/tracheal smooth muscle cells (B/TSMC) were purchased from Cambrex and were positively stained for α-smooth muscle actin and negatively for factor VIII, CD45, and CD3, as indicated by the manufacturer. B/TSMC were grown in their optimal medium (SmGM-2; Cambrex) containing 5% FBS at 37°C in a humidified incubator with 5% CO₂ as recommended by the supplier. The

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second source of ASMC was from human bronchial biopsies. ASMC were isolated and purified from biopsies as described by Labonté et al. (15). Briefly, bronchial biopsies obtained from mild steroid-naïve asthmatic subjects (mean age 25 years, methacholine provocative concentration 20 lower than 24 mg/ml, negative skin prick test for at least one inhaled allergen) and nonasthmatic, nonallergic subjects (mean age 29.3 years, methacholine provocative concentration 20 higher than 24 mg/ml, negative skin prick test for the inhaled allergens) underwent four consecutive cycles of enzymatic digestion with collagenase (Roche) and elastase (Sigma-Aldrich) for 20 min at 37°C. Cells were then plated in flasks and cultured in complete DMEM-F12 medium containing 10% FBS, 20 U/ml penicillin, 20 μg/ml streptomycin, 25 ng/ml fungizone, 5 mg/ml insulin, 10 ng/ml epidermal growth factor, 5 μg/ml transferrin, 10−7 M chola toxin, and 2 × 10−3 M 3,5,7-triiodo-thyronine sodium salt. The medium was replaced every 2–3 days, and cells were passaged with 0.5% trypsin and 1 mM EDTA once confluence was reached. Cell identity was assessed by measuring the expression of α-smooth muscle actin, calponin, smooth muscle myosin H chain (SM-1 and SM-2), tropomyosin, desmin, and vimentin (16). ASMC demonstrated the characteristic hill and valley appearance, with an elongated and spindle shape and possessing a central nucleus.

Cell stimulation

Confluent B/TSMC from Cambrex were used in passages 3–6 while ASMC from biopsies were used in passages 2–3. Cells were growth arrested by FBS deprivation for 24 h before stimulation with cytokines. After serum deprivation, cells were stimulated in fresh, serum-free medium containing either TNF-α, IL-1, IL-13, or IFN-γ (R&D Systems) in a concentration- and time-dependent manner. Cell viability was assessed by the trypan blue dye exclusion test.

RNA extraction and RT-PCR

Total cellular RNA was isolated from B/TSMC, epithelial cells (Calu-3), purified peripheral blood eosinophils and neutrophils, and CCR3-transfected Ghost cells (National Institutes of Health, AIDS reagent program, no. 3682). RNA was extracted using the RNeasy mini kit extraction column (Qiagen) as directed by the manufacturer. RNA was eluted in 30 μl of nuclelease-free water, and cDNA was generated in a 30-μl reaction, using 0.5 μg of total RNA, oligo(dT)12–18 primers (Amersham Pharmacia Biotech), and Superscript II (Invitrogen Life Technologies), in the presence of 0.5 g of total RNA, oligo(dT)12–18 primers (Amersham Pharmacia Biotech), and Superscript II (Invitrogen Life Technologies), in the presence of 0.5 μM each sense and antisense primers, and 1 μl of cDNA. Primers for the housekeeping gene ribosomal protein S9 and CCR3 were commercially generated from Invitrogen Life Technologies (for the following sequences: S9 forward 5‘-TGC TGA CGC TTG ATG AGA AG-3‘ and antisense 5′-CGC AGA GAG AAG TCG ATG TG-3‘ and CCR3 sense 5′-TCC TTC TCT CTT CTC ATC AAT C-3′ and antisense 5′-GGC GTA TTC CTG CTG-3‘). The samples were amplified in a thermal cycler (PTC-100 Programmable Thermal Controller; MJ Research) for 35 cycles, consisting of denaturation at 95°C, annealing at 57°C, and extension at 72°C. The PCR products were visualized on a 2% agarose gel containing 0.2 μg/ml ethidium bromide, and core band size was determined by comparison with a 100-bp DNA ladder (Invitrogen Life Technologies). Amplicons were purified using the QIAquick PCR purification kit (Qiagen), ligated in pGEM-T (Promega), and used for transformation into XL-1 blue bacteria. Plasmids representing each insert were purified using a HiSpeed Plasmid Maxi kit (QIAGEN) and commercially sequenced to confirm integrity and identity (Pavillon de synthèse et d’analyse d’acides nucléiques, Université Laval).

Immunohistochemistry

To determine whether ASMC have the capacity to produce CCR3 in vivo, immunohistochemistry was performed on sections of major airways (large bronchi) from four asthmatics and four normal subjects. The subjects were obtained from the Tissue Bank (MCI/Meakins-Christie Tissue Bank, McGill University). A clinical diagnosis of asthma was based on the evaluation of the patient’s medical file by a respiratory physician. Diagnostic criteria included prior inclusion and treatment for asthma, documented evidence of variable airflow obstruction >15%, and bronchial hyperresponsiveness. Immediately following biopsies, lung specimens were prepared for immunohistochemistry. Briefly, formalin-fixed tissues were paraffin-embedded, and 5-μm-thick sections were processed in xylene, and hydrated through a graded alcohol series. Endogenous peroxidase was quenched by incubating the slides in 0.5% hydrogen peroxide in PBS for 30 min. After rinsing in PBS, sections were blocked with block-

ing solution (DakoCytomation) for 20 min at room temperature (RT). Primary Ab diluted in diluting buffer (DakoCytomation) was applied (5 μg/ml, goat polyclonal Ab anti-CCR3; Santa Cruz Biotechnology), and sections were incubated overnight at 4°C. Control sections were incubated with isotype control (5 μg/ml, normal goat IgG; Caltag Laboratories). After rinsing in PBS, a biotinylated rabbit anti-goat Ab (1/100 dilution; DakoCytomation) was applied, and sections were incubated for 60 min at RT. Sections were thoroughly washed in PBS and incubated with the streptavidin HRP reagent (60 min at RT) and the reaction was revealed using 0.5 mg/ml diaminobenzidine tetrahydrochloride in Tris buffer (pH 7.6) as the chromogen and 0.05% hydrogen peroxide as the substrate for 5 min. Sections were counterstained with hematoxylin and mounted.

Western blotting

Cells were rinsed twice with ice-cold PBS and incubated on ice for 30 min with trypsin buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 0.5% Nonidet P-40) containing a minicomplete protease inhibitor mixture tablet (Roche Diagnostic Systems). Extracts were clarified at 14,000 × g at 4°C for 20 min, and protein concentrations were determined using the Bradford assay (Bio-Rad). With the use of one-dimensional SDS-PAGE, 20 μg of protein extracts were resolved and electrophoretically transferred to Hybond polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). Membranes were blocked in TBBS (0.1% Tween 20, 10 mM TBS, pH 7.5) for 2 h at RT and then washed twice for 2 min with TTBS. Membranes were incubated for 1 h with anti-CCR3 Ab (Santa Cruz Biotechnology; 1/2000) after which a 1-h incubation at RT with an alkaline phosphatase-conjugated anti-goat Ab (1/1000) was performed. The bound secondary Ab was detected with the CSPD chemiluminescence detection kit (Roche Diagnostic Systems). Following a double rinsing with PBS, the signal was visualized on Kodak Bio-Max-x-ray.

Flow cytometric analysis

FACS analysis was performed as follows: B/TSMC and ASMC derived from normal (n = 4) and asthmatic subjects (n = 3) were detached from the flask by addition of a solution of PBS-EDTA (0.5 M) for 20 min at 37°C. Cells were resuspended at a concentration of 1 × 106 cells/ml, washed three times with PBS, and then incubated with purified normal human IgG (Santa Cruz Biotechnology) at 4°C for 20 min to block any nonspecific binding. PE-conjugated anti-CCR3 (FAB155P, clone 61828.111; R&D Systems) or control isotype (rat IgG2A; IC006P, clone 54447; R&D Systems) Abs were incubated with the cells at 4°C for 30 min; after three washes with 0.5% PBS-BSA, cells were then resuspended in PBS at 4°C. Cell-associated immunofluorescence was immediately analyzed using FACS (BD Biosciences) to determine the level of surface expression of CCR3. At least 10,000 cells were counted per analysis, and ASMC were gated to include only viable cells. CCR3 was also identified using fluorochrome-labeled eotaxin (Fluorokine; R&D Systems) and analyzed via flow cytometry. As described above, B/TSMC were prepared for labeling and cells were labeled with a Fluorokine kit for human CCR3 (Cytochrome Flow Cytometry Kit; R&D Systems) according to the manufacturer’s instructions. Briefly, 10 μl of biotinylated recombinant eotaxin reagent was added to 25-μl aliquots of washed cells (106) and incubated for 60 min on ice. Following the incubation period, 10 μl of streptavidin-FITC reagent was added, and cells were incubated for an additional 30 min at 4°C in the dark. Cells were then washed twice, using the buffer provided to remove unreacted streptavidin-FITC, resuspended in 200 μl of PBS, and analyzed by flow cytometry. As a negative control, an identical sample of washed cells was incubated with 10 μl of biotinylated negative control reagent (supplied with the kit). The specificity of the reaction was assessed by mixing 20 μl of anti-human eotaxin blocking Ab with 10 μl of biotinylated eotaxin and incubating for 15 min at RT. The rest of the protocol is as described above.

Immunofluorescence detection of CCR3 in B/TSMC

B/TSMCs were cultured on glass coverslips in six-well plates until 40–50% confluency. Cells were then washed twice with PBS and fixed with IntraPrep (Beckman Coulter) according to the manufacturer’s directions. Cells were incubated overnight at 4°C with either a rat mAb anti-human CCR3 (5 μg/ml; R&D Systems) or the control isotype Ab (rat IgG2A; R&D Systems). After washing with PBS, cells were incubated for 30 min at RT with biotinylated mouse mAb anti-rat IgG2A (2.5 μg/ml; BD Biosciences). Cells were subsequently washed and incubated with Streptavidin conjugated to Alexa 594 (Molecular Probes). After a final wash, nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich), slides were mounted, and cells were imaged on an Olympus
inverted-phase microscope (IX-81) using a mounted digital camera (Cool-SnapPro CF monochrome) equipped with a CRI filter. Images were analyzed using ImagePro Plus software (Carsen Group).

**Measurement of intracellular-free Ca²⁺**

For calcium measurements, glass coverslips with confluent serum-deprived B/TSMC were rinsed twice with prewarmed (37°C) HEPES-buffered (10 mM, pH 7.4) HBSS-1% BSA, and incubated for 1 h at 37°C in a buffer containing the calcium-sensitive dye fura 2-AM (10 μM) as previously described (17). Thereafter, the fura 2-loaded cultures were washed twice with HEPES-HBSS and then incubated in the dark at RT for 30 min before measurement of intracellular calcium. Glass slides were mounted on an inverted microscope (Olympus IX70) equipped with an Olympus TXe3/A/S digital camera controlled through a PC workstation. Cells were alternatively exited at 340 and 380 nm using a X 10 filter (Sutter Instruments). Emitted fluorescence (510 nm) was measured for 350 ms at each excitable wavelength, and collected data were used to calculate calcium concentrations (in nanomolars) at each pixel from an in vitro calibration curve of known free Ca²⁺ (0–1.35 μM) and pentapotassium fura 2 (50 μM). An Olympus UAP0/340 ×200.75 objective was used in all studies, and image size was set to 540 × 540 pixels. Calcium responses within individual cells were determined using UltraView version 4.0 software (Olympus), by circumscribing single myocytes and spatially averaging fura 2 fluorescence within each cell. At the beginning of each experiment, each chamber contained 200 μl of HEPES-HBSS. Intracellular free Ca²⁺ was first recorded for 30 s to establish a baseline; the cells were then stimulated by adding an equal volume of HEPES-HBSS containing either recombinant human eotaxin-1 or MCP-4 (both at 50 ng/ml). Intracellular-free Ca²⁺ was recorded for at least 200 s to characterize peak and plateau responses, and acetylcholine was used as a positive control. Studies were performed in duplicate using three cell lines, each acquired from a different donor.

**Chemotaxis assay**

Migration of B/TSMCs in response to different concentrations of eotaxin (1, 10, 100, and 1000 ng/ml) was assessed in a 24-well microchemotaxis chamber (NeuroProbe) using a polycarbonate filter (8-μm pore size), as previously described (9). Briefly, B/TSMCs were resuspended in Ham’s F-12 medium supplemented with 0.1% BSA, and 5 × 10⁴ cells were then loaded into the upper chambers and tested for chemotraction to medium supplemented with either 0.1% BSA (negative control) or increasing doses of eotaxin. In some of the experiments, cells were incubated for 1 h with 10 μg/ml anti-CCR3 Ab (MAB155, clone 61828; R&D Systems) before the loading of the cells into the chamber. The chambers were incubated at 37°C in 5% CO₂ for 4 h. Cells located on the upper surface of the filter were scraped off, and cells that migrated to the lower face of the membrane were stained with Dif-Quik. The number of migrated cells on the lower face of the filter was counted in five random fields using conventional microscopy.

**Statistical analysis**

Statistical significance was determined using a Student’s t test. Values of p < 0.05 were considered statistically significant.

**Results**

**ASMC constitutively express CCR3 at mRNA and protein levels**

To demonstrate the expression of CCR3 by ASMC at both mRNA and protein levels, we performed RT-PCR and Western blot on RNA and proteins obtained from cultured ASMC, epithelial cells, and CCR3-transfected cells, as well as from eosinophils and neutrophils purified from blood. Gel electrophoresis (Fig. 1A) revealed bands corresponding to the expected size of the CCR3 cDNA product (313 bp). At the protein level, Western blot revealed comparable expressions of CCR3 by ASMC, Calu-3 epithelial cells, and CCR3-transfected cells, whereas eosinophils demonstrated greater signal intensity (Fig. 1B). Surface expression of CCR3 by ASMC was confirmed using flow cytometry and revealed a high percentage of unstarved serum cells expressing the receptor (see Fig. 1C). In the left panel of Fig. 1C, a PE-labeled Ab against CCR3 was used. The specificity of the signal was confirmed by the addition of a CCR3 ligand: FITC-labeled eotaxin (see Fig. 1C, right panel). This signal was completely suppressed when anti-eotaxin was added prior to eotaxin (see Fig. 1C, right panel). Immunofluorescence was performed on unstimulated B/TSMC and demonstrated an increased signal compared with the isotype control. Also, a high percentage of cells stained positive for CCR3 (Fig. 1D), confirming the previous results obtained by flow cytometry (Fig. 1C).
**CCR3 is expressed by ASMC in vivo**

To further investigate the protein expression of CCR3 in human airways, immunocytochemistry was performed. CCR3 immunoreactivity was detected in ASMC in subjects with (Fig. 2A) and without (Fig. 2B) asthma. In asthmatic specimens, CCR3 protein was localized in the smooth muscle bundles, airway epithelium, and infiltrating cells found within the submucosa, as previously demonstrated (data not shown) (13, 18).

**TNF-α up-regulates the expression of CCR3 by ASMC at the protein level**

Expression of chemokine receptors has been shown to be regulated by different cytokines (19). In asthma in particular, the cytokine environment is characterized by increased TNF-α, IL-4, and IL-13 and decreased IFN-γ levels (20). We investigated the effects of the addition of IL-4, IL-13, IFN-γ, and TNF-α on B/TSMC surface expression of CCR3. Addition of IL-4, IL-13, and IFN-γ down-regulated surface expression of CCR3 (33 ± 5% to 23 ± 8% of positive cells for IL-4 (p = 0.14); 33 ± 5% to 13 ± 9% for IL-13 (p = 0.06); and 33 ± 5% to 25 ± 7% for IFN-γ (p = 0.15)), whereas TNF-α was shown to cause significant up-regulation (33 ± 5% to 74 ± 8% of positive cells (p = 0.001)) (Fig. 3). The combination of TNF-α with either IL-4 or IL-13 did not modify the surface expression of CCR3, as seen with TNF-α, IL-4, IL-13, and IFN-γ alone (data not shown).

**ASMC from asthmatics express more CCR3 than control ASMC**

Another objective was to compare the surface expression of CCR3 on ASMC between asthmatic and control patients using flow cytometry. Cells were used soon after initial passaging (passages 2–3), following isolation from asthmatic and nonasthmatic patients. From both groups, we examined the percentage of cells expressing the CCR3 receptor, as well as the intensity of this expression, via a mean assessment of their signal fluorescence. Our observations concluded a higher percentage of CCR3 expression (95 ± 6% vs 75 ± 2%; p < 0.05) as well as a greater mean average fluorescence (21.5 ± 4.3 vs 14.3 ± 0.7; p < 0.01) for ASMC isolated from asthmatic subjects compared with cells isolated from nonasthmatics (Fig. 4).

**Addition of CCR3 ligands induces an increase in intracellular Ca²⁺ concentration**

To assess the functionality of CCR3, we stimulated B/TSMC with eotaxin and MCP-4. We observed a sharp increase in intracellular Ca²⁺ concentration within smooth muscle cells following the addition of both eotaxin and MCP-4 (Fig. 5). However, upon adding MCP-4 alone, we obtained a much weaker signal, presumably due to receptor desensitization. A similar desensitization phenomenon was observed when MCP-4 was used first to stimulate the cells, followed by addition of eotaxin, suggesting that the induction of intracellular Ca²⁺ concentration is through the activation of CCR3 (results not shown).

**Eotaxin induces migration of B/TSMC**

Characteristic of asthma is the increase in smooth muscle mass and the reduction in the distance between the smooth muscle layer and the epithelium, suggested to be associated with the migration of smooth muscle cells toward the smooth muscle layer. Consequently, we wanted to examine whether eotaxin could promote the migration of B/TSMC. As shown in Fig. 6, eotaxin increased the migration of B/TSMC in a dose-dependent manner with a maximal response at 100 ng/ml (2.7 ± 0.32-fold, compared with baseline). No significant differences between 100 ng/ml and 1000 ng/ml (2.06 ± 0.48-fold) were observed when these two doses were compared, suggesting that 100 ng/ml is the peak dose for inducing migration of B/TSMC. Platelet-derived growth factor (PDGF) was used as a positive control and increased cell migration by 2.5 ± 0.30-fold, compared with the medium. Migration of B/TSMC was totally abrogated (1.11 ± 0.07) when the cells were preincubated with blocking anti-CCR3. Collectively, these data demonstrate the

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**FIGURE 2.** Expression of CCR3 in ASMC in vivo. Cross-section of an intermediate airway from asthmatic (A) and control (B) subjects (representative of n = 4 for each group) showing eotaxin immunoreactivity and isotype control (C) in smooth muscle bundle (large arrows). Paraffin-embedded sections were prepared from human lung biopsies, and slides were incubated with anti-CCR3 polyclonal Ab, the appropriate secondary Ab, and a tertiary layer of streptavidin-HRP-conjugated. Sections were developed with diaminobenzidine tetrahydrochloride, with positive cells staining brown.
The capacity of eotaxin to induce the migration of B/TSMC toward an increasing gradient of eotaxin through the activation of CCR3. To address whether the obtained findings were the result of chemotaxis or chemokinesis, eotaxin was added to both upper and lower wells, and migration was examined after identical conditions of stimulation. As shown in Fig. 6, the absence of a concentration gradient did not stimulate the migration of B/TSMC, confirming that eotaxin acts as a chemoattractant for B/TSMC. To assess the viability of the cells, ASMC were incubated with the same concentrations of cytokines or anti-CCR3 for 24 h in six-well plates. No differences in cell viability were observed between the different conditions used.

Discussion
In the last few years, studies have shown that ASMC possess properties that would indicate a potential involvement in airway remodeling and inflammation. These properties include the expression of a variety of cytokines and chemokines as well as their receptors. The CC and CXC chemokines are important chemotactic molecules that control leukocyte trafficking and functions. These molecules also play an important role in regulation of leukocyte development, expression of adhesion molecules, cell proliferation, and angiogenesis. CCR3 is a CC chemokine receptor that has been traditionally associated with recruitment of inflammatory cells implicated in the pathophysiology of asthma (21, 22). At sites of allergic inflammation, increased expression of CCR3 and CCR3 ligands, such as eotaxin and RANTES, by inflammatory cells have been well characterized (14). However, recent work has demonstrated that the expression of chemokine receptors is not restricted to leukocytes. Stellato et al. (18) have shown that airway
epithelial cells could also express CCR3. In this study, we examined the expression of CCR3 by ASMC and showed that the receptor is expressed both in vivo and in vitro. Functional studies demonstrate that CCR3 is a functional receptor, as it transduces intracellular calcium mobilization and induces ASM migration. We have also demonstrated that CCR3 is up-regulated in bronchial smooth muscle cells of individuals with asthma, compared with normal control subjects.

Asthma is a disease characterized by marked structural changes of the airway wall. Benayoun et al. (23) and C. Pepe et al. (manuscript in preparation) have recently demonstrated that there is a decrease in the distance between the airway smooth muscle and epithelial layers of asthmatic individuals. The possible migration of ASM, either from the interstitial compartment or from a circulating precursor stem cell population, has been suggested as a possible mechanism to explain the increase in smooth muscle mass observed in the airways of asthmatics (8, 9). The mediators involved in the migration of ASM, however, must still be determined. We hypothesized that the increased levels of eotaxin in asthmatic airways could promote the chemotaxis of ASM through the activation of CCR3. In the present study, we demonstrate that eotaxin is able to induce the migration of ASM in a dose-dependent manner. Similar observations have recently been made concerning the migration of vascular smooth muscle cells in atherosclerosis (24). In vivo, both epithelial and airway smooth muscle cells are potent producers of eotaxin (25–27) and thus may be responsible for the generation of an eotaxin gradient, allowing migration of ASM toward the smooth muscle bundles and the epithelium. To support this hypothesis, C. Pepe et al. (manuscript in preparation) have recently shown that there is an increased production of eotaxin by ASM in severe asthmatics compared with control subjects. Furthermore, this increased production of eotaxin was seen to correlate significantly with the amount of smooth muscle found in the airways of asthmatic patients.

Recent studies strongly suggest that chemokine receptor expression in many cell types can be modulated by both inflammatory and anti-inflammatory signals (18, 22, 28, 29). Proinflammatory Th1 and Th2 cytokines have been shown to be potent mediators regulating the expression of CCR3 in lymphocytes, eosinophils, and neutrophils, whereas TNF-α was shown to augment the expression of CCR3 transcripts in epithelial cells. We examined the effects of IL-4, IL-13, IFN-γ, and TNF-α on the expression of CCR3 in ASMC. Using flow cytometry analysis, we found that the expression of CCR3 was increased 24 h after stimulation with TNF-α. Interestingly, IFN-γ, IL-4, and IL-13 acted to slightly down-regulate the cell surface expression of CCR3, suggesting that the Th2-biased immunological state observed in asthmatic individuals is not likely to play a role in the migration of ASM within airways. Rather, proinflammatory cytokines, such as TNF-α, are more likely to control the migration of ASM toward the epithelium. Vijh et al. (30) reported that the CCR3 promoter contains several transcription factor-binding sites for AP-1, a transcription factor implicated in the regulation of genes involved in the pathogenesis of asthma (31). Also, AP-1 is strongly activated by TNF-α (32) and may explain the induction of CCR3 expression by ASM. It is also possible that ASM might be able to migrate toward the smooth muscle layer as a result of interactions between eotaxin and CCR3.

We demonstrated in our study that a higher percentage of asthmatic ASM express CCR3 compared with nonasthmatic patients. This increased expression by asthmatic ASM might be an inducible phenomenon related to the augmented expression of TNF-α in the airways of asthmatics (33, 34). The increased expression of CCR3 on the cell surface attributable to an increased expression of TNF-α might render ASM more responsive to the greater eotaxin levels observed in asthmatic airways (35). These observations suggest that the cytokine environment in asthma could contribute to increased CCR3 production by ASM and their increased responsiveness to eotaxin. A different phenotype linked to a genetic duality between asthmatics and nonasthmatics could also explain this increased expression of CCR3 by ASM.

We have also demonstrated that the addition of various CCR3 ligands, such as eotaxin and MCP-4, to cultured ASM induced the release of intracellular calcium, suggesting efficient signaling through CCR3, as shown in epithelial cells (18). Eotaxin alone was able to trigger an increase in intracellular calcium, supporting the requirement of a G protein-coupled receptor (GPCR) for this effect (36). However, because RANTES, MCP-3, and MCP-4 are able to activate other GPCR potentially expressed by ASM, we chose to focus our attention on eotaxin, which solely binds to CCR3. A decrease in the effect of a second ligand stimulus was also observed independent of whether the same chemokine was used in both instances. This phenomenon, known as desensitization, is a well-documented feature of the GPCR response and is an indication of receptor specificity because only ligands that interact with the same GPCR can desensitize its response (36). Further studies are under way to establish the implication of other intracellular pathways possibly involved in the activation of ASM.

The signaling pathways that mediate chemokine-induced trafficking are not well understood. In lymphocytes, activation of Gαi and release of Gβγ subunits have been shown to be crucial for induction of chemotaxis in response to a chemokine (37, 38), whereas, in tracheal smooth muscle, p38MAPK/HSP27 seems to be involved in initiating migration in response to cytokines and growth factor such as TNF-α, IL-1β, and PDGF (39). In eosinophils, multiple signaling pathways activated by CCR3 participate in the inflammatory response and the initiation of migration. Eotaxin stimulates intracellular calcium release, production of reactive oxygen species, and changes in actin polymerization through a pertussis-sensitive pathway. Rho and Rho-associated coiled-coil-forming protein kinase, a protein kinase activated by Rho, regulate actin stress fiber formation and are required for eosinophil chemotaxis (40). MAPK pathways are also involved in

![FIGURE 6. Cellular migration of B/TSMC in response to eotaxin.](http://www.jimmunol.org/)}
chemokinesis (41). However, whether one of these intracellular pathways is involved in the induction of ASM migration toward a gradient of eotaxin will need to be investigated.

In conclusion, we have demonstrated for the first time the expression of functional CCR3 by ASM. Our results suggest that the activation of CCR3 by eotaxin could participate in the increased smooth muscle mass observed in the airways of asthmatic subjects, inducing the migration of ASM toward the smooth muscle layer and contributing to the airway hyperresponsiveness characterizing an episode of asthma. Further work will be required to demonstrate the migration of ASM in an in vivo model of asthma, as well as to determine the intracellular pathways involved in the activation of CCR3 in ASM.

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Disclosures

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