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Expression and Regulation of CCR1 by Airway Smooth Muscle Cells in Asthma[1]

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C-C chemokines such as CCL11, CCL5, and CCL3 are central mediators in the pathogenesis of asthma. They are mainly associated with the recruitment and the activation of specific inflammatory cells, such as eosinophils, lymphocytes, and neutrophils. It has recently been shown that they can also activate structural cells, such as airway smooth muscle and epithelial cells. The aims of this study were to examine the expression of the CCL3 receptor, CCR1, on human airway smooth muscle cells (ASMC) and to document the regulation of this receptor by cytokines involved in asthma pathogenesis. We first demonstrated that CCR1 mRNA is increased in the airways of asthmatic vs control subjects and showed for the first time that ASMC express CCR1 mRNA and protein, both in vitro and in vivo. Calcium mobilization by CCR1 ligands confirmed its functionality on ASMC. Stimulation of ASMC with TNF-α and, to a lesser extent, IFN-γ resulted in an up-regulation of CCR1 expression, which was totally suppressed by both dexamethasone or mithramycin. Taken together, our data suggest that CCR1 might be involved in the pathogenesis of asthma, through the activation of ASMC by its ligands. *The Journal of Immunology,* **2008, 180: 1268–1275.**

Asthma is an inflammatory condition of the airways characterized by bronchial hyperresponsiveness, infiltration of inflammatory cells, and airway remodeling (1–3). Increased airway smooth muscle mass is an important feature of airway remodeling and has been linked in different ways to asthma pathogenesis (4, 5). In the last decade, several studies have shown that airway smooth muscle cells (ASMC)[3] may contribute to airway inflammation through the release of cytokines and chemokines such as TNF-α, IL-1β, CCL11 (eotaxin), and CCL5 (RANTES) (6). ASMC were also shown to respond to a wide variety of immune mediators and to express receptors for several noninflammatory cytokines (7). More recently, expression of CCR3, a receptor for a number of C-C chemokines has been described in ASMC (8, 9). However, little is known regarding the expression of other relevant C-C chemokine receptors by ASMC.

The C-C chemokine subfamily is composed of 28 members and includes CCL3. This chemokine binds to CCR1 and CCR5 and has been shown to be increased in bronchoalveolar lavage and bronchial biopsies of asthmatic patients (10, 11). CCL3 is principally involved in the recruitment of eosinophils, basophils, and mast cells (12, 13). Interest in the role of CCR1, which is one of the CCL3 receptors, in allergy comes from its involvement in the development of the airway remodeling (14, 15).

The aims of this study were to examine the expression and the regulation of CCR1 on ASMC and to assess its expression in asthma. Our findings show that ASMC express CCR1 and that the receptor expression is increased by TNF-α and IFN-γ whereas IL-4 and IL-13 have no effect. Binding of either CCL3 or CCL23 to CCR1 induces the release of intracellular calcium, demonstrating the functionality of the receptor. We have also shown that asthmatic patients express higher levels of CCR1, compared with controls, suggesting a potential function of this receptor in the pathogenesis of asthma.

Materials and Methods

Cell culture

Primary human ASMC were obtained from main bronchial airway segments (0.5–1.0 cm diameter) in pathologically uninvolved segments of resected lung specimens using isolation methods described previously (16, 17). Cells were then seeded at a density of 10^5 cells/cm^2 and grown at 37°C in a humidified incubator with 5% CO_2 in Smooth Muscle Growth Medium (SmGm-2; Clonetics). At confluence, primary human ASMC exhibited spindle morphology and a hill-and-valley pattern characteristic of smooth muscle in culture. In cultures up to passage 5, >90% of the cells at confluence retained smooth muscle-specific α-actin, SM22, and calponin protein expression, and were able to mobilize intracellular Ca^{2+} in response to acetylcholine. Growth rate (determined by cell number) of ASMC from all lung resection donors was similar to that reported previously for ASMC cultures from healthy human transplant donors. Morphologically, the ASMC from lung resection donors and from healthy human transplant donors were indistinguishable. Cell viability was always >95% as assessed by trypan blue dye exclusion.

Cell stimulation

ASMC were growth-arrested by FBS deprivation for 24 h before stimulation with cytokines. After starving, cells were stimulated with fresh, serum-free medium, containing IL-4, IL-13, TNF-α, or IFN-γ (R&D Systems) in a concentration and time-dependent manner. In inhibition experiments,

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[3] Abbreviations used in this paper: ASMC, airway smooth muscle cell; RT, room temperature; Sp1, stimulatory-protein-1; y.o., years old.

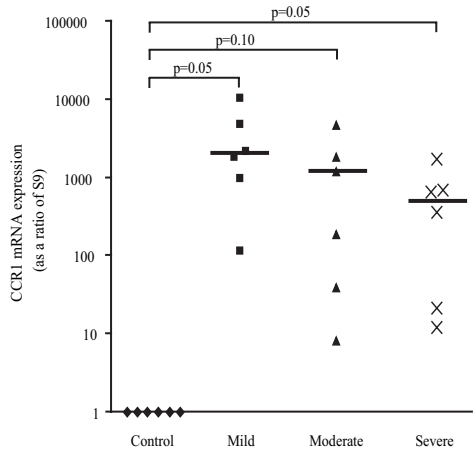

FIGURE 1. CCR1 mRNA expression in asthma. Quantitative RT-PCR analysis of mRNA extracted from airway biopsies obtained from mild (■), moderate (▲), severe (**X**), and control patients (♦).

mithramycin or dexamethasone (Sigma-Aldrich) were added in a dose-dependent manner 1 h before stimulation of the cells with cytokines.

RNA extraction

Total cellular RNA was isolated from human ASMC, epithelial cells (A549), fibroblasts (American Type Culture Collection) purified peripheral blood eosinophils, and endothelial cells (HUVEC; ATCC). RNA was extracted using the RNeasy mini kit extraction columns (Qiagen) as directed by the manufacturer. RNA was eluted in 35 μl nuclease-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 RNAguard (Amersham Pharmacia Biotech).

RNA extraction from human airway biopsies

The biopsies were obtained from the Tissue Bank (MCI/Meakins-Christie Tissue Bank, McGill University, Montréal, Québec). A clinical diagnosis of asthma was made based on the evaluation of the patient's medical file and by a respiratory physician. Individuals in moderate and severe asthma groups were all taking inhaled corticosteroids, while people in the mild group were using B_2-agonist. Age and predicted forced expiratory volume in the first second for each group were as followed: control, 42.3 years old (y.o.) 105 ± 23%; mild, 40.8 y.o., 95.5 ± 20%; moderate, 44.8 y.o., 86 ±9%; severe, 50 y.o., 56 ± 20%. Tissue RNA was extracted using the RNeasy micro kit extraction columns (Qiagen) as directed by manufacturer. RNA was eluted in 12 μl nuclease-free water, and cDNA was generated as described above, using 9 μl of extracted RNA.

RNase protection assay

Total RNA was extracted as previously described. Twenty micrograms of total RNA from cell cultures were used. Riboprobes were synthesized using T7 RNA polymerase and [^{32}P] cytidine 5′-triphosphate (Amersham Biosciences), from human multiprobe set (Riboquant; BD Biosciences) containing template for CCR1 receptor. [^{32}P]-labeled riboprobes were hybridized with RNA samples overnight at 56°C and processed using the manufacturer's protocol. Protected RNA fragments were separated using a 5% acrylamide gel and analyzed by autoradiography (Kodak).

PCR and preparation of standards

Quantification of the housekeeping gene ribosomal protein S9 and CCR1 was achieved by constructing a standard curve from serial dilutions of a known amount of gel-purified cDNA. This latter consisted of the quantified amplicon extracted from a gel. To do so, studied genes were first amplified using conventional PCR. The PCR mixture consisted of 1.5 mM $MgCl_2$, 1× PCR buffer, 0.25 mM dNTP mixture, 2.5 units Platinum Taq polymerase (Invitrogen Life Technology), 0.4 μM each of the sense and antisense primers, as well as 1 μl of cDNA. Primers for S9 and CCR1 were generated by Invitrogen Life Technologies using the following sequences: S9 sense 5′-TGCTGACGCTTGATGAGAAG-3; antisense 5′-CGCAG AGAGAAGTCGATGTG-3′; CCR1 sense 5′-GACAAAGTCCCTTGG AACCA-3′; antisense 5′-ACCAGGATGTTTCCAACCAG. Sequences of the primers were designed in two different exons with a big intronic sequence between the exons, to avoid any possible amplification of genomic contamination. The samples were amplified in a thermal cycler (PTC-100, Programmable Thermal Controller, MJ Research) for 40 cycles consisting of denaturation at 95°C, annealing at 57°C, and extension at 72°C. The PCR products were visualized on a 1% agarose gel containing 0.2 μg/ml ethidium bromide. The correct band size was determined by comparison with a 100 bp DNA ladder (Invitrogen Life Technologies). Amplicons

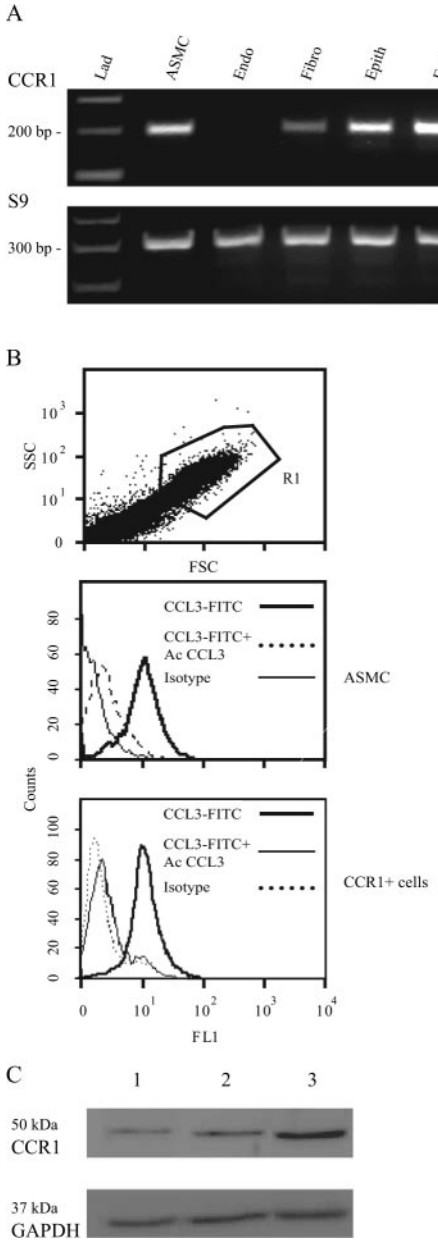

FIGURE 2. Detection of CCR1 expression in ASMC. *A*, RT-PCR analysis of constitutive mRNA CCR1 expression by structural cells. Endothelial cells (Endo), epithelial cells (Epith), fibroblasts (Fibro), and ASMC were examined while eosinophils were used as positive control (representative of $n = 3$). *B*, Determination of CCR1 surface expression by ASMC using flow cytometry. ASMC were cultured and analyzed by flow cytometry for cell surface expression of CCR1. Biotinylated human recombinant CCL3 was added to confluent cultured ASMC (P3-7) in the absence or presence of anti-human CCL3 blocking Ab (representative of $n = 3$). CCR1 protein production was also confirmed by Western blot. Fig. 2*C* shows CCR1 expression in protein extracts obtained from CCR1-transfected cells (line 1), ASMC stimulated with 100 ng/ml IFN-γ for 48 h (line 2) and ASMC stimulated for 48 h with a combination of 10 ng/ml TNF-α and 100 ng/ml IFN-γ (line 3). GAPDH was used as internal control. Western blots are representative of two independent experiments.

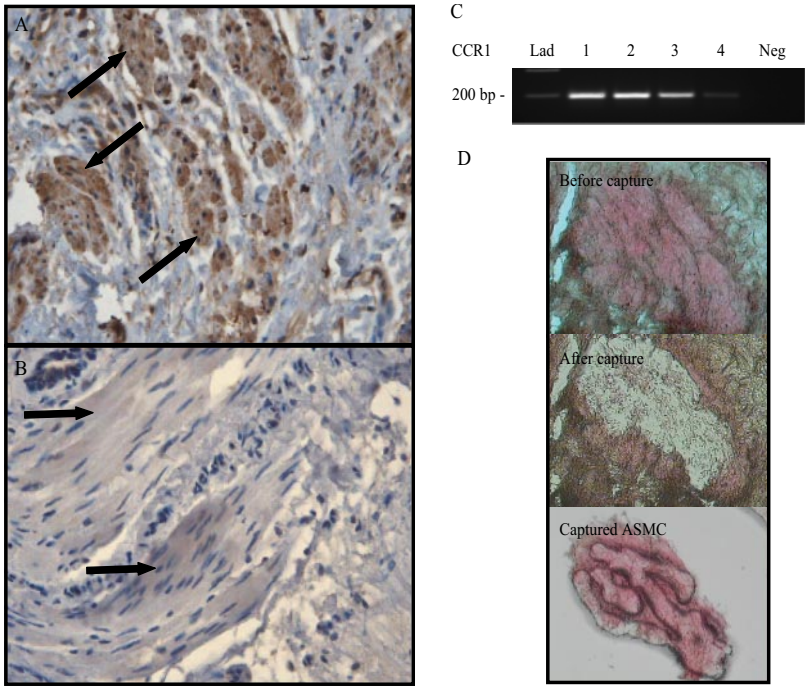

FIGURE 3. Expression of CCR1 in ASMC in vivo. Cross-section of an intermediate airway from asthmatic (*A*) subjects showing CCR1 immunoreacitivity and isotype control (*B*) in smooth muscle bundle (*large arrows*) (representative of $n = 3$). Paraffin-embedded sections were prepared from human lung biopsies, and slides were incubated with anti-CCR1 mAb, the appropriate secondary Ab, and a tertiary layer of streptavidin-HRP-conjugated. Sections were developed with diaminobenzidine tetrahydrochloride, with positive cells staining brown. *C* and *D*, CCR1 mRNA detection of microdissected ASMC from human airway biopsies, using RT-PCR. ASMC were captured using lasercapture microdissection from airway biopsies (D) obtained from four patients (1, 2, 3, and 4 in the figure) with severe asthma.

were purified using the QIAquick PCR Purification Kit (Qiagen) and 10-fold series were prepared in Tris-HCl (pH 8.0). Sequential dilutions ranged from 10^{-1} to 10^{-10} ng/μl.

Quantitative real-time PCR

Quantification of CCR1 and S9 mRNA expression by ASMC was done by quantitative PCR using the LightCycler (Roche Diagnostics) following reverse transcription, as previously described. The same primers as the ones described for preparation of standards were used. PCR were performed in a volume of 20 μl containing 1 μl of cDNA, 0.3 μM of each primer, 10 μl of QuantiTect SYBR Green PCR Master Mix (Qiagen) containing HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, dNTPs, and SYBR Green I. The PCR protocol consisted of three programs: denaturation, amplification, and melting curve analysis for product identification. The denaturation and amplification conditions for both S9 and CCR1 were 95°C for 15 min followed by 45 cycles of PCR. Each cycle included denaturation at 95°C for 10 s, annealing of 30 s at 60°C and extension of 20 s at 72°C. The temperature transition rate was 20°C/s, except when heating at 72°C, when it was at 5°C/s. Fluorescence was measured at the end of every cycle to allow quantification of cDNA. To eliminate the formation of primer dimmers, a melting curve was obtained, after a normal cycle, by slowly increasing temperature of the samples to 95°C with fluorescence detection every 0.2°C following normal cycle.

Western blot

Cells were lysed in lysis buffer (150 mM NaCl; 10 mM Tris-HCl, (pH 7.4); 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 0.5% Nonidet P-40; 100 mM sodium fluoride; 10 mM sodium pyrophosphate; 2 mM sodium orthovanadate) containing a minicomplete protease inhibitor mixture tablet for 30 min on ice. Extracts were clarified at 14,000 $\times$ *g* at 4°C for 20 min, and protein concentration was determined using the Bradford assay. Samples were resolved by SDS-PAGE on 12% (weight-to-volume ratio) polyacylamide gels and transferred on nitrocellulose membranes. CCR1 was determined using rabbit anti-CCR1 polyclonal Ab (4 μg/ml; Abcam). Signals were detected using a goat anti-rabbit peroxidase-conjugated Ab (1/10000; Jackson ImmunoResearch Laboratories) and Supersignal Chemiluminescent (Pierce) as substrate. The levels of the housekeeping protein GAPDH (rabbit anti-GAPDH Abs, 1/500; Abcam) were used as a reference for protein amounts loaded on the gels. Western blots were analyzed using the MultiGenius Bio Imaging System (Syngen).

Immunohistochemistry

To determine whether ASMC have the capacity to express CCR1 protein in vivo, immunohistochemistry was performed on sections of major airways from asthmatics subjects. The biopsies were obtained from the Tissue Bank (MCI/Meakins-Christie Tissue Bank, McGill University). A clinical diagnosis of asthma was made based of the evaluation of the patient's medical file by a respiratory physician. Diagnostic criteria included prior diagnosis and treatment for asthma, documented evidence of variable airflow obstruction greater than 15%, and bronchial hyperresponsiveness. Following resection of the biopsies, lung specimens were prepared for immunohistochemistry. In brief, formalin-fixed tissues were paraffin-embedded and 5-μm-thick sections were prepared, sections were deparaffinized 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 blocking solution (Dako Corporation) for 20 min at room temperature (RT). Primary Ab against CCR1 diluted in diluting buffer (Dako Corporation) was applied (25 μg/ml, MAB145, clone 53504.111; R&D Systems) and sections were incubated overnight at 4°C. Control sections were incubated with isotype control (25 μg/ml, MAB004 clone 20116; R&D Systems). After rinsing in PBS, a biotinylated rabbit anti-mouse Ab (1/100 dilution; Dako Corporation) was applied, and sections were incubated for 60 min at RT. Sections were thoroughly washed in PBS and incubated with the streptavidin-HRP conjugated for 60 min at RT. After PBS washes, 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.

Flow cytometric analysis

FACS analysis was performed as follows: ASMC were detached from the flask by addition of PBS containing EDTA (0.5 M) for 20 min at 37°C. Cells were resuspended at a concentration of 1×10^6 cells/ml and washed once with PBS. ASMC were labeled with a Fluorokine kit for human CCR1 (Fluorokine; R&D Systems) according to the manufacturer's instructions. In brief, 10 μl of biotinylated recombinant CCL3 was added to 25 μl aliquots of washed cells (10^5) 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, 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 CCL3 blocking Ab with 10 μl of biotinylated CCL3 and incubated for 15 min at RT. CCR1 expression was analyzed using fluorochrome-labeled CCL3 (Fluorokine; R&D Systems), and analyzed via flow cytometry (BD FACSCalibur System; BD Biosciences). At least 10000 cells were counted per analysis. As

a positive control, the same protocol was applied to CCR1-transfected Ghost-cells (National Institute of Health, AIDS reagent program, no. 3682).

Lasercapture microdissection of ASMC

To assess the capacity of ASMC in vivo to express CCR1 mRNA, laser-capture microdissection was performed on asthmatic biopsies. The biopsies were obtained from the Tissue Bank (MCI/Meakins-Christie Tissue Bank, McGill University). They were cut into 5-μm sections on a cryostat and placed on charged slides before fixation in 70% ethanol. The slides were stained with H&E, rinsed in an ethanol gradient, and dehydrated in a mixture of xylenes. The smooth muscle cells bundles were carefully captured using the Pixcell laser capture microscope (Arcturus). During this process, cellular material was transferred to CapSure HS LCM Caps (Arcturus Bioscience) and digested in RLT lysis buffer (Qiagen). RNA was extracted using the RNeasy micro kit (Qiagen) following the manufacturer's instruction. mRNA was eluted in 12 μl of water. Reverse transcription was performed as described above. Because of the low amount of cDNA present in the samples, two series of amplification were performed on the sample. The first one consisted of 30 cycles of the PCR program described above. One microliter of the first amplification was then reamplified in a similar PCR for another 45 cycles. The PCR products were visualized on a 1% agarose gel containing 0.2 μg/ml ethidium bromide.

Measurement of intracellular free Ca^{2+}

For the measurement of calcium, cells were loaded with the Ca^{2+}-sensitive dye, fura 2-AM (Molecular Probes) according to the previously described methods (18) and imaged using an intensified charge-coupled device camera (IC200) and PTI software at a single emission wavelength (510 nm) with a double excitatory wavelength (340 and 380 nm). Fluorescence ratio (340/380) was measured in cells stimulated with CCL3 or CCL23 (1, 10, and 100 ng/ml) or appropriate vehicle. Histamine was used as a positive control (10^{-6} M). Intracellular calcium concentration ($[Ca^{2+}]_i$) was calculated according to the formula of Grynkiewicz et al. (19). Each experimental group consisted of 102–115 cells. Studies were performed using three cell lines, each acquired from a different donor.

Statistical analysis

Statistical significance was determined using a Student's t test. p values <0.05 were considered statistically significant.

Results

Biopsies obtained from airways of mild, moderate, and severe asthmatics express higher levels of CCR1 compared with controls

One of CCR1's ligands, CCL3, has been shown to be up-regulated in asthma (20, 21), though expression of this receptor in asthmatic airways has never been documented. We used quantitative real-time PCR to compare the levels of CCR1 mRNA in biopsies obtained from the airways of control, mild, moderate, and severe asthmatics. Fig. 1 shows relative expression of CCR1 mRNA (expressed as function of S9) in the four groups. Mild and severe asthma groups showed a significantly increased expression ($3.4 \times 10^3 \pm 1.7 \times 10^3$ and $5.7 \times 10^2 \pm 2.8 \times 10^2$ for mild and severe asthma groups respectively), while no CCR1 mRNA was detected in the control. Although no significant difference was seen between moderate and control groups, a general trend toward an increased expression of CCR1 mRNA was observed ($p = 0.10$). No significant differences were observed between the asthmatic groups. The housekeeping gene S9 was detected in all the groups, confirming the presence of mRNA in all the samples processed for PCR analysis.

ASMC constitutively express CCR1

To demonstrate the expression of CCR1 by ASMC in vitro at both mRNA and protein levels, RT-PCR, Western blot, and flow cytometry analyses were performed. PCR analyses were conducted on cultured ASMC and different populations of structural cells to assess the expression of CCR1 mRNA, peripheral blood eosinophils were used as a positive control. Gel electrophoresis (Fig. 2*A*) revealed bands corresponding to the expected size of the CCR1 amplicon (197 bp). Surface expression of CCR1 by ASMC was confirmed using flow cytometry, and revealed a high percentage of unstarved serum cells expressing the receptor (Fig. 2*B*, *upper and middle panels*). This signal was completely suppressed when

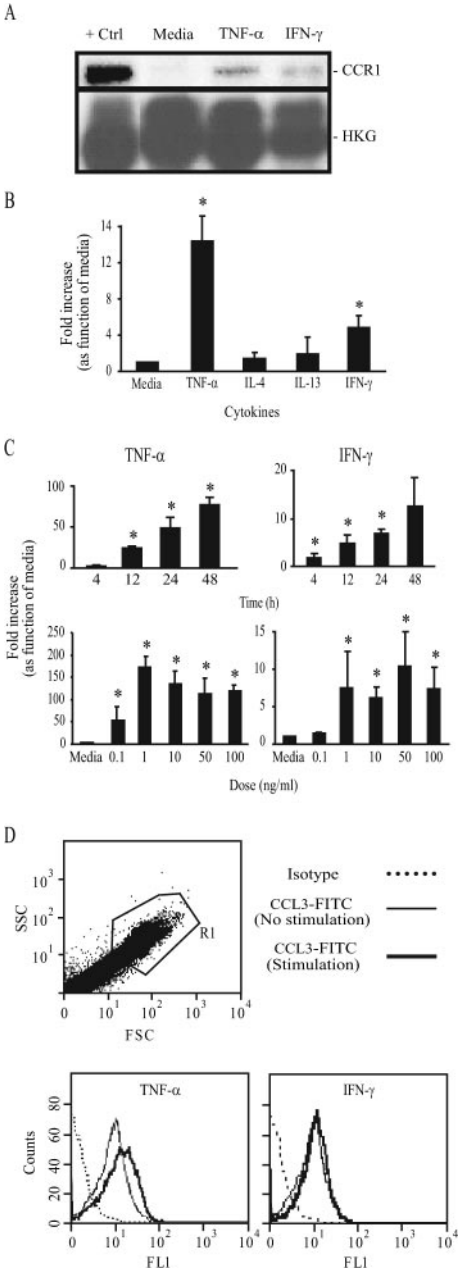

FIGURE 4. Effect of cytokines on CCR1 mRNA and protein expression by ASMC. Regulation of CCR1 mRNA using RNase Protection Assay (RPA) (*A*) and *B*, quantitative PCR using TNF-α (10 ng/ml), IFN-γ (50 ng/ml), IL-4 (50 ng/ml), and IL-13 (50 ng/ml) for 12 h. *, $p < 0.05$ ($n = 4$). *C*, CCR1 mRNA expression using increasing doses of TNF-α and IFN-γ at different time points, using quantitative PCR. Results are expressed as a ratio of housekeeping gene S9. *, $p < 0,05$ ($n = 3$). *D*, Expression of CCR1 was qualitatively evaluated by flow cytometry using a biotinylated human recombinant CCL3. As a negative control, cells were incubated with a biotinylated soybean trypsin inhibitor (supplied by the manufacturer). Cells were stimulated with TNF-α or IFN-γ for 24 h. Viable cells were gated (R1) and analyzed (representative of $n = 3$).

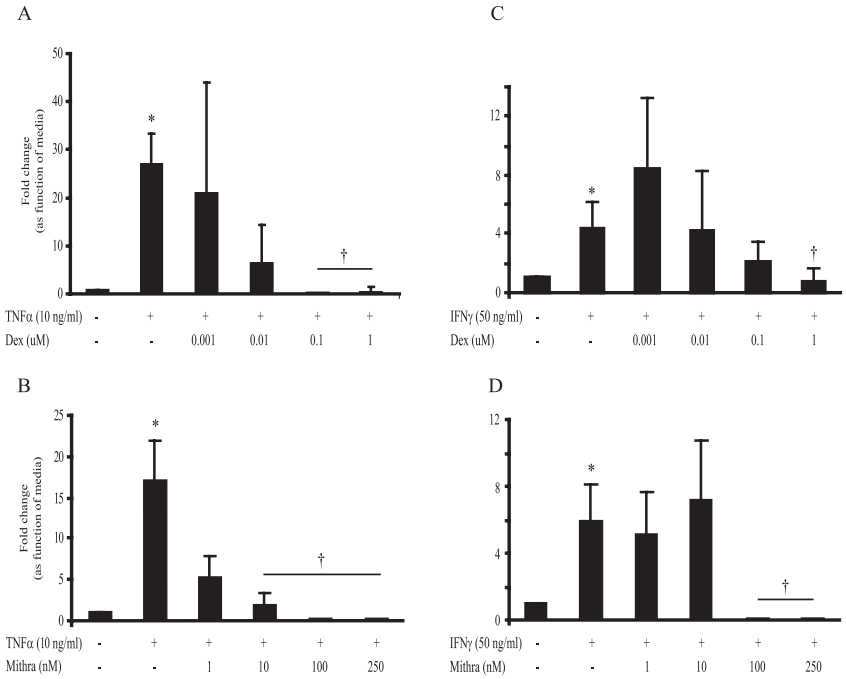

FIGURE 5. Effects of mithramycin and dexamethasone on TNF-α (*A* and *B*) and IFN-γ-induced (*C* and *D*) CCR1 mRNA up-regulation. Confluent ASMC (passages 3-7) cultured in serum-free medium were stimulated with TNF-α (10 ng/ml) or IFN-γ (50 ng/ml) for 24 h. Increasing doses of mithramycin (*B* and *D*) or dexamethasone (*A* and *C*) were added 1 h prior to stimulation with cytokines. mRNA expression was evaluated using quantitative PCR. Results are expressed as a ratio of housekeeping gene S9 expression. *, Different from medium, $p < 0.05$ ($n = 3$); †, different from TNF-α (10 ng/ml) or IFN-γ (50 ng/ml) alone, $p < 0.05$ ($n = 3$).

anti-CCL3 was simultaneously added (Fig. 2*B*, *middle panel*), confirming the specificity of the signal. As positive control, CCR1-transfected cells ($CCR1^+$ cells) were analyzed (Fig. 2*B*, *lower panel*). Protein production was also confirmed using Western blot. Fig. 2*C* shows an increase in CCR1 protein expression when cells were treated with a combination of TNF-α and IFN-γ, as suggested by the results obtained with PCR.

CCR1 is expressed by ASMC in vivo

Using immunocytochemistry, CCR1 immunoreactivity was detected in smooth muscle bundles from bronchial biopsies obtained from subjects with asthma. CCR1 protein was mainly localized in the smooth muscle bundles (Fig. 3*A*), airway epithelium, and some inflammatory cells (data not shown). Expression of CCR1 by ASMC in vivo was confirmed using lasercapture microdissection. Indeed, RT-PCR analysis of mRNA obtained from ASMC microdissected from four severe asthmatic biopsies revealed the presence of the receptor, as presented in Fig. 3*C*, confirming the results obtained in vitro.

TNF-α and IFN-γ up-regulate the expression of CCR1 by ASMC

Expression of chemokine receptors has been shown to be regulated by different inflammatory mediators (22). In asthma, the cytokine environment is characterized by increased TNF-α, IL-4, and IL-13 levels (23). However, there are conflicting results regarding the amount of IFN-γ found in asthmatic airways (24–26). We therefore investigated the effects of IL-4, IL-13, IFN-γ, and TNF-α on ASMC mRNA and surface expression of the CCR1 receptor. Addition of IL-4 and IL-13 did not modulate the expression of CCR1 (1.4 ± 0.6 and 2.2 ± 0.7-fold, respectively), while TNF-α and IFN-γ significantly up-regulated the expression of CCR1 at mRNA level (11.0 ± 4.8 and 4.8 ± 1.4-fold; Fig. 4*B*) and also increase surface expression levels (Fig. 4*D*) after 12 (mRNA) and 24 (surface expression) h of stimulation. The combination of TNF-α with either IL-4, IL-13, or IFN-γ did not potentiate the surface expression of CCR1, as seen with TNF-α or IFN-γ alone (data not shown). Addition of TNF-α or IFN-γ both resulted in a dose and time-dependent augmentation of CCR1 mRNA expression with a plateau dose of 1 ng/ml for TNF-α and 10 to 50 ng/ml for IFN-γ and a maximal expression after 48 h of stimulation (Fig. 4*C*).

CCR1 mRNA up-regulation by TNF-α and IFN-α is sensitive to dexamethsasone and mithramycin

Corticosteroids are widely used for the treatment of asthma, but their exact mechanisms of action remains unclear. However, they have been shown to inhibit signal transduction of proinflammatory cytokines through the inhibition of the NF-κB pathway. We pretreated ASMC with dexamethasone and stimulated with TNF-α and IFN-γ and observed a significant inhibition of CCR1 mRNA expression at low doses of dexamethasone (Fig. 5). The inhibition by dexamethasone was dose-dependent with significant blockade at a concentration of 1 μM (Fig. 4).

The human CCR1 promoter contains several binding sites for stimulatory-protein-1 (Sp1) (27). For this reason, we looked at the potential involvement of this transcription factor in the induction of CCR1 mRNA expression by TNF-α and IFN-γ. We used mithramycin, a potent inhibitor of Sp1 binding, and found that it completely inhibits TNF-α and IFN-γ-induced CCR1 mRNA at concentrations of 100–250 nM (Fig. 5*B*).

Addition of CCR1 ligands induces an increase in intracellular [Ca^{2+}]

To assess the functionality of CCR1, we stimulated ASMC with increasing doses of CCL3 and CCL23. Both triggered calcium responses, indicating that the CCR1 is functional. The number of cells responding and the magnitude of the response were concentration-dependent, with 100 ng/ml CCL23 causing the most pronounced response (Fig. 6). However, even in this group, not all cells were responsive, as suggested by the results obtained with flow cytometry where just a percentage of cells express the receptor. As expected, histamine induced a higher intracellular calcium mobilization when compared with CCL3 and CCL23. Similar results have been reported in ASMC and epithelial cells (8, 28).

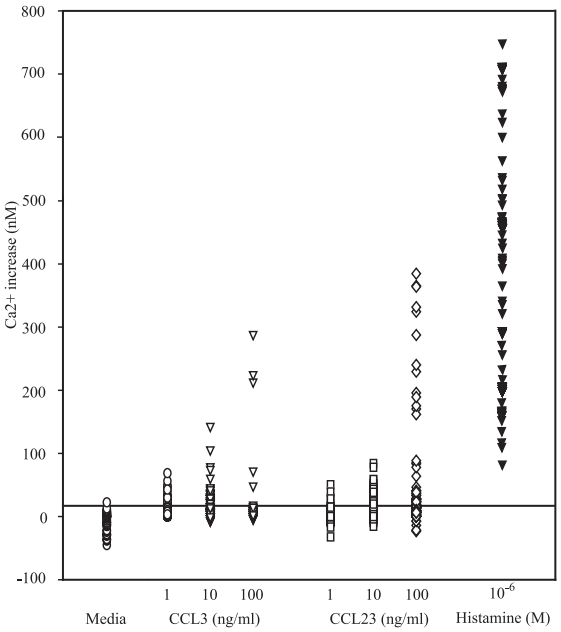

FIGURE 6. Measurement of Ca^{2+} in cultured ASMC in response to three different concentrations of CCL3 or CCL23. For calcium measurements, confluent serum-fed ASMC were loaded with Fura-2 as described in *Materials and Methods*. Cells were stimulated with either CCL3 or CCL23 and intracellular Ca^{2+} was measured for at least 300 s thereafter. Figure shows difference between peak response and baseline level calcium. Histamine (10^{-6}M) was used as a positive control. Data shown are representative of three experiments.

Discussion

In the last few years, the role of ASMC in the pathogenesis of asthma has considerably evolved. A large body of literature has clearly shown that functions of ASMC extend beyond their contractile properties. They can contribute to the airway inflammation through the release of inflammatory mediators, including cytokines and chemokines. They also express a wide variety of receptors, which make them potential targets for the inflammatory mediators involved in the pathogenesis of asthma. Several C-C chemokines have been shown to be up-regulated in the airways of asthmatic patients (29, 30). They are mainly associated with the recruitment of inflammatory cells toward the site of inflammation, although studies have also shown that chemokines can promote angiogenesis and proliferation.

Expression of CCR1 has been primarily described in leukocytes such as macrophages, eosinophils, basophils, and dendritic cells (13), However few studies have demonstrated the expression of CCR1 by cells other than leukocytes, such as osteoclasts and platelets (11, 31). In the present study, we showed for the first time that ASMC express CCR1 both in vitro and in vivo. We also demonstrated that asthmatic airways contain higher levels of CCR1 mRNA, compared with normal airways, which could be of significant importance in the pathogenesis of asthma. Functional studies revealed that the CCR1 is fully operational on ASMC, and its activation by CCL3 or CCL23 induces the mobilization of intracellular calcium. Taken together, our data suggests a potential role for CCR1 on ASMC, in the context of asthma.

Asthma is a disease typically characterized by an increase in Th2 vs Th1 cytokine ratio. Th2 cytokines include IL-4, IL-5, and IL-13 while IFN-γ is the prototypical Th1 cytokine. Both Th1 and Th2 seem to have the potential to modulate the expression of CCR1, depending on the type of cells involved (13, 32, 33). Effects of IFN-γ on CCR1 expression has been reported in monocytes and neutrophils, however, little is known concerning the effects of TNF-α on cell populations expressing CCR1(13). In the present study, we found that both TNF-α and IFN-γ increase CCR1 mRNA and protein while IL-4 and IL-13 had no effect. It is not surprising that TNF-α induces a strong up-regulation of the receptor, and such an effect has been reported with several chemokine receptors, including CCR3 and CCR5 (8, 34). CCR1 is known to be involved in host defense where high levels of TNF-α are usually found. TNF-α has also been shown to be up-regulated in various inflammatory conditions, including asthma (35–37). There is no consensus as to whether or not IFN-γ is diminished in asthmatic airways. Although recent studies have shown that IFN-γ-positive T cells are increased in asthmatic blood and airways (24–26). Therefore, the effect of IFN-γ on CCR1 expression is not necessarily in contradiction to the concept of asthma pathogenesis.

In the present work, we showed that asthmatics express higher level of CCR1 mRNA, which could be related to the higher levels of both TNF-α and IFN-γ asthmatic airways. However, we have also shown that the corticosteroid dexamethasone strongly inhibits the expression of CCR1 mRNA by ASMC that were stimulated with TNF-α. Because severe and moderate asthmatic patients that were used in our study were all treated with corticosteroids, it is possible that the level of mRNA in these patients is not reflecting the real amount of CCR1 in asthma. It is interesting to notice that mild asthmatics without corticosteroid treatment showed the highest level of CCR1 mRNA, suggesting that corticosteroids could down-regulate the expression of CCR1 in asthma. Surprisingly, CCR1 mRNA was undetected in the control group. Because we have been able to detect CCR1 mRNA expression in normal cultured ASMC, it is likely that the amount of CCR1 mRNA present in the RNA extracted from the control airway biopsies is below our detection level.

Using an online promoter analysis program (Consite; http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/) and the published sequence of the CCR1 promoter (27), we identified several Sp1 binding sites. The Sp1 transcription factor binds to guanine-cytosine -rich sequences and is necessary for the activation of several genes, including cytokines (38–40). In our study, we used mithramycin, a DNA-binding antibiotic that binds germinal center-rich regions, to evaluate the contribution of Sp1 to TNF-α and IFN-γ-induced CCR1 up-regulation. We showed that moderate dose of mythramycin (10 nM for TNF-α and 100 nM for IFN-γ) can totally abrogate CCR1 mRNA production, suggesting a preponderant role for Sp1 in activation of the CCR1 transcription in ASMC. Moreover, dexamethasone, which is known to inhibit NF-κB (41), also totally inhibited both TNF-α and IFN-γ-induced CCR1 mRNA expression, suggesting an involvement of this pathway in CCR1 expression. It is noteworthy to mention that a collaboration between Sp1 and NF-κB pathways is required for the induction of CXCL2 in a macrophage cell line (39), raising the possibility that a similar phenomenon occurs in the induction of CCR1 mRNA expression in ASMC. This might explain the complete inhibition obtained with either mithramycin or dexamethasone. It is also possible that the inhibition of CCR1 mRNA expression by dexamethasone occurs through the blockade of Sp1, since this effect of the drug has been described in the expression of CD14 by macrophages, when stimulated with LPS (42). Overall, this suggests that limiting the inflammatory response in the airways would have a strong impact on reducing the expression of CCR1 on structural cells.

Chemokine receptors with defective signaling function have been reported (43). In particular, exposure of dendritic cells to IL-10 and LPS has been shown to suppress the intracellular signal mediated by CCR1(44). In the present study, we have shown that

the addition of CCR1 ligands induces intracellular mobilization of calcium, supporting the requirement of a GPCR (G-protein coupled receptor) for this effect (45). As expected, not all cells responded to CCR1 ligands, presumably because they were not expressing the receptor, as suggested by our flow cytometry results (Fig. 2), or possibly because the receptors expressed at the surface of these cells were not functional or too low in number to induce the intracellular mobilization of calcium.

Functions of chemokines in human diseases have been mainly associated with recruitment and activation of inflammatory cells. Chemokines have also been involved in angiogenesis, Th1/Th2 development, and the release of cytokines (46, 47). Two CCR1 ligands, CCL3 and CCL23, have been associated respectively with recruitment of monocytes and T cells (12), specific inhibition of myeloid progenitor cells, and activation of monocytes and eosinophils (48). Interestingly, in a $CCR1^{-/-}$ model of chronic allergic asthma, the features of airway remodeling were greatly reduced in CCR1-deficient animals, suggesting an association between CCR1 and the development of airway remodeling (14). These results were also strengthened by a similar study in which neutralization of CCR1 using an Ab totally abrogated the pulmonary fibrosis in an animal model of pulmonary fibrosis (49). In these two studies, CCL3 was suggested as one of the potential mediators involved in the activation of CCR1. Because ASMC have been shown to produce extracellular matrix proteins that are involved in airway remodeling (50, 51), such as versican, lumican, and collagen, we initially hypothesized that activation of CCR1 by CCL3 or CCL23 could induce the release of extracellular matrix components by ASMC. However, CCL3 and CCL23 failed to increase message levels of collagen I, decorin, lumican, or versican (data not shown). Because the effects on airway remodeling could be indirectly mediated through the release of profibrotic cytokines by ASMC, we also tested the effect of CCL3 and CCL23 on protein production using a cytokine microarray (Human cytokine array III, RayBiotech). Neither of the two CCR1 ligands had any effect on protein production by ASMC at both 4 and 24 h after stimulation with 100 ng/ml (data not shown). It is also possible that activation of CCR1 in ASMC leads to the modulation of extracellular matrix proteins by other types of cells such as mast cells, fibroblasts, and myofibroblasts (15, 52, 53). It has recently been shown that ASMC can migrate toward a gradient of chemokines, but unlike the report showing this phenomenon with CCL11 and CCL19 (8, 9), CCL3 and CCL23 were unable to induce the chemotaxis of ASMC (data not shown).

In conclusion, we have demonstrated for the first time that ASMC express CCR1. We showed that TNF-α and, to a lesser extend, IFN-γ, up-regulate CCR1 expression at both mRNA and protein levels in a Sp1 and NF-κB dependent pathways. We also documented an increased expression of CCR1 in airways of asthmatic patients, more particularly in patients who are not taking any corticosteroids. The expression of a functional CCR1 by ASMC indicates that CCL3, a chemokine increased in asthmatic airways, might play a role in the pathogenesis of the disease through the activation of ASMC. We are presently investigating whether activation of CCR1 mediates synthetic, proliferating, or migrating responses in ASMC.

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Disclosures

The authors have no financial conflict of interest.

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