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Functional Analysis of the Chemokine Receptor CCR3 on Airway Epithelial Cells

Lisa A. Beck,^{*†} Brian Tancowny,^{*} Mary E. Brummet,^{*} S. Yukiko Asaki,^{*} Stephanie L. Curry,^{*} Margaret B. Penno,[‡] Martyn Foster,[§] Ash Bahl,[§] and Cristiana Stellato^{1,2*}

The function of chemokine receptors on structural cells is only partially known. We previously reported the expression of a functional CCR3 receptor on airway epithelial cells (EC). We speculated that CCR3 might drive wound repair and expression of inflammatory genes in epithelium. The human airway EC lines BEAS-2B, 16-HBE, and primary bronchial EC were used to test the effect of in vitro challenge with the CCR3 ligands CCL11/eotaxin, CCL24/eotaxin-2, or CCL26/eotaxin-3 on 1) wound repair, using an established wound model; 2) cell proliferation and chemotaxis, using specific fluorometric assays; and 3) gene expression, using pathway-specific arrays for inflammatory and profibrotic cytokines, chemokines, and chemokine receptor genes. Agonist specificity was tested by cell pretreatment with an AstraZeneca CCR3 antagonist (10^{-8} – 10^{-6} M). CCL24 challenge significantly accelerated epithelial wound closure, with similar effects exerted by CCL11 and CCL26. This effect was time dependent, sub-maximal at 1 nM, and comparable in potency to epidermal growth factor. CCL24 induced a concentration-dependent increase in EC proliferation and chemotaxis, with significant effects observed at 10 nM. The AstraZeneca compound selectively inhibited these CCL24-mediated responses. CCL11 induced the up-regulation of several profibrogenic molecules such as fibroblast growth factor 1 and 5 and of several CC and CXC chemokines. Epithelial immunostaining for CCR3 was stronger in bronchial biopsies of asthmatics displaying marked inflammatory changes than in nondiseased samples. Epithelial CCR3 participates in key functions for wound repair, amplifies the expression of profibrogenic and chemokine transcripts, and appears up-regulated in inflamed asthmatic airways. *The Journal of Immunology*, 2006, 177: 3344–3354.

It is well established that chemokines play a central role in many homeostatic and pathological processes. Chemokines coordinate the recruitment, activation, and homing of leukocytes relevant for both innate and adaptive immune responses (1, 2). However, recent evidence indicates that the effects of chemokines extend well beyond their ability to modulate cell trafficking and involve actions on other fundamental processes of the inflammatory reaction, such as angiogenesis and tissue remodeling (3). The role of chemokines in these biological processes is supported by reports of the expression of functional chemokine receptors on structural cells such as endothelial cells, epithelial cells (EC),³ fibroblasts, and smooth muscle cells (3). We have reported previously that airway EC express CCR3 in vivo in normal and diseased lung samples and that this receptor is functional, based on in vitro studies showing agonist-induced increases in intracellular Ca^{2+} and phosphorylation of ERK proteins (4).

The airway epithelium provides a host of functions that crucially contribute to both the innate and the adaptive mucosal immunity (5). Airway EC are known to be a key source of homeostatic and inflammatory chemokines (6). Among the latter, several CCR3 ligands, such as eotaxins (CCL11, CCL24, and CCL26/eotaxin-3) and CCL13/MCP-4, CCL5/RANTES, and CCL28/MEC, are produced by these cells both in vitro in response to inflammatory and Th2-derived cytokines, or in vivo after allergen challenge or in asthma (6, 7). Additionally, the epithelium also is exposed to chemokines produced by neighboring cells and infiltrating leukocytes within the inflamed airway mucosa. Therefore, the expression of the CCR3 receptor on EC suggests that chemokines might regulate EC function and gene expression in homeostatic or inflammatory settings. Alterations of airway epithelium are often observed in asthmatics and consist of goblet cell hyperplasia, basement membrane thickening, and epithelial denudation (8, 9). It is now well established that airway epithelium is more susceptible to damage in asthmatic patients, and that normal repair processes are compromised in asthma (reviewed in Ref. 5). In this context, we have investigated whether CCR3 is involved in the epithelial response to wounding and in mediating some of the epithelial-derived inflammatory responses.

The wound healing process is a complex biological response involving multiple events, including profound changes in EC phenotype, activation of chemotaxis of cells from the epithelial basal layer, followed by cell proliferation and differentiation, as well as by changes in gene expression. All these processes are controlled by receptor-mediated signals generated by growth factors such as the tyrosine kinase receptor ligand, epidermal growth factor (EGF) (10). Our present study indicates the novel involvement of the chemokine network in the epithelial wound repair process in the airways, as we report that a specific CCR3 ligand, CCL24, is able to accelerate EC wounding in vitro by promoting the chemotaxis

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³ Abbreviations used in this paper: EC, epithelial cell; AZ XX, AstraZeneca CCR3 antagonist; BMP, bone morphogenic protein; EGF, epidermal growth factor; FGF, fibroblast growth factor; HVEM-L, herpes virus entry mediator-ligand; PBEC, primary bronchial EC; TNFSF, TNF (ligand) superfamily; RT, room temperature; MFI, mean fluorescence intensity.

as well as the proliferation of airway EC, a response that can be blocked by a selective CCR3 antagonist. Accordingly, studying gene expression following epithelial challenge with the CCR3 ligand, CCL11, we found an increase in the expression of a host of genes involved in wound repair, as well as of several chemokines and chemokine receptors. Immunohistochemical analysis of biopsy samples from asthmatic airways show more intense CCR3 staining of airway epithelium, compared with control samples.

These data reveal a novel chemokine-driven pathway that targets EC functions, such as proliferation and chemotaxis, which are key components of the wound repair process and might be altered in several diseases in which EC play a pathogenetic role. Additionally, our data strongly suggest the existence of an epithelial-derived, CCR3-mediated network aimed at amplifying chemokine-mediated responses.

Materials and Methods

Culture of human EC

The cell line BEAS-2B was maintained in F12/DMEM (Invitrogen Life Technologies) containing 5% heat-inactivated FCS (HI-FBS; HyClone), 2 mM L-glutamine (Invitrogen Life Technologies), penicillin/streptomycin (100 U/100 mg/ml) (Invitrogen Life Technologies), and 0.5% fungizone (PSF; Invitrogen Life Technologies) (11). The 16-HBE cell line was maintained in DMEM containing 10% HI-FBS, 2 mM L-glutamine, and PSF and in Ultraculture serum-free medium (BioWhittaker) for serum starvation. Primary bronchial EC (PBEC) were isolated by enzymatic digestion with pronase (Sigma-Aldrich) from the bronchi of cadaveric lungs and maintained in serum-free LHC-9 medium (Biofluids) (12).

Cell stimuli

CCL11 and CCL24 were purchased from R&D Systems. Chemokines were reconstituted in PBS/0.1% BSA in 100 μ g/ml stocks. According to the manufacturer, endotoxin levels as determined by the *Limulus* ameocyte lysate method were <0.1 ng/ μ g CCL24, <1 EU/ μ g CCL11. EGF and vitronectin, used as positive controls, were purchased from Clonetics and BD Biosciences, respectively. All stimuli were divided into single-use aliquots, kept at -80°C , and used within their suggested shelf life. The AstraZeneca CCR3 antagonist (AZ XX) was dissolved in DMSO as 0.01 M stock and further dissolved in medium for the concentrations used in the experiments.

Wounding assay

The assay has been set up as modification of an established model (10). Epithelial 16-HBE cells (0.5×10^6) were grown to full confluence on collagen-coated 60×15 -mm petri dishes with grids. Cells were serum starved for 24 h before wounding. A linear wound was made with a 22-gauge, sterile needle in monolayers of cells cultured at 37°C in serum-free medium in the absence or presence of the indicated stimuli. Wound areas were monitored for wound repair and photographed at fixed time points (0, 2, 4, and 6 h) after wounding. Wounds were photographed (100 \times) using an Olympus Microscope Model BX60 mounted with a Pixera Penguin 600CL digital camera. The wound area was measured using Scion Image software (National Institutes of Health, Bethesda, MD) and expressed in pixels square. The data were expressed as the percent of wound area, compared with baseline wound area at time 0.

EC chemotaxis

A fluorescent-based chemotaxis assay optimized for EC was used, which uses rhodamine-labeled cells (13). Briefly, the upper wells of a 96-well chemotaxis chamber (NeuroProbe) were blocked with 30 μ l of 2% BSA in PBS for 30 min at 4°C . After removal of excess BSA, the membrane was washed with PBS and allowed to air dry. Medium, CCL24 (1–100 nM), or vitronectin (50 ng/ml = 0.7 nM), used as positive control for epithelial chemotaxis (13), were dispensed in 30 μ l-volume aliquots to lower wells. Cell suspension (100,000 cells per well) was added to upper wells, and the chemotaxis chamber was incubated for 4 h at 37°C . At the end of the incubation, unbound cells in the upper wells were removed by pipetting, and residual cells were removed by wiping the wells with a cotton swab. Subsequently, the membrane was fixed in 15 ml of 1% formaldehyde in PBS with continuous agitation by a shaker (150 rpm for 15 min). Cells were then permeabilized with 15 ml of 0.2% Triton X-100 in PBS on a shaker (150 rpm for 15 min). Migrated cells were stained with 15 ml of

rhodamine-phalloidin dye (5 U/ml PBS; Molecular Probes), prepared from a 200 U/ml stock in methanol) for 30 min under agitation, and covered with aluminum foil to avoid degradation of the fluorescent signal. Excess dye was removed, and the membranes were rinsed twice with PBS. The membranes were air dried and analyzed in a microtiter fluorometer at 530 nm excitation/590 nm emission. Migrated cells were visualized with fluorescence microscopy (model BX60; Olympus). Each experimental condition was performed in quadruplicates.

Proliferation assay

Cell proliferation was assayed using the Cyquant cell proliferation assay (Molecular Probes). BEAS-2B cells were grown to 50% confluence in 96-well plates in serum-free bronchial EC growth medium (Cambrex), deprived of EGF for 24 h before challenge, and subsequently stimulated according to the experimental protocols. As per the manufacturer's instructions, at the end of the experiment, the cell supernatants were removed and cell lysis was performed by a freeze-thaw cycle. Cells were then incubated for 5 min at room temperature (RT) with Cyquant lysis buffer containing the Cyquant-GR fluorescent dye. Fluorescence was measured by a Cytofluor 4000 fluorescence reader (Applied Biosystems). Each experimental condition was assessed in quadruplicates.

Analysis of gene expression by cDNA arrays

Changes in gene expression were monitored using pathway-specific arrays (GEArray Q Series: Human Common Cytokine, and Chemokine and Receptors Gene arrays; SuperArray). Gene accession numbers are provided by the manufacturer. Total RNA was extracted using the Qiagen RNeasy reagent following cell treatment. Reverse transcription and labeling of cDNA probes, hybridization, and washing of the arrays were performed following the manufacturer's protocol. The gene expression profile was detected by a Typhoon 8600 Imager (GE Healthcare) using ImageQuant software (GE Healthcare). Gene analysis was performed using the GEArray Analyzer software. Briefly, gene expression was taken into consideration when significantly different from background. A gene was considered up-regulated when a difference >2-fold was detected between unstimulated and treated samples, in at least two of three independent experiments.

RT-PCR

Reverse transcription of total RNA and amplification of target mRNAs were performed using the Gene Amp Kit and the TaqMan reagent kit (PerkinElmer), respectively. The following gene targets were validated by RT-PCR using commercially available optimized, gene-specific primers designed for end-point validation of genes present on GEArray platforms (PCR Primer single gene kit; Superarray): CCL2/MCP-1, CCL26/eotaxin-3, CXCL5/ENA-78, FGF-1, FGF-5, bone morphogenic protein 6 (BMP-6), TNF superfamily 14 (TNFSF-14), and IL-6. The reaction conditions used for all primer sets were: 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 60°C for 60 s, for 30 cycles. Expression of FGF-5, TNFSF-14, and IL-6 mRNA was additionally detected using specific primers designed for GEArray target validation by real-time PCR (PCR Primer single gene kit). Real-time PCR also was used to detect CXCL8, CCL11, and CCR3. The PCR primers, designed by Primer Express software (PerkinElmer), are as follows: for CXCL8, CTGGCCGTGGCTCTCTTG (forward) and TTAGCACTCCTGGCAAACTG (reverse), used for detection with sybr green; for CCL11, AGGAGAATCACCAGTGGCAAA (forward) and GGAACTCCTGCACCCACTTCTT (reverse), and the probe sequence is FAM-TCCCCAGAAAGCTGTGATCTTCAAGACC-TAMRA; for CCR3, AAAGCTGATACCAGAGCACTG (forward) and CCAA GAGGCCACAGTGAAC (reverse), and the probe sequence is FAM-TGCCCCCGCTGTACTCCCTGG-TAMRA; for the housekeeping gene, GAPDH, GAAGGTGAAGGTCGGAGTC (forward), and GAAGATGG TGATGGGATTC (reverse), and the probe sequence is JOE-CAAGTGT TCCCCTTCTCAGCC-TAMRA. The reaction conditions used for all primer sets were as follows: 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 60°C for 60 s, for 40 cycles. Resulting PCR products were measured by the sequence detector ABI 7700 (PerkinElmer). Quantitation of gene expression by real-time RT-PCR was evaluated using the Comparative C_T Method as per PerkinElmer's guidelines (14).

ELISA

Secreted CXCL8 and FGF-1 were detected in the supernatants of BEAS-2B cells using sensitive and specific ELISAs (R&D Systems). The manufacturer excluded cross-reactivity after testing each ELISA with the full panel of human chemokines and with other members of the FGF superfamily, respectively. The sensitivity of the assays was 0.122 and 1.19 pg/ml, respectively.

FACS analysis

EC were lifted with Versene (Invitrogen Life Technologies), labeled by indirect immunofluorescence as described (4), and analyzed using a FACS Calibur flow cytometer (BD Biosciences). Cells were incubated in saturating concentrations of the mouse monoclonal anti-human CCR3 Ab 7B11 (R&D Systems), or an equivalent concentration of isotype-matched control Ig. A FITC-conjugated goat F(ab')₂ anti-mouse IgG (BD Pharmingen) or FITC-conjugated goat F(ab')₂ anti-hamster IgG (BioSource International) was used as the secondary Ab. Cells were analyzed using propidium iodide (Sigma-Aldrich) exclusion to separate live and dead cells. Cell viability, detected by propidium iodide exclusion, was routinely $\geq 85\%$, and only live cells were analyzed. Mean fluorescence intensity (MFI) from the isotype-matched control Ig control sample was subtracted from the MFI of the Ab-labeled sample to give the net MFI.

Western blot

Whole cell lysates were prepared from BEAS-2B cells using Triton lysis buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM sodium orthovanadate). Insoluble material was removed by centrifugation at $14,000 \times g$ for 5 min. Lysed cells were loaded at 1×10^6 cells per lane, separated by SDS-PAGE, and transferred to a Sequi-blot polyvinylidene difluoride membrane (Bio-Rad). Blots were blocked in $1 \times \text{PBS}/5\% \text{BSA}/0.1\% \text{Tween 20}$ overnight and incubated with the anti-CCR3 Ab 7B11 (1 mg/ml). After washing with $1 \times \text{PBS}/0.1\% \text{Tween 20}$, blots were incubated with HRP-conjugated secondary Ab. Immunoreactive bands were visualized using ECL (Amersham Biosciences).

CCR3 immunohistochemistry

Endobronchial biopsies were selected from the AstraZeneca tissue pathology archive and were originally obtained as part of ongoing clinical collaborations. All subjects were ethically consented according to the United Kingdom Department of Health and AstraZeneca Bioethics policy. The original asthma diagnosis was based on clinical history. In total, nine biopsies were selected that, based on histological criteria, were graded as: 1 ($n = 3$), 2 ($n = 4$), and 3 ($n = 2$). Grading was based on degree of inflammatory cell influx, mucosal reactive changes, and mucus gland hyperplasia (see below). For comparison purposes, three postmortem samples of upper airways from individuals with no history of airways disease also were studied.

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections. Four-micron-thick sections were mounted on glass slides, dewaxed, and rehydrated. Endogenous peroxidase was blocked with 3% H₂O₂ in methanol, and nonspecific Ig-binding sites were blocked with 20% normal rabbit serum. Briefly, sections were incubated in rat anti-human CCR3 (5 $\mu\text{g}/\text{ml}$; R&D Systems) for 60 min at RT. A rat IgG2a was used as a negative control (5 $\mu\text{g}/\text{ml}$; BD Pharmingen). Sections were washed in buffer (three times for 2 min) and then incubated with biotinylated rabbit anti-rat serum (1/200 dilution; DakoCytomation) for 20 min at RT. Thereafter, sections were incubated with Strept ABC/HRP complex (DakoCytomation) for 20 min at RT. To allow visualization of CCR3, sections were incubated with 3,3'-diaminobenzidine (DakoCytomation) and then counterstained with Gills Hematoxylin (Pioneer Research Chemicals). Sections were then dehydrated, cleared and mounted using Hystomount (Hughes & Hughes).

The bronchial samples were scored using the following scoring system: Grade 0, Essentially normal histology. Some diffuse presence of inflammatory cells, mainly in lamina propria and rarely in the epithelium. Non-consistent epithelial changes, with occasional goblet cell hypertrophy or acute epithelial loss; Grade 1, Inflammatory cell influx into lamina propria, mainly constituted by eosinophils and mononuclear cells. Consistent presence of fibrotic thickening of reticular layer, epithelial hypertrophy, and focal hyperplasia of variable degree. Increased numbers of dense basal cells. Goblet cell hypertrophy; Grade 2, Marked, confluent loss of epithelium with marked basal cell hyperplasia in areas of mature cell loss. Focal areas of EC cuboidal metaplasia with variable surrounding inflammatory cell presence. Overall inflammatory cell influx more marked that Grade 1, with marked focal accumulation around the epithelium-lamina propria interface, around smooth muscle bundles and within EC. Variable degree of goblet cell hypertrophy and very marked fibrosis of reticular layer; Grade 3, Markedly greater loss of epithelium. Foci of significant metaplasia in areas of epithelial restitution, in many areas characterized as squamous metaplasia. Variable degree of inflammatory cell influx, mainly represented by mononuclear infiltrates in the lamina propria and in the epithelium. High incidence of myolysis and mucus gland hyperplasia, with inflammatory cell influx within gland acini.

Statistical analysis

Data were analyzed using ANOVA test with Fisher's post hoc analysis. Comparisons among groups were further analyzed with the Kruskal-Wallis or Wilcoxon nonparametric tests. When appropriate, a Student paired *t* test was applied.

Results

We first set out to investigate the role of epithelial-bound CCR3 using an established in vitro wound model (10). Confluent monolayers of 16-HBE cells were wounded and exposed to medium alone, CCL24 (1–100 nM), or EGF (16.5 nM = 100 ng/ml), used as positive control, for 2, 4, or 6 h. CCL24 significantly accelerated wound closure, showing a submaximal effect when used at 1 nM (percent area of original wound after 6 h, 38 ± 2) (Fig. 1). The effect of CCL24 was similar to that exerted by EGF in time course, potency, and efficacy (Fig. 1; $34.1 \pm 4\%$ area of original wound after 6 h). We also compared the effect of an equimolar concentration (10 nM) of the CCR3 ligands CCL11 (eotaxin) and CCL26

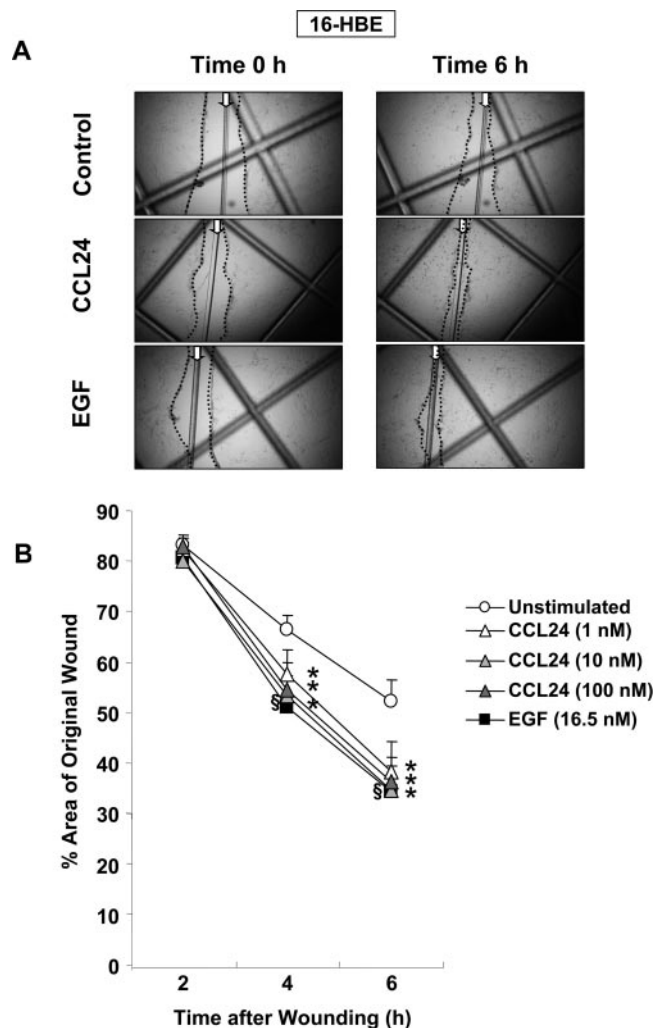


FIGURE 1. CCL24 accelerates wound closure in vitro. Confluent monolayers of 16-HBE cells were wounded and exposed to either medium, CCL24 (1–100 nM) or EGF, (16.5 nM) used as positive control. Photographs of the wounded areas were taken after 2, 4, and 6 h. **A**, Photographs (100 \times) taken at time of wound (time 0 h) and 6 h later (time 6 h) for each condition. White arrows denote the abrasion made in the petri dish from the wounding needle. The dotted lines outline the wound margins. The grid lines were used to identify the identical wound location in each petri dish. **B**, Plotting of the wound area (mean \pm SEM of $n = 7$ –15). *, $p < 0.05$ CCL24; §, $p < 0.001$ EGF vs unstimulated cells.

(eotaxin-3) to that exerted by CCL24 on wound repair. Similarly to CCL24 ($34.5 \pm 5\%$ area of original wound at 10 nM), these chemokines accelerated wound closure (18 ± 11 and $20 \pm 5\%$ area of original wound after 6 h, $n = 3$ and 2, respectively) when compared with wounded monolayers exposed to medium alone ($53 \pm 5\%$ area of original wound after 6 h).

To test the specificity of such effect, cells were pretreated for 30 min in the absence or presence of increasing concentrations of AZ XX. Cells were subsequently cultured with medium only or challenged for 6 h with CCL24 or EGF at the indicated concentrations (Fig. 2). As previously observed, at the end of the incubation time, the wound area in the monolayers treated with either stimuli was significantly smaller, in the absence of the AZ XX compound, than the one measured in unstimulated cells. However, the acceleration

of the wound closure mediated by CCL24 was abolished by pretreatment with the CCR3 antagonist, while the effect of EGF was not influenced by the AZ XX compound (Fig. 2).

Restoration of epithelial damage is a complex, multistep process in which basal EC contiguous to the wounded area first flatten and then migrate toward the denuded area, to create a temporary barrier. Subsequently, migrated cells start to proliferate, restoring the original, fully differentiated pseudostratified layer (5). Therefore, we examined the effect of CCL24 on airway EC chemotaxis and proliferation. To test CCR3-mediated epithelial chemotaxis, PBEC were placed in a 96-well microplate-format chemotaxis chamber in the presence of buffer, CCL24 (1–100 nM), or vitronectin (0.7 nM = 50 ng/ml) used as a positive control. Migration was allowed to occur for 4 h at 37°C. Fluorometric reading from rhodamine-labeled migrated cells indicated that CCL24 concentration-dependently increased EC chemotaxis, with an efficacy similar to that displayed by vitronectin. The chemotactic effect of CCL24 was statistically significant at 10 and 100 nM (Fig. 3A). Checkerboard analysis confirmed that cell migration occurred only in the presence of a chemotactic gradient, ruling out a chemokinetic component in the observed response (Fig. 3B). Migrated cells also were visualized with a fluorescent microscope (Fig. 4A) in a second set of experiments, where PBEC were pretreated with 10^{-6} M AZ XX for 30 min before challenge with CCL24 or vitronectin, to evaluate the specificity of the chemotactic response. Cell pretreatment with

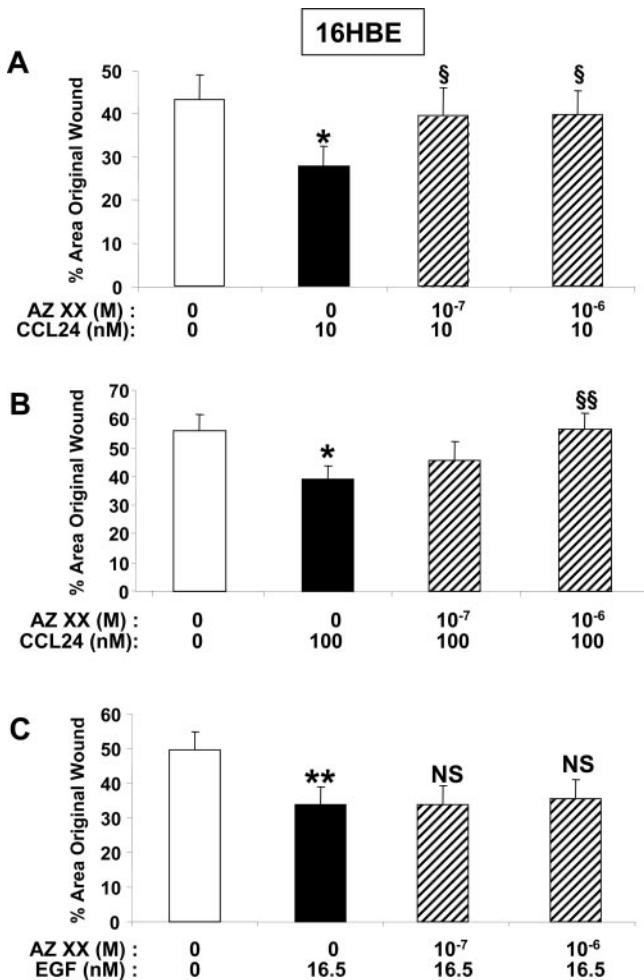


FIGURE 2. Selective ablation of CCL24 effect on wound closure in vitro by a CCR3 antagonist. Confluent monolayers of 16-HBE cells were wounded and incubated for 30 min in the absence or presence of increasing concentrations of the AstraZeneca CCR3 antagonist 133620XX (AZ XX). Cells were subsequently cultured with medium alone or medium plus CCL24 (10 (A) and 100 nM (B)) or EGF (16.5 nM (C)) for 6 h. Mean \pm SEM ($n = 6-10$) of percent of the original wound area, measured at the time of wounding (time 0). *, $p < 0.05$ for CCL24 response vs unstimulated cells and **, $p < 0.001$ for EGF response vs unstimulated cells; §, $p < 0.05$, response to 10 nM CCL24 challenge after AZ XX pretreatment, compared with cell challenge with CCL24 without pretreatment; §§, $p < 0.01$ response to 100 nM CCL24 challenge after AZ XX pretreatment, compared with cell challenge without pretreatment. NS, Not significant change in response to EGF challenge after AZ XX pretreatment compared with cell challenge without pretreatment.

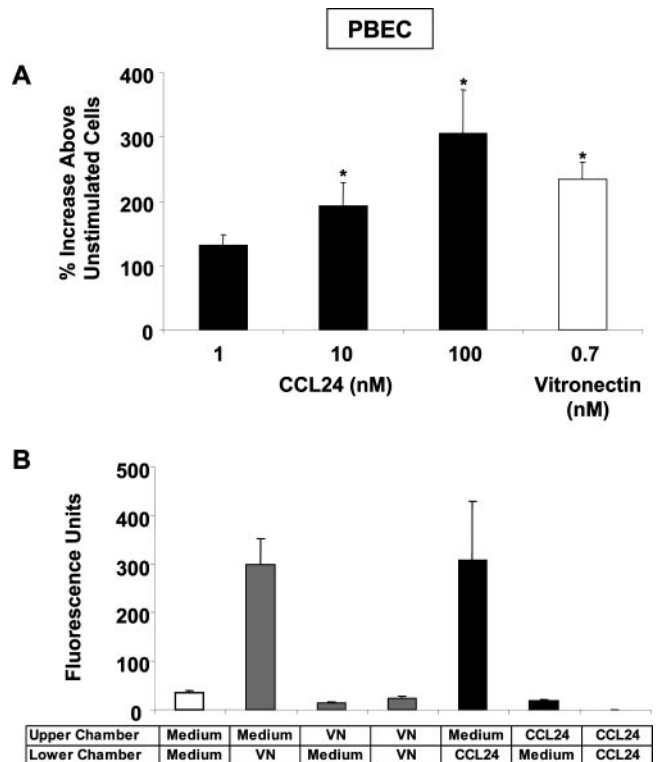


FIGURE 3. CCL24 induces chemotaxis of airway EC. A, Chemotaxis of PBEC assayed by a fluorometric assay following cell treatment with CCL24 (10–100 nM) and vitronectin (0.7 nM), used as positive control, for 4 h. Results are shown as mean \pm SEM of $n = 6$. *, $p < 0.05$, compared with unstimulated cells. Mean \pm SEM fluorescence units (FU) in unstimulated cells: 122.8 ± 27.4 . B, For the checkerboard analysis of chemotaxis response, cells were placed in the upper side of the chemotactic chamber in the presence of medium or the indicated stimuli in different gradient conditions (indicated by the table at the bottom) to rule out chemokinesis. Results expressed as mean \pm SEM FU of quadruplicate readings. VN, Vitronectin.

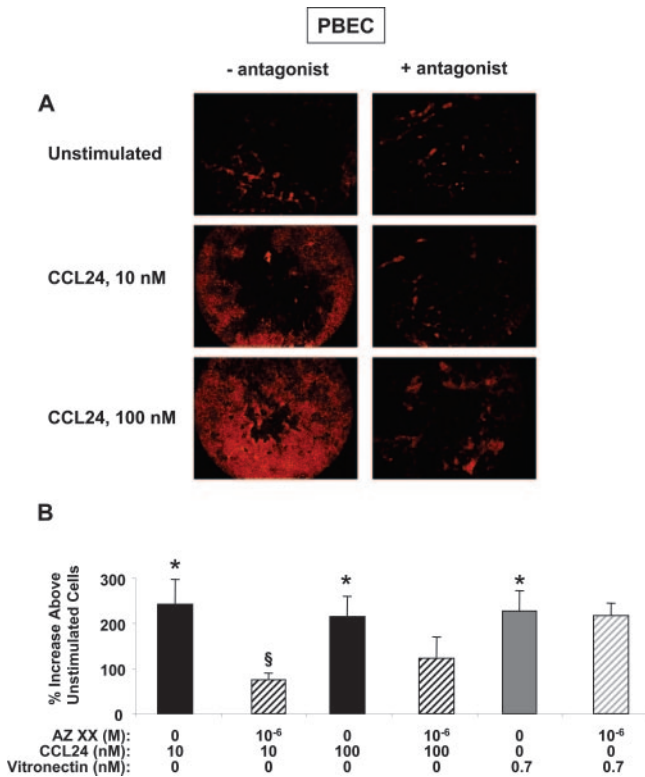


FIGURE 4. CCL24 effect on epithelial chemotaxis is antagonized by a specific CCR3 antagonist. PBEC were incubated for 30 min in the absence or presence of 10^{-6} M AZ 133620XX (AZ XX). Cells were subsequently placed in the upper chamber of a 96-well chemotaxis chamber, where medium, CCL24 or vitronectin at the indicated concentrations were previously loaded in the bottom chamber; chemotaxis was allowed to occur for 4 h. **A**, Representative photomicrographs (100 \times) of chemotaxis membranes showing rhodamine-labeled EC migrated in response to medium (unstimulated) or to increasing concentrations of CCL24, in the absence (left column) or presence (right column) of 10^{-6} M of the CCR3 antagonist. **B**, Fluorometric reading of the chemotaxis experiments (mean \pm SEM of $n = 4$) showing epithelial chemotaxis induced by CCL24 (10 and 100 nM) and vitronectin (0.7 nM) in the absence (solid bars) or presence (striped bars) of pretreatment with 10^{-6} M AZ XX compound, showing the selective effect of the CCR3 antagonist. *, $p < 0.05$ chemotaxis response, compared with that of unstimulated cells, §, $p < 0.05$, 10 nM CCL24 response after 10^{-6} M AZ XX pretreatment vs stimulated cells without pretreatment. Mean \pm SEM FU in unstimulated cells: 119.7 ± 15.8 .

AZ XX selectively blocked CCL24-mediated and not vitronectin-mediated chemotaxis (Fig. 4B). The inhibition was most demonstrable at the 10 nM concentration, and with the inhibitory effect still being present, although to a lesser degree, in cells stimulated with the highest concentrations (100 nM) of CCL24 (Fig. 4).

We then investigated whether the proliferative response that contributes to restoration of the epithelial layer after wounding was also affected by CCR3-mediated signals, as observed for the chemotactic response. Primary bronchial EC and the EC line BEAS-2B were seeded at low-density, serum starved, and then challenged for 24 h with increasing concentrations of CCL24 (1–100 nM) and EGF (1.6–16.5 nM = 1–100 ng/ml) used as positive control (10). In both cell models, CCL24 concentration-dependently increased cell proliferation, with a significant effect observed by 10 nM, similarly to the chemotactic response (Fig. 5). The efficacy of CCL24 was comparable to that obtained with EGF stimulation (Fig. 5). The specificity of this response was demonstrated by pretreating PBEC with increasing concentrations of AZ XX (10^{-8} – 10^{-6} M) for 30 min before challenge with CCL24 or

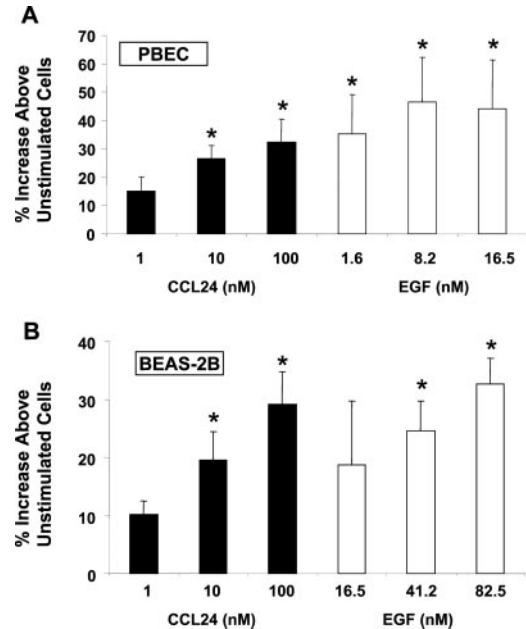


FIGURE 5. CCL24 Concentration-dependently induces proliferation of airway EC. Fluorometric assessment of the proliferation of PBEC and BEAS-2B cells following treatment with increasing concentrations of CCL24 and EGF (used as positive control), for 24 h. Results are shown as mean \pm SEM of $n = 3$ –12 for PBEC and of $n = 3$ –11 for BEAS-2B. For PBEC, *, $p < 0.01$ for CCL24, compared with unstimulated cells; $p < 0.05$ for 1.6 and 8.25 nM (10 and 50 ng/ml) EGF, and < 0.01 for 16.5 nM EGF, compared with unstimulated cells. For BEAS-2B, *, $p = < 0.01$ for CCL24, $p < 0.01$ and < 0.05 for 41.2 and 82.5 nM (250 and 500 ng/ml) EGF, respectively, compared with unstimulated cells. Mean \pm SEM FU in unstimulated cells: 514.3 ± 79 (PBEC) and 932.6 ± 95.4 (BEAS-2B).

EGF (Fig. 6). The AZ XX inhibition of CCL24-induced proliferation was concentration dependent and was observed even at the highest concentration of CCL24 (100 nM). The AZ XX compound did not affect basal or EGF-mediated proliferation (Fig. 6).

We next tested whether challenge of EC with a specific CCR3 ligand would induce the expression of genes relevant to the CCR3-mediated wounding activity, and whether epithelial CCR3 also

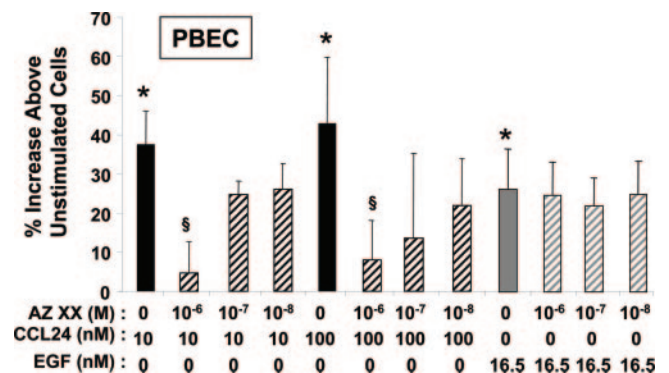


FIGURE 6. CCL24 effect on epithelial proliferation is antagonized by a specific CCR3 antagonist. PBEC were incubated for 30 min in the absence or presence of increasing concentrations of AZ XX. Cells were subsequently cultured with medium only or challenged with CCL24 or EGF at the indicated concentrations for 24 h. Mean \pm SEM of $n = 5$. *, $p < 0.05$ CCL24 and EGF-induced responses vs unstimulated cells; §, $p < 0.05$, 10, and 100 nM CCL24-induced response after 10^{-6} M AZ XX pretreatment, compared with cells without pretreatment. Mean \pm SEM FU in unstimulated cells: 322.8 ± 96 .

Table I. Up-regulation of cytokine/cytokine receptor genes detected upon challenge of BEAS-2B cells with CCL11 (1 and 10 nM)

CCL11 (nM)	Challenge Time 3 h		Challenge Time 18 h	
	Name (alternative nomenclature)	Mean Fold Induction (range)	Name (alternative nomenclature)	Mean Fold Induction (range)
1	<i>Leptin</i>	17.2 (2.2–32.1)	<i>FGF-1*</i>	22.7 (3.1–42.3)
			<i>FGF-5</i>	7.6 (7.3–13.3)
			<i>FGF-6</i>	5.4 (3.6–7.2)
			<i>IL-4</i>	3.7 (2.2–5.1)
			<i>IL-6</i>	3 (2.5–3.5)
			<i>IL-9</i>	3.2 (2.8–3.5)
			<i>TNFSF-4</i>	15.4 (2.1–28.7)
			<i>VEGF-B</i>	2.3 (2.2–2.5)
			<i>VEGF-C</i>	2.6 (2.4–2.7)
10	<i>BMP-6</i> <i>FGF-6</i> <i>FGF-10</i> <i>IL-22</i> <i>PDGF-α</i> <i>TNFSF-4 (OX40 ligand)</i> <i>TNFSF-14 (LIGHT, HVEM)</i>	9.8 (7.3–12.3) 2.2 (2.1–2.4) 2.5 (2.4–2.6) 5.4 (3.2–7.6) 5.1 (3–7.13) 2.9 (2–3.9) 7.2 (2.4–11.9)	<i>None</i>	

^a Genes (listed in alphabetical order) were considered up-regulated when a difference >2-fold was detected between unstimulated and treated samples in at least two of three independent experiments.

would support, through an autocrine mechanism, the expression of chemokine receptors, CCR3 ligands, or other chemokines from these cells.

Total RNA was extracted from BEAS-2B cells stimulated with CCL11, at 1 and 10 nM, or medium alone for 3 or 18 h. These concentrations of CCL11 were chosen as they are in the range of optimal receptor binding (4). Labeled cDNA was hybridized to the Human Common Cytokine Gene GEArray, which includes several genes involved in wounding and inflammatory tissue remodeling, and the Human Chemokine and Receptors Gene Array, which includes all chemokine ligands and receptors. A gene was considered up-regulated when a difference >2-fold was detected between unstimulated and treated samples, in at least two of three independent experiments. Tables I and II summarize the genes that displayed increased expression following challenge with CCL11 for the two-array series, and indicate their mean fold increase over unstimulated cells, as well as the range of fold induction.

The first set of data on gene expression (Table I) showed that CCL11 induced in BEAS-2B cells the expression of two distinct subsets of cytokines, cytokine receptors, and growth factors ac-

ording to the concentration and timing of stimulation. In cells stimulated for 3 h with 1 nM CCL11, the expression of only one cytokine, leptin, was increased, while nine genes were up-regulated after an 18-h challenge. Conversely, response to 10 nM CCL11 induced a response exclusively after 3 h, with seven genes being up-regulated. Strikingly, the majority of the up-regulated genes are involved in the wound healing process. Fig. 7A shows validation by end-point RT-PCR of the up-regulation of FGF-1, FGF-5, TNSF-14, IL-6, and BMP-6 following CCL11 stimulation (representative of $n = 2-3$). The CCL11-induced up-regulation shown for FGF-5, TNSF-14, and IL-6 mRNA in Fig. 7A was quantitated, using the same cDNA, by real-time RT-PCR. Fold mRNA induction over unstimulated cells was 7.0 for FGF-5, 4.2 for IL-6, and 4.7 for TNSF-14. Secretion of FGF-1 also was significantly increased in the supernatants of BEAS-2B cells treated with 10 nM CCL11 for 24 h (259 ± 92 vs 47 ± 29 pg/ml in unstimulated cells, $n = 4$, $p < 0.05$ by Student's paired t test) (Fig. 7B).

In the second set of arrays, chemokine mRNA expression occurred only in response to 10 nM CCL11 and for some genes, the magnitude of the induction varied greatly between experiments.

Table II. Upregulation of chemokine and chemokine receptor genes upon challenge of BEAS-2B cells with CCL11 (10 nM)^a

Chemokine Class	Challenge Time 3 h		Challenge Time 18 h	
	Name (former nomenclature)	Mean Fold Induction (range)	Name (former nomenclature)	Mean Fold Induction (range)
Chemokine Receptors	<i>CXCR1</i>	187.8 (6.3–369.3)	<i>CCR3</i>	7.5 (3.0–12.1)
	<i>CCR2</i>	2.4 (2.7–2)	<i>XCRI</i>	5.7 (3.6–7.8)
	<i>HM74</i>	2.5 (2.3–2.7)		
CXC	<i>CXCL5 (ENA-78)</i>	3.3 (3.1–3.4)	<i>CXCL5 (ENA-78)</i>	22.6 (2.9–42.4)
	<i>CXCL6 (GCP-2)</i>	83.2 (164.3–2.1)	<i>CXCL7 (NAP-2)</i>	4.4 (2.9–5.9)
	<i>CXCL11 (I-TAC)</i>	11.2 (2.1–20.3)	<i>CXCL8 (IL-8)</i>	5.7 (3.5–8)
	<i>CXCL12 (SDF-1)</i>	3.0 (3.1–2.7)	<i>CXCL9 (MIG)</i>	3.2 (2.9–3.6)
			<i>CXCL12 (SDF-1)</i>	6.3 (3.6–9.1)
CC	<i>CCL2 (MCP-1)</i>	37.0 (3.1–71)	<i>CCL8 (MCP-2)</i>	2.4 (2.3–2.5)
	<i>CCL16 (HCC-4)</i>	16.1 (4.5–27.8)	<i>CCL11 (Eotaxin-1)</i>	4.2 (2.9–5.5)
	<i>CCL18 (PARC)</i>	50.7 (3.8–97.6)	<i>CCL16 (HCC-4)</i>	11.8 (2.2–21.4)
	<i>CCL26 (Eotaxin-3)</i>	9.0 (3–18.1)		
	<i>CCL27 (C-TACK)</i>	3.3 (2.9–3.8)		
	<i>CCL28 (MEC)</i>	5.9 (2.4–9.3)		

^a Genes (listed in numerical order of the chemokine nomenclature) were considered up-regulated when a difference >2-fold was detected between unstimulated and treated samples in at least two of three independent experiments.

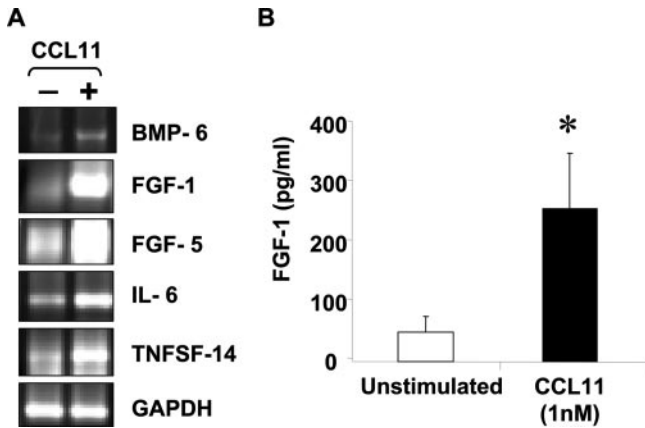


FIGURE 7. CCL11 induces the expression of genes involved in wound healing and inflammation in the airway EC line BEAS-2B. Validation of CCL11-induced gene expression detected by the Human Common Cytokine Gene Array Q series, and the Human Chemokine and Receptors Gene Array. **A**, End-point RT-PCR (representative of $n = 2-3$) showing expression of the indicated genes (for abbreviations, see Table I) in unstimulated cells (-), and after 18 h stimulation with 1 nM CCL11 (+), with the exception of BMP-6, which was up-regulated after 3-h stimulation with 10 nM CCL11. Shown at the bottom is the housekeeping gene GAPDH. **B**, FGF-1 protein levels detected by ELISA in the supernatants of BEAS-2B cells treated with CCL11 (10 nM) for 24 h. Mean \pm SEM of $n = 4$. *, $p < 0.05$, compared with unstimulated cell, Student's paired t test.

Up-regulated genes included seven CXC and nine CC chemokine members, and five chemokine receptors (Table II). The pattern of chemokine expression changed in a time-dependent fashion, with only two CXC chemokines (CXCL5 and CXCL12) showing up-regulation at both time points. We validated the expression of CXCL5, CCL2, and CCL26 mRNA using end-point RT-PCR ($n = 2-3$) (Fig. 8A). Induction of CCL11 and CXCL8 mRNA was confirmed by real-time RT-PCR (fold induction over unstimulated cells, 2.6 and 2.1, respectively; $n = 2$). A significant increase in the protein levels of CXCL8 also was detected by ELISA in the supernatants of cells stimulated with 10 nM CCL11 for 24 h (523 ± 93 pg/ml vs 236.3 ± 54 pg/ml in unstimulated cells, mean \pm SEM of $n = 5$, $p < 0.01$ by Student's paired t test) (Fig. 8B). In the same conditions, CCL26 and CCL2 protein levels also were increased, although without reaching statistical significance due to a high baseline level of the proteins found in the supernatants of unstimulated cells (data not shown).

Among the chemokine receptors, CCR3 itself was found in the array study to be up-regulated by cell challenge with CCL11. CCL11-induced up-regulation of CCR3 mRNA was confirmed by real time RT-PCR (fold induction over unstimulated cells, 14.9 ± 10.9 ; $n = 5$, $p < 0.05$ by Wilcoxon test). Modulation of expression of CCR3 on the cell surface was investigated by FACS analysis. In the present study, CCL11 did up-regulate surface expression of CCR3 in PBEC, although with high variability, which precluded statistical significance ($n = 6$) (Fig. 8C). Importantly, our data show that primary cell cultures are quite heterogeneous not only in response to CCR3 ligands, but also in their basal CCR3 expression levels, which suggest a complex network of regulation of this pathway in vivo. Potential changes in intracellular pool of CCR3 following chemokine stimulation were investigated by Western blot analysis, using whole cell lysates of BEAS-2B cells unstimulated or treated for 18 h with 10 nM CCL11 or CCL24 (1×10^6 cells per lane). Densitometric analysis of the blots indicated that there was no detectable change in CCR3 levels following challenge with the two CCR3 ligands (Fig. 8D). This is in agreement with our pre-

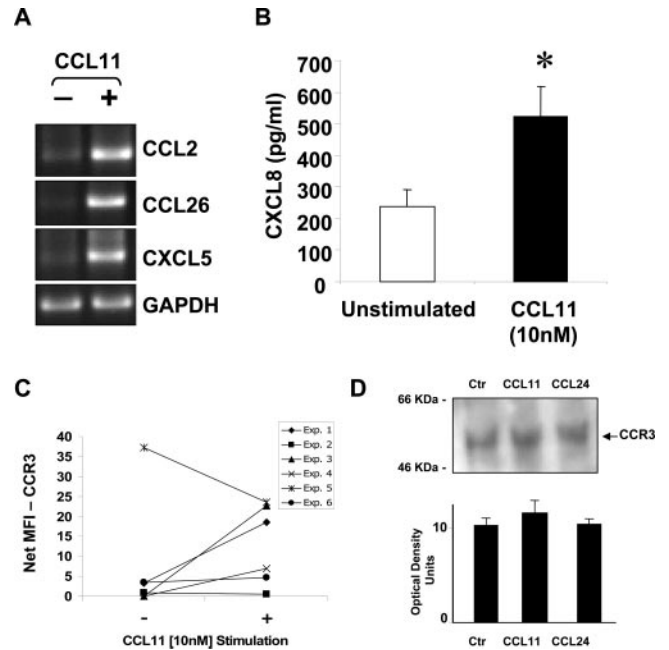


FIGURE 8. CCL11 induces chemokine and chemokine receptor gene expression in the airway EC line BEAS-2B. Validation of CCL11-induced gene expression detected by the Human Chemokine and Receptors Gene Array Q series. **A**, End-point RT-PCR (representative of $n = 2-3$) showing expression of the indicated chemokines in unstimulated cells (-) and 3 h after stimulation with 10 nM CCL11⁺. Shown at the bottom is the housekeeping gene GAPDH. **B**, CXCL8 protein levels detected by ELISA in the supernatants of BEAS-2B cells treated with CCL11 (10 nM) for 24 h. Mean \pm SEM of $n = 5$. *, $p < 0.05$ compared with unstimulated cells, Student's paired t test. **C**, FACS analysis of PBEC demonstrating up-regulation of surface expression of CCR3 upon cell treatment with CCL11 (10 nM) for 18 h. Results are expressed as Net Mean Fluorescence Intensity ($n = 6$). **D**, Western blot analysis of BEAS-2B whole cell lysates (1×10^6 cells per lane, representative of $n = 3$) and densitometric analysis showing CCR3 expression in cells unstimulated or challenged with CCL11 or CCL24 (10 nM) for 18 h.

vious data showing lack of modulation of CCR3 surface expression in cytokine-stimulated EC, despite detection of increased CCR3 mRNA expression (4), and points at possible regulation of CCR3 at the translational level.

Surprisingly, we could not demonstrate antagonism (with the AZ XX compound) of the CCR3 ligand-induced gene expression, which is in contrast with the dose-dependent antagonism of CCL24-mediated effects observed in chemotactic, proliferative and wound responses. The mRNA levels of FGF-1, FGF-5, CXCL5, CCL2, or CCL26 as well as CCR3 remained unchanged after the same pretreatment protocol with AZ XX used for the aforementioned cellular assays (data not shown). These results suggest an indirect effect of chemokine on gene expression in BEAS-2B mediated by a CCR3-independent mechanism and provide further support to data showing that CCL11 is a ligand for other chemokine receptors, such as CCR5 (15).

Participation of epithelial-bound CCR3 to the remodeling process is further suggested by the pattern of CCR3 expression found in bronchial biopsies of asthmatic subjects, compared with airway samples from nonasthmatic individuals. CCR3 receptor expression was present on airway epithelium in both nondiseased airways (Fig. 9A) and samples from asthmatics (Fig. 9B). CCR3 was expressed throughout the pseudostratified epithelium, with both columnar cells and basal cells showing marked staining. The staining

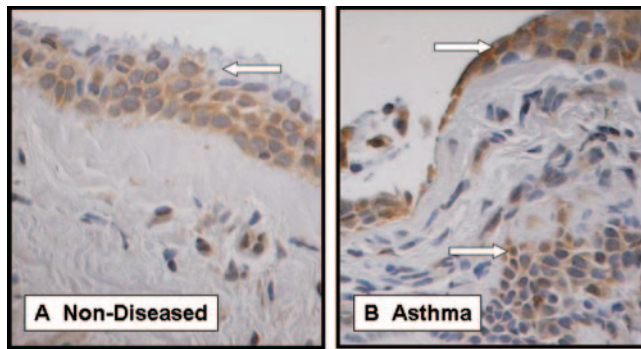


FIGURE 9. Bronchial epithelial CCR3 immunoreactivity is observed in normal and diseased airways. *A*, CCR3 expression in the airway mucosa from a nondiseased sample histologic grade 0 (200 \times). This is a representative example of an $n = 3$. *B*, CCR3 receptor expression in a representative biopsy from an asthmatic subject with histologic grade 3 ($n = 2$, total asthmatic biopsies; $n = 9$) demonstrating more intense CCR3 expression on basal “epithelioid” cells and punctuate staining of epithelial metaplasia (200 \times). Note CCR3-positive leukocytes also can be seen in the lamina propria in the asthmatic sample. Arrows denote areas of CCR3 immunoreactivity. Matched isotype controls were negative (data not shown).

was more intense and had a granular pattern in asthmatic epithelium, which was more disorganized and frequently characterized by epithelial metaplasia (Fig. 9*B*). The asthmatic group in which inflammatory changes were most represented (Fig. 9*B*) showed a marked heterogeneity in CCR3 staining, with evidence of a diffuse as well as of a punctuate staining in areas of epithelial metaplasia. There was marked staining of basal cells, similar to that observed in biopsies with moderate inflammation group, in addition to intense staining of areas of disorganized epithelium. In all groups, there were areas of marked expression of the CCR3 receptor on mucus gland epithelium.

Discussion

Structural cells in the airways actively participate in the regulation of leukocyte trafficking in the lung under homeostatic conditions as well as in response to a variety of proinflammatory events, through the release of specific patterns of chemokines (3). Numerous *in vitro* studies have shown that structural cells also are relevant chemokine targets. In fact, airway EC, keratinocytes, endothelial cells, fibroblasts, and smooth muscle all possess a diverse array of functional chemokine receptors (4, 16–20), suggesting that chemokines have autoregulatory or juxtaregulatory functions on structural cells. The expression of chemokine receptors on these cells can be regulated *in vitro* by inflammatory signals such as TNF- α , IL-1 β , IL-4, or LPS (4, 16, 17, 19) and by other environmental changes, such as hypoxia (21), indicating that the function(s) they convey likely contribute to the altered phenotype induced by inflammation in several disease states. *In vivo*, chemokine receptors have been identified on structural cells in normal tissue and in a variety of pathological conditions. For example, expression of CCR3 on airway epithelium has been detected in bronchial biopsies from patients with asthma, hypereosinophilic syndrome as well as in normal airways (22), and was highly up-regulated in atherosclerotic lesions (23). The CCR2 receptor was increased in fibroblasts isolated from fibrotic pulmonary granulomas (19) and from synoviocytes of rheumatoid arthritis patients, which also showed constitutively high levels of several other chemokine receptors (24).

The characterization of the biological functions of structural cell-bound chemokine receptors is an active area of investigation,

situated at the crossroads of research in cancer, inflammation and infectious diseases. Several studies indicate that the main biological functions mediated by chemokine receptors on structural cells are: 1) the regulation of cell survival and/or cell proliferation; 2) the promotion of cell chemotaxis; and 3) the induction of gene expression. According to the cell type involved and the nature of the genes induced, it appears that chemokines' contribution to the inflammatory process is broadening to include effects on wound healing, tissue remodeling and fibrosis (25, 26). In line with this hypothesis, recent reports have shown CCR3-mediated proliferation, chemotaxis and changes in gene expression in lung smooth muscle cells and fibroblasts (27–29). The involvement of CCR3 in angiogenesis, which is another feature of airway remodeling, through promotion of endothelial cell chemotaxis (30, 31), further supports this hypothesis. As airway EC are key players in the regulation of many of remodeling processes in the lung (5), we tested the hypothesis that epithelial-bound CCR3 would participate in mechanisms of airway mucosal repair. Accordingly, our data indicate that CCL24, a specific CCR3 ligand, accelerated the repair of a wound created in a monolayer of airway EC and that CCR3 mediated this process, as pretreatment with the AstraZeneca small-m.w. CCR3 antagonist significantly and selectively blocked CCL24-induced wound closure. Interestingly, the other two eotaxins, CCL11 and CCL26, displayed similar effects in promoting the repair response. Although we did not test the specificity of their response, this observation suggests that the effect on wounding could be a class-specific rather than a chemokine-specific effect.

In PBEC, stimulation with CCL24 induced both cell chemotaxis and proliferation in a concentration- and CCR3-dependent fashion. These two biological functions are very relevant to the epithelial wound healing, as both migration of cells from the wound edge toward the denuded area, as well as subsequent cell proliferation and differentiation, need to occur for an appropriate wound closure (5). On a molar basis, both EGF and vitronectin displayed higher potency than chemokines in mediating EC proliferation and chemotaxis, respectively. However, the concentrations of CCL24 eliciting EC chemotaxis and proliferation (10 and 100 nM) are equal to those at which other CCR3 ligands, such as CCL11 and CCL13 (MCP-4), induce eosinophil chemotaxis *in vitro* (12). Expression of CCR3 ligands is significantly up-regulated in the airway epithelium of asthmatics and strongly correlates with the influx of inflammatory cells, in particular, eosinophils. Indeed, we showed previously that immunostaining for CCR3 and for its ligands, CCL5 and CCL11, in airway mucosa followed a similar pattern (4). Therefore, it is conceivable that the chemokine levels yielding an appropriate chemotactic signal within the airway epithelium *in vivo* also would be sufficient to modulate the wounding response.

Chemotactic and proliferative responses have been previously reported to be mediated by CCR2 in the tracheal cell line BEAS-2B stimulated with CCL2 (MCP-1), while CXCR3-mediated chemotaxis has been reported in 16-HBE cells (17, 32). A direct comparison of chemokine-mediated chemotactic and proliferative response in airway EC would likely yield relevant information on the general role of chemokine receptors in epithelial wound repair, and its relative role, compared with other established, potent epithelial growth factors, such as epidermal growth factor (EGF) (10).

Our evaluation of gene expression in airway EC following challenge with CCL11 further supports the emerging role of chemokines in mediating the epithelial wound response and the associated remodeling process in the airways. According to the validated results of our array analysis of gene expression conducted in BEAS-2B cells, CCL11 induced a strong up-regulation of members of the fibroblast growth factor (FGF) family. These molecules

are important regulators of the proliferation, chemotaxis and survival of numerous structural cells, including fibroblasts, myofibroblasts, and smooth muscle cells (33). It is well established that EGF plays a key role in the remodeling process through activation of myofibroblasts, enhancement of cell proliferation, and collagen production (34). In a recent study, expression of FGF-1, FGF-2, and their receptor, FGFR-1, was found significantly up-regulated in EC and smooth muscle cells in the airways of chronic obstructive pulmonary disease patients, compared with normals. Their expression correlated with markers of disease activity, and both FGF-1 and FGF-2 induced proliferation of smooth muscle cells *in vitro* (35). Similarly, other factors up-regulated in EC following CCL11 stimulation, such as IL-6, CCL2, CXCL8, leptin, and IL-22, could convey chemokines' action on wound healing. In fact, using cutaneous or vascular *in vitro* or *in vivo* wounding models, these molecules have been recognized for their role in wound repair (26, 36–39). Therefore, by promoting the secretion of a host of profibrogenic factors, the effect of chemokines could expand beyond the injured epithelial surface toward the submucosa, potentially affecting myofibroblasts and smooth muscle cells, and thereby enhancing lung remodeling. Interestingly, CCL11 also induced the expression of TNFSF4 and TNFSF14, two members of the TNF- α superfamily. The costimulatory molecule TNFSF4 (or OX40 ligand) induces IL-4 release by T cells, which transiently express its receptor, OX40, following TCR stimulation (40). Recently, OX40 ligand has been described on human airway smooth muscle cells, and its expression was found to be significantly up-regulated by TNF- α in smooth muscle cells from asthmatics vs control subjects (41, 42). Another member of the TNF family, TNFSF14, binds to stromal cell-expressed LT β receptor and displays important costimulatory functions in T cell activation through binding to the T cell-expressed HVEM receptor (43). Expression of these costimulatory molecules on airway EC have never been reported so far, although members of the B7 family have been identified on EC (44–46), further supporting the notion that airway epithelium may play an active role in T cell activation.

For most targets, the amplitude of the response to CCL11 challenge in the arrays varied significantly. The signaling of chemokine receptors is influenced by a complex and not fully characterized network of chemokine ligands with both agonistic and antagonistic effects (47). It is possible that other chemokines derived from epithelium, or present in the serum contained in the medium, could have affected the basal level of CCR3 in EC, as well as the response to CCL11.

Alteration of airway epithelial signaling for wound repair and remodeling is increasingly viewed as one of the key initial pathogenetic events that lead to self-sustaining mucosal injury and remodeling in asthma (34). We propose that, following the initiation of such epithelial response by environmental stimuli, this process can be further sustained by chemokines produced in response to ongoing inflammation. It can be envisioned that CCR3 ligands, secreted either by EC themselves or by infiltrating cells in response to inflammatory signals, could sustain a prolonged repair response and aberrant activation of remodeling responses by promoting EC chemotaxis and proliferation through the epithelial-bound CCR3.

The lack of inhibition of CCL11-induced gene expression in BEAS-2B by a specific CCR3 antagonist suggests that this process, differently from the chemokine-induced EC chemotaxis and proliferation, occurs through the activation of other epithelial-derived mediators that indirectly amplify chemokine action. It also can be hypothesized that the lack of an inhibitory effect by CCR3 antagonism of CCL11-mediated responses could be due, at least in part, by agonistic effect of CCL11 on CCR5 (15), as we have evidence of robust expression of this receptor on BEAS-2B cells (48).

The strong immunostaining in bronchial biopsies of asthmatics showing consistent inflammatory changes, compared with the weak staining in nondiseased, noninflamed samples, further suggests that epithelial CCR3 is well represented in an inflammatory milieu to perform its functions. A similar increase in CCR3 expression has been also reported in airway smooth muscle cells (28). The inconsistent up-regulation of epithelial CCR3 surface expression observed *in vitro* after cell challenge with CCL11 and CCL24, together with the highly variable basal receptor levels, points at the existence of a level of regulation *in vivo* of higher complexity, possibly involving translational mechanisms and genetic factors influencing the basal CCR3 expression levels. Another intriguing possibility is that CCR3 could be shed from the EC surface in the context of microparticles. These small membrane vesicles are released from several immune cell types and can activate a host of cells by transferring cell surface proteins as well as functional cytoplasmic components of the original cell. The chemokine receptor CCR5 was found to be released through microparticles from PBMC and transferred to CCR5⁻ cells, rendering them CCR5⁺ and susceptible to HIV infection (49).

It still remains to be established, by studying well-characterized groups of asthmatic patients, whether epithelial CCR3 expression or function bears any correlation with the degree of asthma severity, and whether it is in particular associated with histological signs of remodeling. Similarly, it will be important to establish its role in other diseases characterized by increased epithelial proliferation, inflammation, and remodeling, such as chronic obstructive pulmonary disease. Furthermore, studies are needed to characterize the signaling pathways responsible for the proliferative and chemotactic responses and for the activation of gene expression.

The induction of selected chemokines and chemokine receptors by EC stimulation with CCL11 also suggests that epithelial CCR3 may have a role in further sustaining other chemokine-mediated functions in the mucosa. Although chemokine up-regulation induced by CCL11 was not inhibited in this study by CCR3 antagonism, autoregulation of CCR3 by CCL11 and CCL26 has been previously reported in normal bronchial EC and in the alveolar EC line A549, respectively (50, 51), suggesting the existence of a complex regulatory feedback system, similar to that documented for other receptors, such as the high-affinity IgE receptor (52, 53). A complex role of CCR3 in regulating local chemokine levels is further suggested by the reported involvement of the CCR3 expressed on human dermal microvascular EC in mediating the suppression of TNF- α -induced CXCL8 in cells treated with CCL11 (54).

In summary, we propose that CCR3 ligands play a novel and important role in airway inflammation by promoting epithelial wound repair, and subsequent tissue remodeling, through CCR3-driven enhancement of epithelial proliferation and chemotaxis, associated with the induction of a pattern of gene expression consistent with profibrotic and proinflammatory activities. In concert with similar functions conveyed on CCR3-bearing smooth muscle, fibroblasts, and endothelial cells (4, 27–30), epithelial-bound CCR3 could participate to the development of the structural remodeling changes observed in asthmatic airways and possibly, in airway remodeling observed in other inflammatory lung diseases. Recent key studies are corroborating these findings *in vivo*, as CCL11^{-/-} mice showed a significant reduction of fibrosis in a model of bleomycin-induced lung injury (55). The growing recognition of the role of CCR3 in epithelium and in other structural cells in the lung, together with the key role of CCR3 and its ligands established for eosinophil recruitment in experimental asthma (56), indicates that targeting CCR3 in the lung would have an effect broader than previously appreciated. In fact, by affecting CCR3-driven functions in lung structural cells, such as those we describe

in this study, such treatment could help controlling key pathophysiological components of airway inflammation and remodeling, and such effect would synergize with those accomplished by blockade of CCR3-driven leukocyte recruitment.

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