Autoregulation of CCL26 synthesis and secretion in A549 cells: a possible mechanism by which alveolar epithelial cells modulate airway inflammation

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Abonyo, B. O., M. S. Alexander, and A. S. Heiman. Autoregulation of CCL26 synthesis and secretion in A549 cells: a possible mechanism by which alveolar epithelial cells modulate airway inflammation. *Am J Physiol Lung Cell Mol Physiol* 289: L478–L488, 2005. First published April 29, 2005; doi:10.1152/ajplung.00032.2005.— Eotaxins (CCL11, CCL24, CCL26) originating from airway epithelial cells and leukocytes have been detected in bronchoalveolar lavage of asthmatics. Although the alveolar epithelium is the destination of uncleared allergens and other inflammatory products, scanty information exists on their contribution to the generation and regulation of the eotaxins. We envisioned a state whereby alveolar type II cells, a known source of other inflammatory proteins, could be involved in both the production and regulation of CCL24 and CCL26. Herein, we demonstrated that all three eotaxins are constitutively expressed in A549 cells. IL-4 and IL-13 stimulated a concentration-dependent secretion of CCL24 and CCL26. The cytokines did not act synergistically. Cycloheximide and actinomycin D abrogated IL-4- and IL-13-dependent CCL26 but not CCL24 secretion. Both IL-13 and IL-4 stimulated CCL26 synthesis that was inhibited in a concentration-dependent manner by CCL26 but not CCL24. Only CCL26 reduced expression of CCR3 receptors by 30–40%. On the other hand, anti-CCR3 pretreatment reduced IL-4+IL-13-dependent CCL26 secretion, implying autoregulation. A CCR3-specific antagonist (SB-328437) significantly decreased IL-4-dependent synthesis and release of CCL26. Eosinophils treated with medium from IL-4-stimulated A549 cells preincubated with anti-CCL26 showed a marked decrease of superoxide anion production compared with anti-CCL24 treated. These results suggest that CCL26 is a major eotaxin synthesized and released by alveolar epithelial cells and is involved in autoregulation of CCR3 receptors and other eotaxins. This CCL26-CCR3 ligand-receptor system may be an attractive target for development of therapeutics that limits progress of inflammation in airway disease.

eotaxins; airway epithelial cells; CCR3 receptors

VARIETIES OF PATHOLOGICAL, STRUCTURAL, and functional changes occur in the airway epithelium in inflammation associated conditions such as acute respiratory distress syndrome (ARDS), alveolar reperfusion injury [acute lung injury (ALI)], and asthma (10, 24, 26). Creola bodies in sputum of asthmatics (11) and increased numbers of epithelial cells in bronchoalveolar lavage (BAL) of subjects with asthma (15) are strong indications that the airway epithelium (including alveolar epithelium) may be involved in asthma progression. Airway epithelium is composed of specialized cells performing specialized functions, hence, each region of the airway epithelium could participate and respond differently when exposed to inflammation-inducing agents.

The involvement of conducting zone airway epithelial cells in asthma progression is well documented. Apart from acting as a physical barrier to allergens and infiltration of cells from the circulation, they also secrete cytoprotective molecules and engage in communication with cells of the immune and inflammatory systems. Bronchial epithelial cells in asthma, for example, have increased expression of membrane markers such as ICAM-1 or human leukocyte antigen-DR (43) and increased release of proinflammatory mediators (8). The respiratory zone airway epithelium including the alveolar epithelium is the terminal destination for uncleared allergens as well as proinflammatory chemicals produced locally and those originating from the conducting zone. The epithelium in this region is a thin simple squamous epithelial wall composed of alveolar type I and alveolar type II cells. The multifunctional cuboidal alveolar type II cells produce surfactant, are important for active alveolar liquid clearance, and represent the progenitor cells that regenerate the alveolar epithelium after injury. Several studies have demonstrated the importance of the alveolar type II cells in the pathogenesis of and recovery from severe ALI and ARDS. Such recovery processes have been associated with regulation of release and synthesis of cytokines such as IL-1β (for review see Ref. 18). With respect to asthma, less is known about alveolar epithelial type II cell activation and responses. To improve treatment of airway diseases, it is therefore important to elucidate the interactions between signaling chemicals produced by the alveolar epithelial cells and other resident and recruited cells.

CC chemokines and T helper (Th) 2-derived cytokines appear to be crucial for progression of asthma and other inflammatory diseases. Of all the Th2-derived cytokines, recent evidence suggests that a dominant signaling cascade involving IL-4 and IL-13 plays a critical role in the development and pathogenesis of asthma (33). Both cytokines are produced at elevated levels in the asthmatic lung and allergic tissues. Although the amino acid homology of the two cytokines is only 25%, they are structurally similar, partially share the IL-4/IL-13 receptor complex and signal pathways, and thus have overlapping effector functions (25). IL-13 is considered a key effector cytokine in asthma because it regulates eosinophilic inflammation and mucus production, promotes epithelial damage and airway hyperresponsiveness, and may stimulate airway fibrosis (12, 22, 47).

IL-4 and IL-13 released by Th2 cells induce the expression of CC chemokines including CCL11, CCL24, and CCL26 that act through CCR3 receptors to attract eosinophils into the airway (32, 50). During inflammation, recruited cells, includ-

ing macrophages, lymphocytes, and eosinophils may well become significant sources of these chemokines (51) and possess CCR3 receptors through which the eotaxins may exert further effects. The fact that a single cell possesses both the receptor and its specific ligands implies existence of a complex regulatory mechanism (39a). However, a detailed underlying inflammation regulatory mechanism involving these cytokines and chemokines is currently being unveiled. It simply starts by Th2 cytokines, predominantly IL-13 and IL-4, acting upon resident cells to stimulate release of eotaxins and other chemokines. In response to these selective signals, eosinophils emigrate into pulmonary tissues. The activated eosinophils further contribute to inflammation by producing mediators including IL-4, IL-13, and eotaxins that functionally interact (39). These mediators signal resident smooth muscle and endothelial and epithelial cells to engage in cyclic chemokine-cytokine target/effector cell responses that perpetuate the inflammation in airway diseases.

There is compelling evidence that the airway epithelium is a cytokine-stimulated source of CCL11, the earliest known eotaxin family member (17, 31). In bronchial biopsies and BAL fluid cells obtained from normal control subjects, asthmatics, and challenged asthmatics, CCL11 gene expression was upregulated in asthmatic subjects but was not elevated during the 24-h postchallenge periods. In contrast, eotaxin-3 mRNA expression in asthmatics was dramatically enhanced only after challenge. These data suggest that CCL26 rather than CCL11 or CCL24 may account for the ongoing eosinophil recruitment to asthmatic airways (6). Recent reports indicate that bronchial epithelial cells had increased levels of CCL11, CCL24, and CCL26 when stimulated by IL-4 and IL-13 (29). In an attempt to understand regulation of cytokines and eotaxins by the alveolar epithelium during asthma, we recently reported that cytokine-stimulated alveolar type II epithelial cells are also a pulmonary source of CCL11 and a rich source of CCL24 and CCL26. However, the cells appeared to release very large quantities of CCL26, triggering a question as to whether it had a special role in alveolar epithelial cells. We also reported that alveolar epithelial cells possess constitutive eotaxin CCR3 receptors that are increased by the TNF-α, IL-1β, and IL-4 cytokine stimulation that concomitantly stimulated the release of the eotaxins (20). These results support the postulation that alveolar autocrine/paracrine CCR3-ligand interactions may contribute to the perpetuation of the underlying inflammation in pulmonary diseases such as asthma.

In this regard we hypothesized that expression and release of eotaxins, particularly CCL24 and CCL26 by alveolar type II cells, are regulated by their own interaction with CCR3 receptors as well as stimulant cytokines. The data presented here indicate that the predominant eotaxin synthesized and secreted by alveolar type II epithelial cells in response to IL-4 stimulation is CCL26. CCL24 synthesis was not affected by either IL-4 or IL-13. CCL26-treated cells showed a marked decrease in IL-4-dependent synthesized and secreted CCL26 as well as CCR3 receptors, implying the involvement of CCL26 in autoregulation. A CCR3-specific antagonist significantly decreased IL-4-dependent release of CCL26 but not CCL24, further confirming cyclic regulation of CCL26 and CCR3 in an IL-4-dependent manner. Eosinophils treated with medium from IL-4-stimulated A549 cells preincubated with anti-CCL26 showed a marked decrease in superoxide anion ($O_2^-\cdot$) produc-

tion compared with anti-CCL24-treated media, indicating that CCL26, derived from alveolar epithelial cells, has the capacity to induce eosinophil hyperresponsiveness. Apart from establishing that CCL26 may be an appropriate target for limiting progression of inflammation in the alveoli of asthmatics, these findings also place alveolar type II epithelial cells at the forefront of the first inflammatory responders capable of contributing to progression of inflammation in asthma and other inflammatory diseases.

MATERIALS AND METHODS

Culture and stimulation of airway epithelial cells. Human A549 alveolar type II epithelium-like cells (ATCC CCL-185) were purchased from American Type Culture Collection and grown in RPMI-1640/F-12K (50:50 vol/vol) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% carbon dioxide at 37°C. Trypsin/EDTA- or PBS/EDTA-dispersed cells were suspended in fresh medium in flasks or wells at 0.2×10^6 or 1×10^6 cells/ml, respectively. Experiments were performed after subcultured cells had reached ~80% confluence. Viability of cells used for experiments was assessed by trypan blue exclusion and the LIVE/DEAD viability-cytotoxicity calcein AM/ethidium homodimer-1 fluorescence assay (Molecular Probes, Eugene, OR). Only populations of cells with viability >93% were used for experiments. Before stimulation, cells were incubated in serum-free medium and then stimulated in fresh serum-free medium with indicated concentrations and combinations of chemokines and/or cytokines (Atlanta Biologicals, Atlanta, GA) (3, 20).

Detection of eotaxins in alveolar epithelial cells by immunocytochemistry. A549 alveolar type II epithelium-like cells (1×10^5 cells/0.1 ml) were cultured in 24-well plates for 24 h. Cells were treated with PBS or 30 ng/ml of IL-4 and/or IL-13 in serum-free media for 24 h. They were fixed in 0.4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X (TX)-100 for 5 min. Nonspecific antibody binding was blocked by treatment with 10% normal goat serum for 1 h followed by incubation with 15 μg/ml of goat anti-human CCL11, CCL24, CCL26, no primary antibody, or goat IgG. Cells were then washed three times with PBS and incubated with 1:100 dilution of FITC-conjugated anti-goat secondary antibody. Cell images were visualized and captured by an Olympus fluorescent microscope ($\times$40 objective) fitted with an Olympus DP70 camera. Images were documented using Adobe Photoshop.

CCL24 and CCL26 detection by specific ELISA. After stimulation of A549 epithelium-like cells for 24 or 48 h with the indicated cytokines, supernatants were removed and centrifuged at 100 g for 5 min at 4°C. Resulting supernatants were immediately assessed for presence of each of the eotaxins by specific ELISA (R&D Systems, Minneapolis, MN). To detect nonsecreted CCL24 and CCL26, cells were lysed in lysis buffer [20 mM Tris (pH 7.4), 2 mM EDTA, 150 mM NaCl, and one tablet of protease inhibitor cocktail/10 ml; Sigma-Aldrich Chemical, St. Louis, MO] and sonicated for 3 s. The lysates were then incubated in lysis buffer plus 2% TX-100 at 4°C for 30 min to extract proteins. Samples were diluted 10-fold and centrifuged at 50,000 g for 1 h to separate the supernatant from the membranes. CCL24- and CCL26-specific ELISAs were then conducted on 50 μg of supernatant proteins. These ELISAs recognize both natural and recombinant human CCL24 and CCL26. No cross-reactivity of recombinant human CCL24 and CCL26 was noted when the chemokines were tested at 100 ng/ml. Secreted and synthesized eotaxins were quantified with a Power Wave X 340 microplate reader equipped with KC4 version 3.0 PowerReports software (Bio-Tek Instruments, Winooski, VT).

Detection of alveolar epithelial cell CCR3 receptors by flow cytometry. Cells were detached with 0.5 mM EDTA in PBS, centrifuged at 100 g, 4°C for 5 min, washed twice in cold FACSflow buffer (BD

Biosciences, San Jose, CA), and resuspended in PBS to a final concentration of 5×10^6 cells/ml. Aliquots of cells were stained with 500 ng/ml biotinylated human recombinant eotaxin or the equivalent amount of negative control biotinylated soybean trypsin inhibitor for 60 min at 4°C followed by 1 μg/ml fluorescein conjugated avidin (Fluorokine flow cytometry reagents, R&D Systems) for 30 min in the dark at 4°C. After two washes in cold FACSflow buffer, stained cells were maintained at 4°C and then subjected to flow cytometry on a FACSCalibur, and data were analyzed using CellQuest software (BD Biosciences, San Jose, CA) (20, 50).

Western immunoblotting of the CCR3 receptor. To assess effects of cytokines on CCR3 receptor expression, A549 airway epithelium-like cells (1×10^6 cells/well in 24-well cluster plates) were stimulated for 24 h with the indicated cytokines. After stimulation, cells were trypsin/EDTA detached, resuspended in RPMI-1640 containing 10% fetal calf serum, and centrifuged at 100 g, 4°C for 5 min. Supernatants were removed, and cells were washed with 1 ml of cold, sterile Hanks' balanced salt solution (HBSS) and centrifuged as described above. All sample tubes were placed on ice and incubated for 5 min after adding 20 μl of lysis buffer. Cell lysates were centrifuged at 50,000 g, 60 min, 4°C, and supernatants were collected for protein analysis. Protein concentrations in the lysates were assessed by the Bio-Rad "DC" protein assay (Bio-Rad Laboratories, Hercules, CA) and quantified with the microplate reader described above. Cell lysates were separated by electrophoresis on 10% SDS-polyacrylamide gels (35 μg protein/lane) and then transferred to Immobilon-P polyvinylidene difluoride membranes. Equal loading was verified by staining with Ponceau S (Sigma-Aldrich Chemical Co, St. Louis, MO). Blots were blocked at 4°C overnight in 5% Carnation Instant Milk in Tris-buffered saline with 0.05% Tween 20 in PBS (PBST) and then incubated overnight at 4°C with a 0.75 μg/ml rabbit anti-human CCR3 affinity-purified antibody (IMGENEX, San Diego, CA). Membranes were washed three times with PBST and incubated with 1:2,000 goat anti-rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) in PBST overnight at 4°C. The membranes were washed three times in PBST and rinsed twice with PBS. Immunoblot images were obtained using a Fluor-s Max Multimager (Bio-Rad Laboratories).

RNA extraction and CCL24, CCL26, and CCR3 PCR. A549 airway epithelium-like cells (0.25×10^6 cells/well in six-well cluster plates) were treated with 20 ng/ml CCL26 or CCL24 for 30 min followed by stimulation for 24 h with the indicated cytokines. Cells were washed three times with cold RPMI containing 10% fetal calf serum, lysed with Trizol (Life Technologies, Rockville, MD), and RNA extracted according to the manufacturer's protocol. DNA contamination, purity, and quality were determined with an ultraviolet spectrophotometer and by gel electrophoresis. First-strand cDNA was synthesized from 5 μg of total RNA in a 100-μl reaction volume by reverse transcription using an iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's protocol. cDNA of 2 μl was amplified by PCR in a 50-μl reaction volume containing 10 mM dNTP, 2.5 units *Taq* polymerase, 0.3 μM CCR3 primers, and 0.5 μM CCL24/CCL26 primers and was inner controlled with GAPDH. CCR3 and GAPDH R&D Systems primer sequences are proprietary. Primer sequences for CCL24 and CCL26, designed according to the Promega OligoPerfect Designer program were as follows: CCL24 forward primer: 5′-CACATCATCCCTACGGGCTCT-3′, reverse primer: 5′-GGTTGC-CAGGATATCTCTGACAGGGGG-3′. CCL26 forward primer: 5′-GCCTGATTTGCAGCATCATGATGG-3′, reverse primer: 5′-CG-GATGA CAATTCAGCTGAGTCAC-3′. PCR conditions for CCR3 were: predenaturation 94°C for 5 min, followed by 32 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s followed by final extension at 72°C for 10 min, whereas those of CCL24 and CCL26 were 94°C for 5 min, then 34 cycles of amplification at 94°C for 30 s, at 55°C for 30 s, and at 72°C for 30 s, and extension at 72°C for 10 min. Aliquots of the PCR products (20 μl) were separated and visualized with ethidium bromide staining after electrophoresis in a 2% agarose gel in

Tris borate ethylenediamine tetra-acetic acid buffer, pH 8.3, at 100 V for 1 h, and expression was captured by a Fluor-s Max Multimager (Bio-Rad Laboratories).

Culture and stimulation of human clone 15 HL-60 eosinophilic cells. Clone 15 HL-60 eosinophils (ATCC CRL-1964) were purchased from American Type Culture Collection. Final differentiation and culturing were carried out in RPMI-1640 (Cellgro by Mediatech, Herndon, VA) supplemented with 10% fetal calf serum, 10 ng/ml IL-5 (Atlanta Biologicals), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% carbon dioxide at 35°C as previously described (2). Viability of cells harvested for experiments was assessed by trypan blue exclusion, and populations of cells with viability >95% were used for generation of superoxide. For experiments, cells were centrifuged (50 g, 5 min) washed two times in HBSS. $O_2^- \cdot$ generation was assessed in microtiter plates with 1×10^5 cells/well in a total volume of 0.1 ml HBSS containing 0.2% bovine serum albumin, 80 μg ferricytochrome C with and without 30 μg superoxide dismutase (2), and 50 μl A549 cell culture medium. A549 cells were cultured as indicated above and stimulated with IL-4/IL-13 (0–100 ng/ml) for 48 h. Culture medium was collected and centrifuged. Supernatant aliquots received no further pretreatment or were pretreated with mouse anti-CCL24 or isotype control mouse IgG, goat anti-CCL26, or isotype control goat IgG (R&D Systems). In indicated experiments, aliquots of eosinophils were pretreated with 55 nM of the CCR3 antagonist (S)-methyl-2-naphthoylamino-3-(4-nitrophenyl)-propionate (SB-328437) (EMD Biosciences, La Jolla, CA) for 2 min (5). Microtiter plates were incubated at 35°C in an atmosphere of 5% CO_2 for 2 h, and absorbances were read at 550 nm to determine superoxide dismutase-inhibitable reduction of ferricytochrome C (2, 40).

Data handling and analysis. Unless otherwise stated, experiments were conducted in triplicate and repeated on at least three or four separate occasions (flow cytometer experiments were performed in duplicate on several different occasions). Unless otherwise stated, all data are expressed as means ± SE with the mean of triplicates from one experiment serving as one observation. When indicated, one-way analysis of variance followed by either the Tukey multiple comparisons or Dunnett posttest, as appropriate, was applied to experimental results to determine statistical significance ($P < 0.05$) between indicated groups.

RESULTS

Eotaxins CCL11, CCL24, and CCL26 are constitutively expressed in A549 alveolar type II epithelial cells and released by stimulation with IL-4 and/or IL-13. We have recently reported that in response to TNF-α, IL-1β, or IL-4 alone or in combination, alveolar type II epithelium-like cells differentially release all three of the eotaxins. Considering the recent evidence suggesting that the IL-4/IL-13 signaling cascade plays a central role in the development and pathogenesis of airway diseases such as asthma, the effects of these cytokines on alveolar type II cells require clarification. To explore the possibility that alveolar type II cells synthesize and store the cytokines, cells were cultured and probed for the eotaxins with specific antibodies. The panels depicted in Fig. 1 demonstrate that unstimulated A549 cells in culture do endogenously express the eotaxins. Differences in fluorescence intensity of cells following stimulation with IL-4 and/or IL-13 could not easily be discerned (not shown). Because small changes in fluorescence may have been due to cytokine-induced release of the eotaxins, cells were stimulated with IL-4, IL-13, and the two in combination for 24 or 48 h. CCL24- and CCL26-specific ELISAs were used to quantify released eotaxins. Both IL-4 and IL-13 induced concentration-dependent increases in extracel-
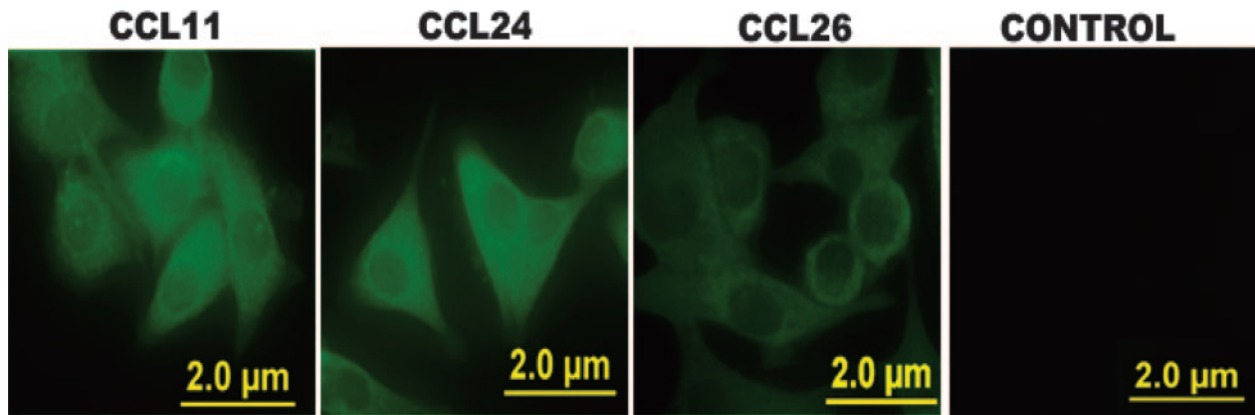
Fig. 1. CCL11, CCL24, and CCL26 are constitutively expressed in unstimulated A549 cells. A549 cells ($1 \times 10^5/100$ μl) were cultured in RPMI-1640/F-12K plus 10% FBS for 24 h. The monolayers were bathed in PBS then cultured in serum-free media for 24 h, fixed in 0.4% paraformaldehyde for 20 min, and permeabilized with 0.2% Triton X (TX)-100 in PBS for 5 min. Nonspecific binding was blocked by treatment with 10% normal goat serum for 1 h, then cells were incubated with 15 μg/ml of goat anti-human CCL11, CCL24, CCL26, or goat IgG. Cells were washed three times with PBS and incubated with 1:100 dilution of FITC-conjugated anti-goat secondary antibody. Images are representative of several pictures taken from duplicates of 3 separate experiments.

lular CCL24 and CCL26, but only CCL24 was released from unstimulated cells (data not shown). As illustrated in Fig. 2, significant amounts of both chemokines were released during the 24- to 48-h interval of cytokine stimulation, and IL-4 and IL-13 did not act synergistically.

IL-4 and IL-13 differentially regulate expression of CCL24 and CCL26 in A549 cells. The robust increases in release of the chemokines suggested that the stimulating cytokines may be modulating CCL24 and CCL26 synthesis. To explore this possibility, cells were treated with IL-4 and/or IL-13 for 24 and 48 h. Cell lysates were then prepared, and intracellular CCL24 and CCL26 were quantified by ELISA. Results are depicted in Fig. 3. In Fig. 3*A*, results with 24-h lysates indicate significant IL-4- and IL-13-stimulated increases in CCL26 but not CCL24. Further increases in CCL26 synthesis were noted in lysates prepared from cells treated with IL-4 for 48 h as seen in Fig. 3*B*. In contrast, much smaller changes were noted in amounts of intracellular CCL24. To further investigate the effects of IL-4 and IL-13 on chemokine transcription and protein synthesis, cells were pretreated with actinomycin D or cycloheximide, and released eotaxins were measured. Results are depicted for CCL24 in Fig. 4*A* and CCL26 in Fig. 4*B*. Actinomycin D treatment did not alter untreated cell release of CCL24 but did inhibit IL-4- and IL-13-stimulated increases in released CCL24. Protein synthesis of CCL24 was equally inhibited in untreated and all treated groups. In contrast, pretreatment of cells with actinomycin D or cycloheximide completely abrogated release of CCL26 induced by either IL-4 or IL-13.

These results indicate that airway epithelial cells constitutively transcribe, synthesize, store, and release CCL24, whereas CCL26 transcription and synthesis occur predominantly after IL-4 and IL-13 stimulation.

IL-4 and IL-13 regulate the expression of CCR3 receptors in A549 alveolar type II epithelial cells. In previous investigations we have reported that A549 cells possess CCR3 cell surface receptors that were upregulated by stimulation with combinations of TNF-α, IL-1β, and IL-4. Central to the present investigations are the effects of IL-4 and/or IL-13 on the eotaxin receptor. Representative results are shown in Fig. 5, *A–F*. The histogram overlays in Fig. 5*A* depict negative con-

trols and untreated and treated cells and demonstrate constitutive expression of CCR3 and cell surface upregulation 4 h after stimulation with cytokines alone and in combination. Increased fluorescence intensity was found in 47% of unstimulated cells (Fig. 5*B*), indicative of constitutive CCR3 expression. IL-4 treatments increased fluorescence intensity on 55% of A549 cells (Fig. 5*C*), whereas IL-13 alone did not upregulate cell surface CCR3 expression (Fig. 5*D*). IL-4 and IL-13 together increased fluorescence on 65% of cells in 4 h (Fig. 5*E*). Similar studies were conducted with 24-h treatments in serum-free medium with and without IL-4 and IL-13 (data not shown). IL-13-treated cells showed 32% increased fluorescence, whereas IL-4-treated cells showed 46%. When the cytokines were administered together, 37% of cells exhibited increased fluorescence following a 24-h cytokine exposure. Collectively, these results suggest that stimulation of alveolar type II cells with the Th2 cytokines IL-4 and IL-13 modulates the eotaxin CCR3 cell surface receptor.

Inhibition of CCR3 function regulates IL-4- and IL-13-dependent CCL26 synthesis and release in alveolar epithelial cells. Presence of the CCR3 eotaxin receptor and endogenously expressed and released CCL24 and CCL26 in alveolar type II cells provide conditions for existence of an autoregulatory receptor-ligand loop. To explore this possibility using the A549 cell culture model, cells were pretreated with 0–30 μg/ml monoclonal anti-human CCR3 antibody or 0–100 nM of the CCR3 antagonist SB-328437 then stimulated with 30 ng/ml of IL-4/IL-13. Results with antibody pretreatments, depicted in Fig. 6, demonstrate a concentration-dependent 28–50% decrease in CCL24 or 10–40% decrease in CCL26 release compared with cells stimulated in the absence of antibody or presence of the antibody isotype control. On the other hand, pretreatment with SB-328437 inhibited IL-4-dependent CCL26 secretion by 61% and synthesis by 55% at 100 nM concentration (Fig. 7, *A* and *B*, respectively) compared with 30 and 3% inhibition of CCL24 secretion and synthesis, respectively (data not shown). IL-13-dependent synthesis and secretion of CCL26 were inhibited by 28 and 72%, respectively. The effect of the CCR3 antagonist on CCL24 secretion and synthesis was minimal (data not shown), even though SB-328437 competitively inhibits binding of all CC chemo-
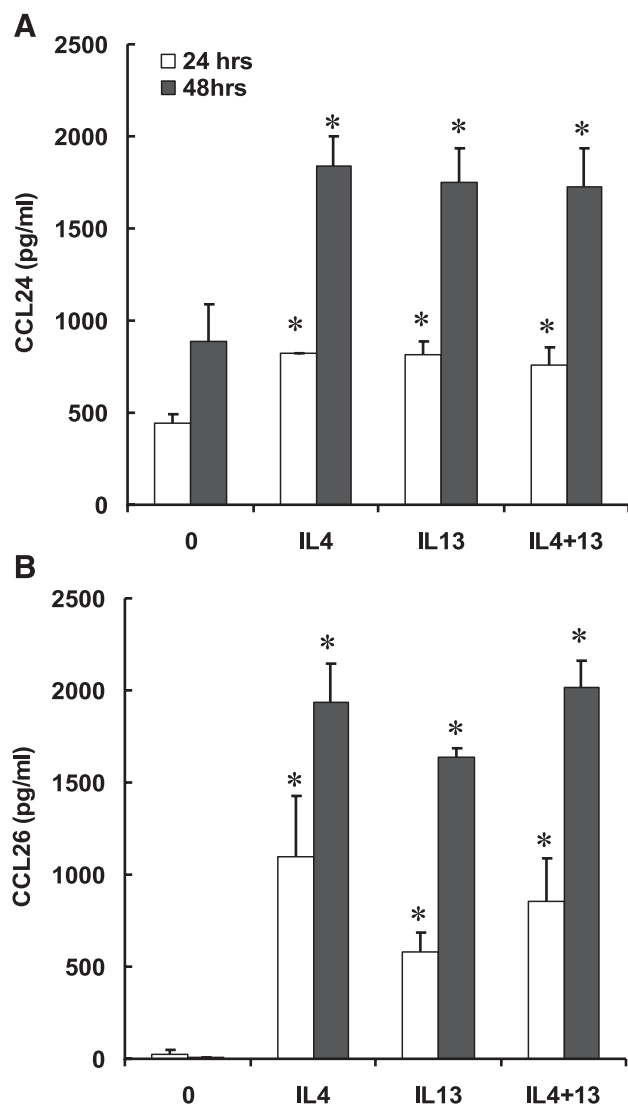
Fig. 2. IL-4- and/or IL-13-stimulated release of CCL24 and CCL26 is time dependent. A549 cells (0.5×10^6/ 0.5 ml of RPMI-1640/F-12K plus 10% FBS) were cultured overnight. Cells were then treated with 30 ng/ml of IL-4 and/or IL-13 in serum-free media for 24 or 48 h. The medium was collected and centrifuged at 1,000 rpm for 5 min to remove cell debris. Supernatants (100 μl) were used to quantify released CCL24 or CCL26 by specific ELISA. The data presented are an average of 3 separate experiments each conducted in triplicate. All treated groups were significantly different from their time-matched controls at $P < 0.05$. *All treated 48-h groups were significantly different ($P < 0.05$) from similarly treated 24-h groups.

effects of CCL26 on its own synthesis and that of CCL24. Results of experiments in which cells were treated with 0–10 ng/ml CCL24 or CCL26 alone or with IL-4 or IL-13 are shown in Fig. 8. CCL26 did not significantly affect its own synthesis when present as the sole agent but did significantly decrease its own synthesis by 48% in cells treated with 10 ng/ml IL-4 (Fig. 8B). Interestingly, CCL26 also decreased CCL24 synthesis by 32% in IL-4-treated cells. However, CCL24 increased its own synthesis in a concentration-dependent manner (Fig. 8A) but failed to produce any notable effect on CCL26 synthesis. Further analysis by Western blot showed a decrease in band intensity in A549 cells treated with 30 ng/ml IL-4 plus 10 ng/ml CCL26 as shown in Fig. 9, *lane 14*. CCL24 did not show
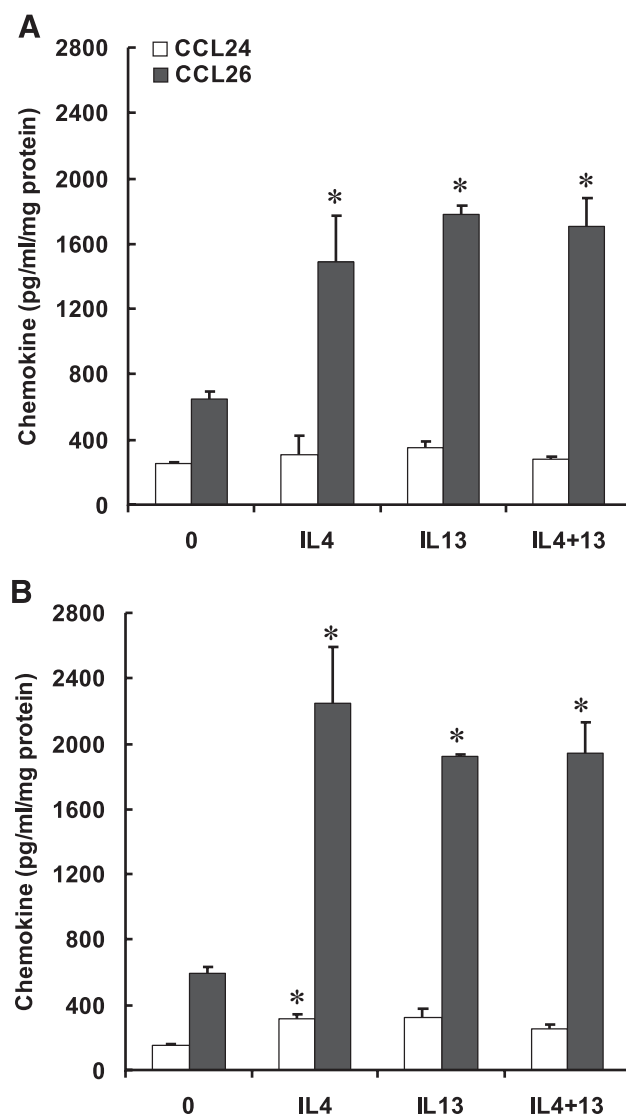

Fig. 3. IL-4 and IL-13 stimulation of A549 cells differentially regulate synthesis of CCL24 and CCL26. A549 cells (5×10^5/500 μl) were cultured for 24 h in RPMI-1640/F-12K supplemented with 10% FBS. Cells were then treated with 30 ng/ml of IL-4 and/or IL-13 in serum-free media for 24 (*A*) and 48 h (*B*), respectively. To extract proteins, the cells were lysed and treated with 2% TX-100 for 30 min in ice. Cell extracts were centrifuged at 50,000 *g* for 1 h to remove membranes. Supernatant was diluted $10\times$ in PBS, and 50 μg of protein were used to quantify cellular CCL24 and CCL26 by specific ELISA. The data presented are an average of 3 separate experiments each conducted in triplicate. *Groups that differed from controls at $P < 0.05$.

kines to CCR3 receptors. It is therefore accurate to suggest that CCR3-CCL26 receptor-ligand interactions play a more central role in alveolar epithelial cell autoregulation than do CCR3-CCL24 interactions. It also implies that IL-4-dependent CCL26 synthesis and secretion are more tightly regulated in A549 cells than CCL24. Collectively, these results suggest that blocking the CCR3 receptors with an antibody or an antagonist prevents released eotaxins from receptor occupancy, which may be needed to maintain prolonged release or synthesis of the chemokines.

Regulation of CCL26 and CCR3 transcription and translation by CCL26. Because IL-4 and IL-13 were shown to increase synthesis of CCL26, it was of interest to explore the
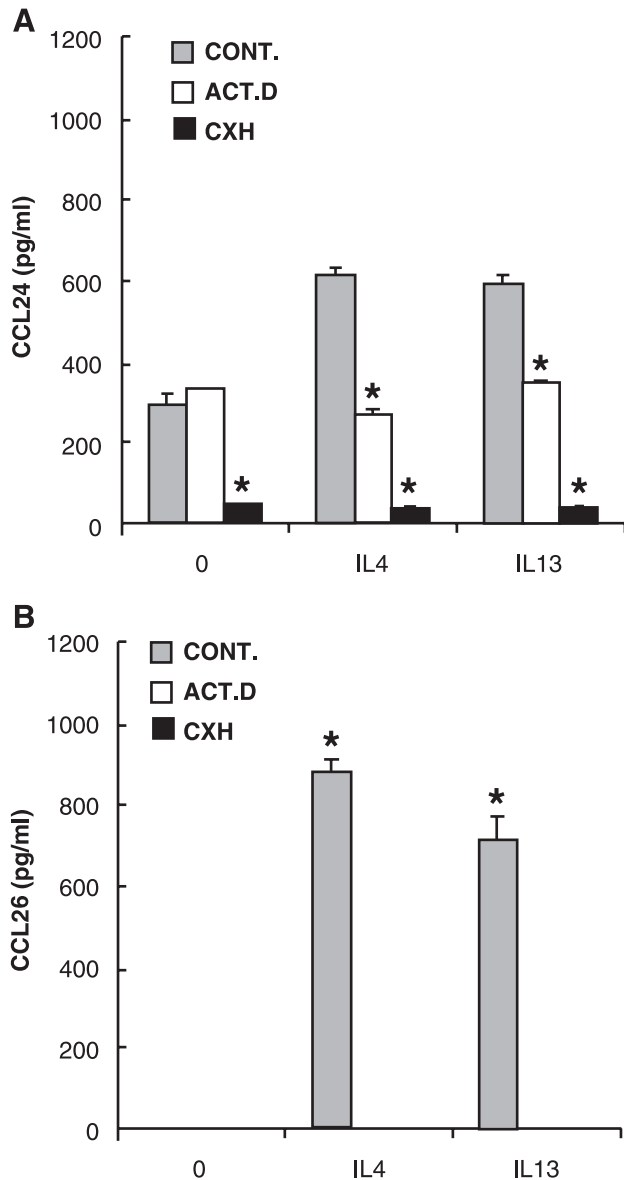
Fig. 4. Actinomycin D (Act.D) and cycloheximide (Cxh) inhibit IL-4- and IL-13-induced release of CCL24 (A) and CCL26 (B) from A549 cells. Cells were cultured as described in Fig. 2 and then treated with 10 μg/ml Chx or 100 nM Act. D for 30 min before stimulation with 30 ng/ml of IL-4 and/or IL-13 in serum-free media for 24 h. The medium was collected and centrifuged at 1,000 rpm for 5 min to remove cell debris. Aliquots (100 μl) of the supernatants were used for analysis of released CCL11, CCL24, or CCL26 by ELISA. Data presented are an average of 3 separate experiments each conducted in triplicate. *Values that differed significantly ($P < 0.05$) from their respective controls.

any effect on the expression of CCR3 protein (Fig. 9). To establish that CCL26 regulated itself and CCR3 at the transcriptional level, we performed RT-PCR on RNA extracted from A549 cells treated with 30 ng/ml IL-4 with or without 10 ng/ml CCL24 or CCL26. As shown in Fig. 10, *lane 4*, CCL26 plus IL-4 reduced the transcription of CCL26 and CCR3 but did not affect the transcription of CCL24. In the absence of Th2-type cytokines, CCL26 exhibited a small downregulation of its CCR3 receptor transcription. Together these results indicate that CCL26 not only tightly regulates its own transcription and hence translation during an allergic response but

also regulates expression of CCR3 in the A549 alveolar type II cell culture model.

CCL26 released by A549 cells mediates eosinophil activation. In asthma, inflammatory processes generate toxic levels of reactive oxygen species (ROS) including $O_2^-\cdot$ by activated eosinophils, alveolar macrophages, and neutrophils (13). To confirm that stimulated A549 cells release CCL26, which may activate the eosinophils and further compound inflammation, we exposed eosinophils to medium of IL-4- or IL-13-treated cells preincubated with anti-CCL26, anti-CCL24, or IgG. In data not shown, $O_2^-\cdot$ generation was initially assessed in clone 15 HL-60 eosinophils treated with 0–100 ng/ml human recombinant CCL24 or CCL26. Results indicated a concentration-dependent generation of $O_2^-\cdot$, reaching a maximum of 2.22 ± 0.19 nmol/10^6 cells with CCL24 and 2.33 ± 0.17 mol/10^6 cells with CCL26. $O_2^-\cdot$ generation by recombinant CCL24- or CCL26-stimulated and CCR3 antagonist-pretreated eosinophils was reduced by 54 and 41%, respectively. Phorbol myristate acetate (1 nM)-stimulated cells served as the positive control and generated 15.7 ± 0.57 nmol $O_2^-\cdot/10^6$ cells. Results of $O_2^-\cdot$ generation by eosinophils treated with media from A549 cells stimulated with IL-4, IL-13, and medium preincubated with anti-CCL26 and anti-CCL24 are shown in Table 1. To demonstrate that chemokine stimulation via the CCR3 receptor was responsible for $O_2^-\cdot$ generation, eosinophils were pretreated with 55 nM SB-328437. These data confirmed that the medium obtained from stimulated A549 cells contained proinflammatory mediators, CC chemokines included, that act through eosinophil CCR3 receptors to generate $O_2^-\cdot$ anion.

DISCUSSION

Interactions of chemokines with their respective receptors are emerging as important events in the selective recruitment, priming, and activation of leukocytes at sites of allergy and/or inflammation. Discovery of cellular sources of the chemokines, their agonist and antagonist activities, signal transduction, and regulatory pathways continue. We have carried out these current studies to test the hypothesis that alveolar epithelial cells engage in cytokine and chemokine (CCL24 and CCL26) mediated target/effector proinflammatory cyclic responses via autoregulatory pathways that perpetuate inflammation in airway diseases such as asthma. The findings demonstrate that alveolar type II cells endogenously express CCL24 and CCL26 and are a constitutive source of low levels of CCL24 and a rich source of CCL24 and CCL26 when stimulated with the Th2-type cytokines IL-4 and IL-13. Constitutive expression of the CCR3 receptor through which the eotaxins exert their agonist activities was also documented as was the dynamic modulation of the receptor by treatment with the chemokines and/or IL-4 and IL-13.

IL-4, produced at elevated levels in the asthmatic lung and thought to be a central regulator of the hallmark features of the disease (51), elicited maximal release of the chemokines from alveolar type II cells. IL-13 also stimulated robust synthesis and release of CCL26 and release but not synthesis of CCL24. These responses could be predicted since IL-4 and IL-13 reportedly elicit overlapping, though not identical, biological effects in a number of settings since the two can utilize a common receptor, composed of IL-4Rα and IL-13Rα1 (25, 44). The emerging paradigm for involvement of IL-13 in
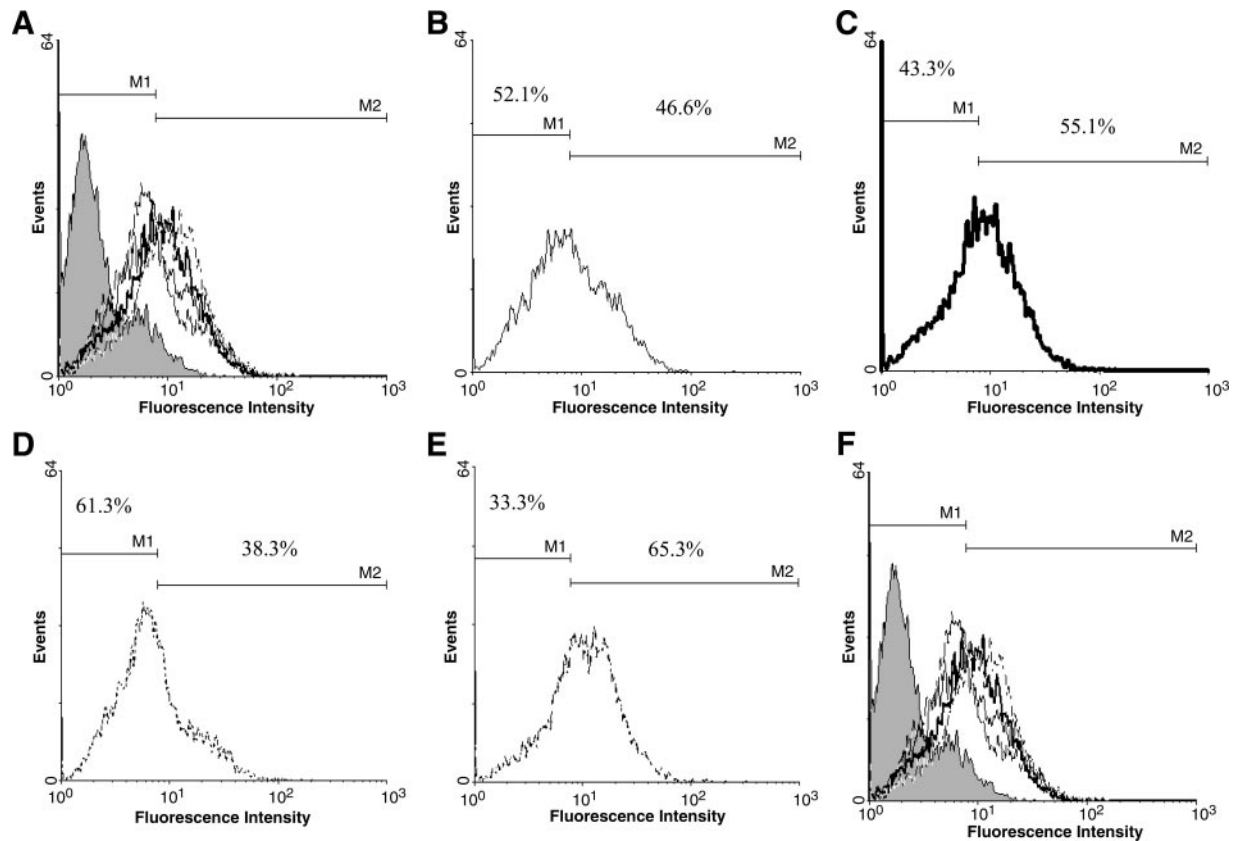
Fig. 5. IL-4 and IL-4/IL-13 upregulated CCR3 receptors on the surface of A549. A549 cells were detached with PBS/0.5 mM EDTA, washed, placed in serum-free DMEM, and aliquoted to 24-well plates at 0.5×10^6 cells·ml^{-1}·well^{-1}. Cells were treated with vehicle (medium), Il-4, IL-13, or the two together at 100 ng/ml. The plate was placed on a microtiter plate rocker, and cells were incubated at 37°C for 4 h, then washed, and stained with biotinylated eotaxin followed with FITC-avidin and analyzed by flow cytometry. *A*: histogram overlay. The filled histogram is the negative control overlaid with *B–E*, where M1 is defined as the negative population and contains 96% of the negative control events acquired, and M2 is defined as the increased fluorescence population. *B*: unstimulated cells. *C*: IL-4 (100 ng/ml, 4 h). *D*: IL-13 (100 ng/ml, 4 h), *E*: IL-4+IL-13 (100 ng/ml, 4 h). *F*: specificity control. Filled histogram, biotinylated soy bean trypsin inhibitor negative control; bold line, biotinylated eotaxin; dashed line, mouse IgG blocked then treated with biotinylated eotaxin that had been incubated with human anti-eotaxin. Data presented are representative of 4 separate experiments each conducted in duplicate.

pathogenesis of airway disease is via a complex array of actions on resident airway cells rather than through traditional effector pathways involving eosinophils and IgE-mediated events (30, 46). Although it has been previously reported that human bronchial epithelial cells and immortalized BEAS-2B bronchial epithelial cells produce various members of the eotaxin family after stimulation with IL-13 and IL-4 (29, 49), results of the present investigations extend the effect to alveolar epithelial cells. An active role for the alveolar epithelium in eosinophilic inflammation in asthma has been postulated and is supported by these present studies (41). Results with the alveolar type II cells suggest that the two cytokines appear to have a more additive rather than synergistic role in release of the eotaxins. An additive effect has also been suggested for IL-4 and IL-13 in an animal model of asthma (45). In humans, polymorphisms in the IL-4/IL-13 cytokine-receptor axis pathway activities may contribute to individual susceptibility and manifestation of asthma (9); therefore, an inhibitor/antagonist that blocks activities of both cytokines may be a very useful agent for treatment of diseases including asthma and other allergic conditions (14, 28).

The eotaxins CCL11, CCL24, and CCL26 are considered specific agonists for the CC chemokine receptor CCR3 (37). Constitutive expression of the CCR3 receptor has been de-
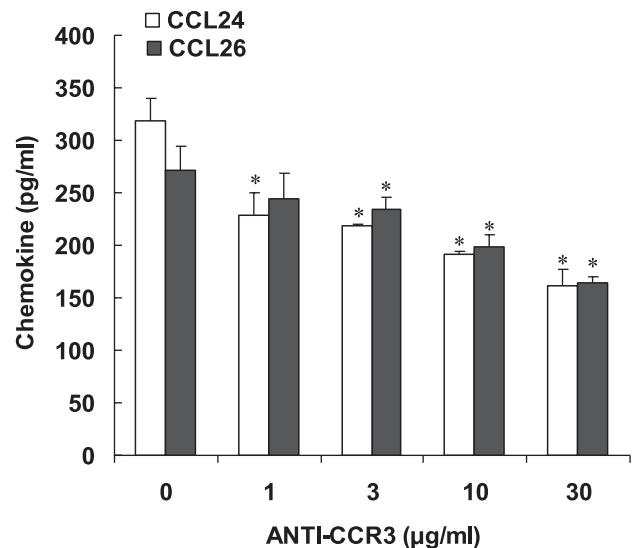

Fig. 6. Human anti-CCR3 antibody modulates cytokine-induced release of CCL24 and CCL26 in A549 cells. Experiments were carried out as described in Fig. 2. Cells were pretreated with 0–30 μg/ml human anti-CCR3 antibody for 30 min before IL-4+IL-13 (30 ng/ml) stimulation. Rat IgG isotype control (30 μg/ml) had no effect on cytokine-induced release of either CCL24 or CCL26 (data not shown). Data shown are means ± SE of 3 experiments each conducted in triplicate. *Groups that differed significantly from cells stimulated in the absence of anti-CCR3 at $P < 0.05$.
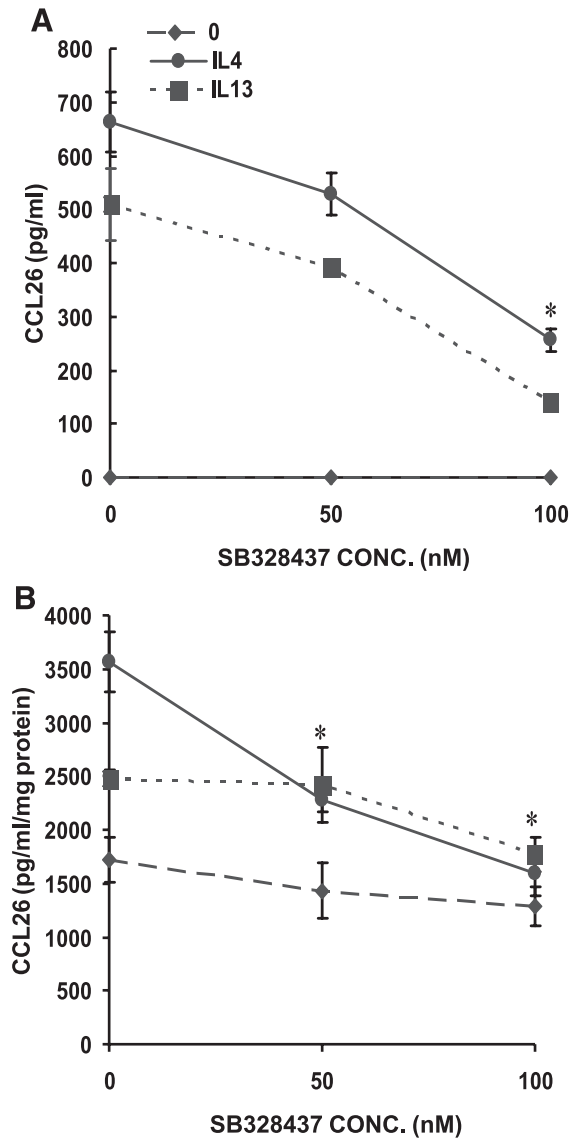
that CCL26-CCR3 pathways significantly modulate the IL-4-stimulated CCR3 expression, whereas CCL24-CCR3 transduction pathways were not involved. Interestingly, CCL24 may be involved in downmodulation of CCR3 in the absence of cytokines. These results suggest that the chemokines CCL24 and CCL26 are involved in modulation of their CCR3 receptor by parallel mechanisms. This finding may be important in pointing out stimulus-dependent signaling pathways that may lead to development and maintenance of the airway epithelium in the "activated phenotype" seen in the asthmatic state. Inflam-

Fig. 7. Pretreatment with the CCR3 antagonist SB-328437 regulates CCL26 secretion. Cells were cultured as described in Fig. 2, pretreated for 30 min with 0–100 nM SB-329437 in serum-free media, and then stimulated with 30 ng/ml of IL-4 or IL-13 for 24 h. The final concentration of DMSO was 0.1% and did not alter release of the eotaxins. *A*: medium was collected as described in Fig. 2, and 100 µl of each supernatant were used to quantify released CCL26 by specific ELISA. *B*: cell lysates were prepared as described in Fig. 3, and 50 µg of protein were used to quantify cellular CCL26 by specific ELISA. The data presented are an average of 3 separate experiments each conducted in duplicate. *Values that differed significantly from cells stimulated in the absence of the antagonist at $P < 0.05$. (*Significance for IL-4-treated samples only).

tected on normal human bronchial epithelial cells, bronchial epithelial cell lines, and A549 human alveolar type II epithelial cells (1, 20, 23, 39a, 42). IL-4 and IL-13, alone and in combination, upregulated surface CCR3 receptors on A549 cells. Binding of anti-CCR3 to cell surface receptors significantly decreased CCL24 and CCL26 release in a concentration-dependent manner, suggesting that CCR3-chemokine ligand autoregulatory signal transduction pathways may be activated. Differential regulation of CCR3 receptors was noted in CCL24- and CCL26-treated cells. Results of experiments with recombinant CCL24- and CCL26-specific antibodies indicate
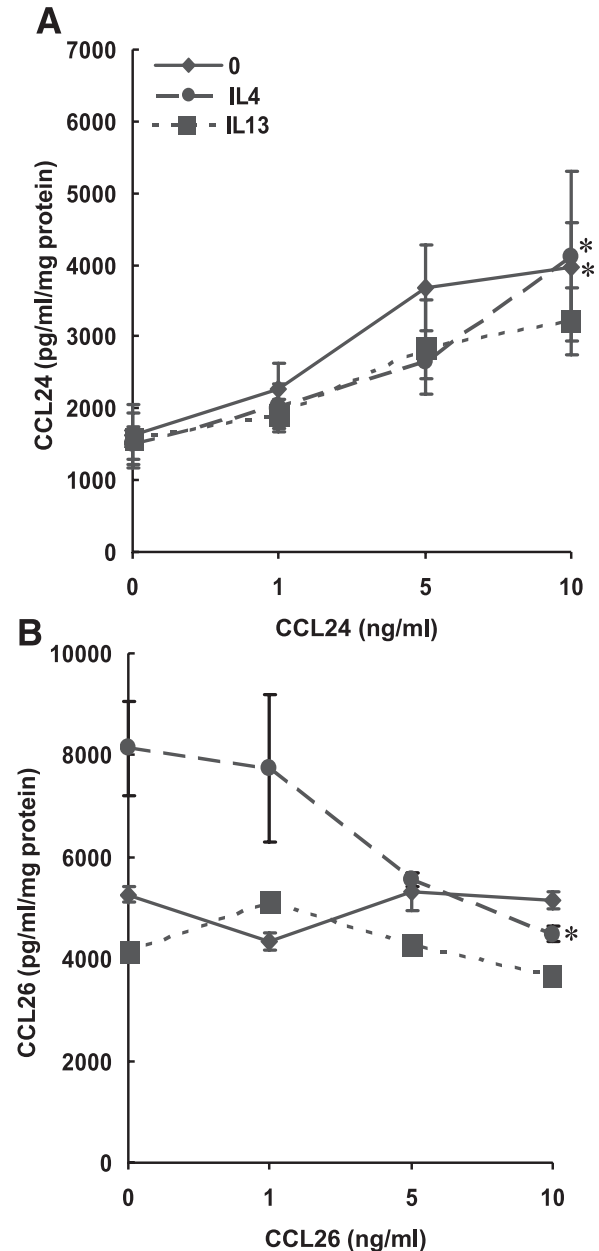
Fig. 8. CCL26 inhibited IL-4-dependent CCL26 synthesis in A549 cells. Cells were cultured as described in Fig. 2 and then treated with 0–10 ng/ml CCL24 (*A*) or CCL26 (*B*) for 30 min before treatment with 30 ng/ml of IL-4 or IL-13 in serum-free media for 24 h. Cell lysates were prepared as described in Fig. 3, and 50 µg of protein were used to quantify cellular CCL24 and CCL26 by specific ELISA. The data presented are an average of 3 separate experiments each conducted in triplicate. *Groups that differed significantly ($P < 0.05$) from controls.
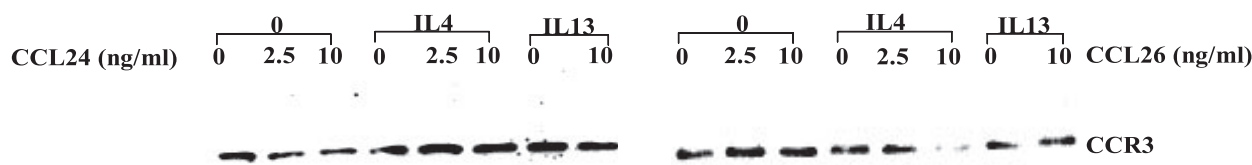
Fig. 9. CCL26 slightly suppressed the expression of CCR3 receptors in A549 cells. Cells were cultured as described in Fig. 2 and then treated with 0–10 ng/ml CCL24 or CCL26 for 30 min before treatment with 30 ng/ml of IL-4 or IL-13 in serum-free media for 24 h. Cells were lysed in lysis buffer, and 40 μg of proteins were used for detection of CCR3 by Western blot. The data presented are representative of 3 separate experiments each conducted in triplicate.

mation may be amplified by local responses of the epithelium, smooth muscle, and fibroblasts through further release of chemokines, cytokines, and proteases that in turn promote persistent disease, amplify inflammation, and cause disease progression (12).

We have recently reported that significant amounts of CCL26, the most recently discovered member of the family of CC chemokines, are released from A549 alveolar type II cells stimulated with combinations of TNF-α, IL-1β, and IL-4 (20). Results of the present investigations indicate that the Th2-type cytokines IL-4 and IL-13 stimulate both the synthesis and release of CCL26. In the absence or presence of Th2-type cytokines, CCL26 exhibits a concentration-dependent downregulation of its CCR3 receptor. In contrast to CCL11 and CCL24, CCL26 did not stimulate its own synthesis and was involved in modulation of both CCL26 and CCL24 in IL-4-stimulated cells. These results are similar to those found in bronchial epithelial cells, endothelial cells, and dermal fibroblasts. CCL26 mRNA expression is reportedly not present in unstimulated NCI-H727 lung epithelial cells. However, IL-4 and IL-13 did induce CCL26 expression in a time- and concentration-dependent manner with IL-4 demonstrating greater potency than IL-13, supporting the conclusion that modulation of CCL26 expression is different from that of CCL11 and CCL24 (4, 34). CCL26 was shown to be upregulated by IL-4 and IL-13 in endothelial cells and its transcription and protein expression likewise stimulated by Th2 cytokines in human dermal fibroblasts (16, 21). Synthesized CCL26 is stored in the Weibel-Palade body from which it is rapidly released after exposure to agents such as histamine and thrombin. CCL26 is also expressed on the endothelial cell surface where it is thought to play a critical role in CCL26-induced transmigration (36). Stimulated release may enable rapid recruitment of leukocytes and paracrine/autocrine modulation of resident cell responses at inflammatory sites. Together, these results suggest that CCL26 may have biological roles distinct from the other two eotaxins. However, the mechanism by which CCL26 regulates itself, other eotaxins, and CCR3 is yet to be established.
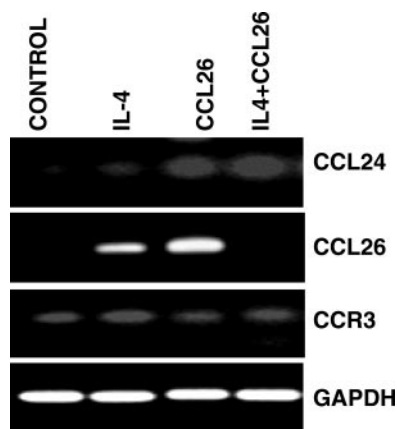

Fig. 10. CCL26 inhibited IL-4-dependent CCL26 and CCR3 RNA expression in A549 cells. A549 airway epithelial cells (0.25×10^6 cells/well in 6-well cluster plates) were treated with 20 ng/ml CCL26 or 24 for 30 min followed by stimulation for 24 h with 30 ng/ml IL-4 or IL-13. The medium was removed, and cells were washed three times with cold RPMI-1640 containing 10% FCS. Cells were then lysed with Trizol and RNA extracted according to the manufacturer's protocol. First-strand cDNA was synthesized from 5 μg of total RNA in a 100-μl reaction volume by reverse transcription using an iScript cDNA synthesis kit. CDNA of 2 μl was amplified by PCR in a 50-μl reaction volume, containing 10 mM dNTP, 2.5 units *Taq* polymerase, 0.3 μM CCR3 primers, and 0.5 μM CCL24/CCL26 primers and was inner controlled with GAPDH. PCR conditions for CCR3 were predenaturation 94°C for 5 min, then 32 cycles of amplification at 94°C for 45 s, at 55°C for 45 s, at 72°C for 45 s, and extension at 72°C for 10 min, whereas those of CCL24 and CCL26 were 94°C for 5 min, 34 cycles of amplification at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and extension at 72°C for 10 min. Aliquots of the PCR products (20 μl) were separated and visualized with ethidium bromide staining after electrophoresis in a 2% agarose gel in Tris borate ethylenediaminetetraacetic acid buffer, pH 8.3, at 100 V for 1 h. The figure is a representative of 3 figures from 3 separate experiments.

Table 1. *Effect of anti-CCL24, anti-CCL26, or SB-327438 on superoxide generation by clone 15 HL-60 eosinophilic cells exposed to IL-4- or IL-13-treated A549 airway epithelial cells*

A549 Cell Treatments	nmol Superoxide/10^6 Eosinophils			
	None	Anti-CCL24, 10 μg/ml	Anti-CCL26, 10 μg/ml	SB-328437, 55 nM
None	3.49±0.418	2.98±0.379†	3.12±0.314†	3.26±0.413
IL-4				
1 ng/ml	5.19±0.519	3.92±0.559	6.03±0.475	3.85±0.495
10 ng/ml	8.49±0.306	6.35±0.919	5.12±0.829*	5.08±0.506*
100 ng/ml	10.28±0.471	7.38±0.372*	4.33±0.712*	4.56±0.606*
IL-13				
1 ng/ml	5.16±0.662	7.67±0.141	4.17±0.412	4.17±0.667
10 ng/ml	5.20±0.774	5.72±0.241	4.96±0.254	2.26±0.773*
100 ng/ml	9.61±0.271	6.23±0.397*	4.80±0.137*	4.41±0.327*

Values are means ± SE. A549 cells were cultured and treated with indicated cytokines for 48 h as described in Fig. 2. Supernatants were collected, centrifuged, and immediately used to stimulate eosinophils. Supernatants (50 μl) were used to stimulate 1×10^5 eosinophils in a total volume of 100 μl containing 80 μg of ferricytochrome C with and without 30 μg of superoxide dismutase. Results of a typical experiment performed in quadruplicate. Supernatants were pretreated with mouse anti-CCL24 or goat anti-CCL26 for 30 min at room temperature. Eosinophils were pretreated for 2 min with 55 nM SB-329437. Supernatants received no pretreatments. Final concentration of DMSO in the assay was 0.1%. †Contains the anti-CCL24 or anti-CCL26 antibody isotype control mouse or goat IgG, respectively. *Values that differed significantly from untreated supernatants at $P < 0.05$.

ROS such as $O_2^-\cdot$, hydrogen peroxide, and hydroxyl radicals contribute to inflammation in the asthmatic airway (40). Asthma attacks and experimental antigen challenge are both associated with immediate formation of $O_2^-\cdot$ (7), which persists throughout late asthmatic response (40a). Using clone 15 human eosinophilic HL-60 cells, we have recently reported that each of the three eotaxins stimulate a concentration-dependent $O_2^-\cdot$ generation, and their bioactivity occurs through occupation of the eosinophil CCR3 receptor. It was suggested that CCL11, CCL24, and CCL26 may play an important role not only in attracting eosinophils to the site of inflammation but also in damaging tissue by their capacity to induce release of ROS (2). This eosinophil experimental paradigm was employed in these present investigations to explore the hypothesis that alveolar epithelial cells indirectly contribute to the high pool of ROS in asthmatic lungs by producing eotaxins that activate eosinophils to produce $O_2^-\cdot$. The data presented here support the hypothesis that A549 alveolar epithelial cells synthesize and release bioactive CCL24 and CCL26 capable of leukocyte activation.

Although asthma symptoms may be acutely controlled, there is a need for therapies that address the underlying immune dysfunction and provide control of chronic airway inflammation. Therapeutic strategies targeting IL-4 and IL-13 or their specific signaling pathways are the current focus of new treatment developments for allergy and asthma. Of course differences in signaling pathways and target genes of these cytokines are not yet clear (19, 27). Similarly, CCR3-ligand signaling pathways are also more complicated than initially proposed as evidenced by emerging data on chemokine receptor-ligand system cross talk. For example, CCL11 may act as a natural antagonist for CCR2 and will bind to CCR5 (35). CCL26 has also been identified as a natural antagonist for CCR2 as well as CCR1 and CCR5 (32). Ligands for the CXCR3 receptor antagonize CCL11 effector cell functions. Although all three eotaxins are inactive at the CXCR3 receptor, CCL11 will bind with high affinity, thus CXCR3 may be a decoy receptor for sequestering the eotaxin (48). It is known, from all these studies, that the eotaxins and a variety of other chemokines interact with CCR3 and that this receptor is expressed on eosinophils, basophils, mast cell subpopulations, activated Th2 cells, macrophages, airway epithelial cell, endothelial cells, and dermal fibroblasts. The report on their synthesis and release, as well as regulation in alveolar epithelial cells in these findings, suggests that the alveolar epithelium may be an attractive target for controlling inflammation as well as limiting eosinophil infiltration and activation. Because CCR3 is closely associated with asthma and allergic conditions, a focused regulation of CCL26 synthesis and release as well as blockade of the CCR3-ligand pathway may have pronounced safe and beneficial effects in controlling these diseases.

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