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CCL26-Targeted siRNA Treatment of Alveolar Type II Cells Decreases Expression of CCR3-Binding Chemokines and Reduces Eosinophil Migration: Implications in Asthma Therapy

Younes J. Errahali, Equar Taka, Barack O. Abonyo, and Ann S. Heiman

The underlying inflammation present in chronic airway diseases is orchestrated by increased expression of CC chemokines that selectively recruit leukocyte populations into the pulmonary system. Human CCL26 signals through CC chemokine receptor 3 (CCR3), is dramatically upregulated in challenged asthmatics, and stimulates recruitment of eosinophils (EOSs) and other leukocytes. CCL26 participates in regulation of its receptor CCR3 and modulates expression of a variety of chemokines in alveolar type II cells. Utilizing the A549 alveolar type II epithelial cell culture model, we carried out studies to test the hypothesis that CCL26-siRNA treatment of these cells would ameliorate Th2-driven release of the eotaxins and other CCR3 ligands that would, in turn, decrease recruitment and activation of EOSs. Results demonstrate that CCL26-siRNA treatments decreased interleukin-4-induced CCL26 and CCL24 expression by $> 70\%$. CCL26-directed small-interfering RNA (siRNA) treatments significantly decreased release of CCL5 (RANTES), CCL15 (MIP-1δ), CCL8 (MCP-2), and CCL13 (MCP-4). In bioactivity assays it was shown that EOS migration and activation were reduced up to 80% and 90%, respectively, when exposed to supernatants of CCL26-siRNA-treated cells. These results provide evidence that CCL26 may be an appropriate target for development of new therapeutic agents designed to alleviate the underlying inflammation associated with chronic diseases of the airways.

Introduction

Chemokines are a superfamily of over 50 small secreted proteins named for their precise orchestration of leukocyte trafficking in both homeostatic and disease states. Within the recently devised systematic nomenclature, chemokines are divided into four groups based on the arrangement of their amino-terminal cysteine residues. The largest groups are designated CC and CXC for their adjacent cysteine amino acids or cysteine residues separated by a single amino acid moiety, respectively (Bacon and others 2002). Chemokine effects are mediated through over 20 G protein–coupled receptors also grouped as CC chemokine receptors (CCRs) that generally bind with the CC chemokines and CXCRs that generally limit their binding to CXC ligands (Murphy 2002; Pease and Williams 2006). Bioactivities and functions of the chemokines extend well beyond their initially identified chemotaxis properties. In addition to their integral roles in both the innate and adaptive arms of the immune system, chemokines are implicated in the pathogenesis of diseases including rheumatoid arthritis, atherosclerosis, multiple sclerosis, inflammatory diseases of the skin and lung, chronic obstructive pulmonary disease (COPD), allergic rhinitis and asthma (Charo and Ransohoff 2006). In addition to their specifically defined pathologies, one pulmonary problem common to emphysema, chronic bronchitis, COPD, and asthma is underlying and complex lower airway inflammation regulated by chemokine receptor/ligand signaling. Airway structural or constituent cells that include fibroblasts, myofibroblasts, endothelial cells, smooth muscle cells, and airway epithelial cells are important sources of cytokines and chemokines that play pivotal roles in the perpetuation of the underlying airway inflammatory responses.

Studies with asthmatics have shown a strong correlation between eosinophil (EOS) numbers in bronchoalveolar lavage, endobronchial biopsies and sputum, and

College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, Florida.

hyper-responsiveness of the airways (Green and others 2002). This characteristic trafficking of EOS is directed, in part, by EOS-selective chemokines and their receptors, most notably the three eotaxins CCL11, CCL24, and CCL26. They exert EOS bioactivities by signaling predominantly through the CC receptor three (CCR3) which is highly expressed on human EOSs but also found on basophils, mast cells, subpopulation of Th2 cells, and dendritic cells (Ponath and others 1996; Phillips and others 2003; Garcia and others 2005). CCL11 was the first highly EOS-selective chemokine discovered by protein purification and peptide sequencing of bronchoalveolar lavage fluid. CCL24 was then identified followed shortly by discovery of CCL26 during a systematic search of the CCL24 genomic region (Kitauri and others 1999). Work with animal models has delineated the importance of CCR3-CCL11 and CCR3-CCL24 receptor–ligand signaling in airway disease (Humbles and others 2004; Lee and others 2004). Hallmark features of asthma confirmed were: the importance of CCR3 and eotaxins on EOS recruitment, a regulatory role of EOSs in Th2 cytokine production, diminished airway mononuclear cell accumulation, reduced mucus production and the EOS/CCR3–ligand dependence of allergen-induced overexpressed gene transcripts (Fulkerson and others 2006).

CCL26, however, is a human chemokine, therefore understanding its signaling, functions, and bioactivities must be gathered through clinical, *ex vivo* and *in vitro* experimentation. The following is a synopsis of recent studies focusing on the presence, regulation, and function of the CCL26-CCR3 system in the human pulmonary system. These findings underpin the rationale for the present investigations. With respect to chronic airway diseases, several resident cell types selectively produce eotaxins and express CCR3 including bronchial and alveolar type II epithelial cells (Stellato and others 2001; Abonyo and others 2005), fibroblasts (Puxeddu and others 2005), monocytes, dendritic cells (Moon and others 2007; Regamey and others 2007), smooth muscle cells (Joubert and others 2005; Odaka and others 2007), and endothelial cells (Shinkai and others 1999; De Lucca 2006). In an *in vitro* transepithelial chemotaxis system, it was found that CCL26 was the only eotaxin that remained bound to the cell surface, an association critical for EOS transepithelial migration (Yuan and others 2006). In comparative clinical studies of asthmatics it was found that the elevated prechallenge CCL11 and CCL24 messenger RNA (mRNA) levels did not increase following allergen challenge. In contrast, CCL26 expression was dramatically upregulated (to 100-fold) in asthmatics 24 h following allergen exposure (Berkman and others 2001). Numbers of biopsy cells expressing CCL26 and CCL24, but not CCL11, correlated significantly with the magnitude of the late asthmatic response (Ravensberg and others 2005). Th2-type cytokines induce release of significant amounts of the eotaxins from both bronchial and alveolar type II cells (Komiya and others 2003; Yamamoto and others 2004; Heiman and others 2005). In contrast to the other eotaxins, CCL26 mRNA is reportedly not present in unstimulated lung epithelial cells. Its comparative time- and concentration-dependent induction by interleukin-4 (IL-4) support the conclusion that CCL26 expression is modulated differently than CCL11 or CCL24 (Nakamura and others 2001; Banwell and others 2002). Differential regulation is also noted by inclusion of NF-κB inhibitors that abrogate CCL11 mRNA induction but stimulate CCL26 expression (Kobayashi and others 2004). Collectively, these results suggest that CCL26 may indeed be playing central and differential roles in the persistence of eosinophilic pulmonary inflammation in humans.

The expression of the eotaxin CCR3 receptor on airway epithelial cells suggests that CCR3–ligand interactions may modulate epithelial cell function and gene expression. To date, there are more chemokines capable of binding and transducing signals through CCR3 than any of the other CCRs (Pease and Williams 2006). Other agonists that bind CCR3 with varying potency and efficacy include RANTES (CCL5), MEC (CCL28), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13), and MIP-1δ (CCL15) (Fan and others 2005). There is a remarkable overlap between this CCR3 agonist list and the increased expression of CC chemokines implicated in recruitment of leukocyte populations into the pulmonary system. Indeed, biopsies from asthmatic lungs have confirmed increased levels of Th2 cytokines (IL-4, IL-13), CCR3 and several of its ligands (Komiya and others 2003; Zimmermann and others 2003; Kobayashi and others 2004). Interleukin-4 is known to upregulate CCR3 and expression and release of CCL24 and CCL26 in both bronchial and alveolar type II cell culture systems (Stellato and others 2001; Heiman and others 2005). Presence of both the receptor and its high-affinity ligands in the pulmonary epithelium provide conditions for autoregulatory mechanisms. Using the alveolar type II cell system, we have previously shown that transfection of the A549 cells with CCR3-siRNAs followed by IL-4 stimulation resulted in concomitant reductions in CCR3 and CCL26 release (Taka and others 2008). Thus, with respect to CCL26, results imply that multiple receptor/ligand pathways are involved in its signaling and modulation, CCL26 release from activated cells is robust and sustained, and that CCL26 may signal leukocyte trafficking in an organized and coordinated manner to elicit specific disease stage–specific bioactivities.

In this regard, we hypothesized that post-transcriptional silencing of CCL26 in Th2 cytokine–stimulated alveolar type II cells would also downregulate CCL24 and other CC chemokines that bind with CCR3 and this would favorably decrease migration and activation of EOSs. The data presented here demonstrate that CCL26-directed small-interfering RNAs (siRNAs) specifically and significantly suppress CCL26 mRNA and protein expression in IL-4-stimulated A549 alveolar type II cells. Using the CCL26-siRNA-treated A549 alveolar type II cell system, we demonstrate for the first time that siRNA-induced inhibition of CCL26 significantly suppresses IL-4-stimulated release of CCL24 by >80%. Six additional chemokines capable of binding to CCR3 were then assessed. Of these, increased release in response to IL-4 treatment of alveolar type II cells was noted for CCL5 (RANTES), CCL15 (MIP-1δ), CCL8 (MCP-2), and CCL13 (MCP-4). CCL26-directed siRNA treatments significantly decreased release of CCL5, CCL15, CCL8, and CCL13. Interestingly, levels of the known CCR3 antagonists MIG (CXCL9) and I-TAC (CXCL11) were also suppressed in cells treated with CCL26-targeted siRNAs. In bioactivity assays it was shown that silencing of CCL26 decreased both migration and activation of EOSs exposed to alveolar type II cell supernatants. These results provide evidence that CCL26 may be an appropriate target for curtailing the underlying inflammation associated with chronic diseases of the airways.

Materials and Methods

Cell culture

Human A549 alveolar type II epithelial-like cells [American Type Culture Collection (ATCC Manassas, VA) CCL-185] were 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 phosphate-buffered saline (PBS)/EDTA-dispersed cells were suspended in fresh medium in flasks or wells at 0.1×10^6 cells/mL (Abonyo and others 2005). Clone 15 HL-60 EOSs (ATCC CRL-1964) were cultured in RPMI 1640 (Cellgro by Mediatech, Inc., Herndon, VA, USA) supplemented with 10% fetal calf serum, 10 ng/mL IL-5 (Atlanta Biologicals, Atlanta, GA, USA), penicillin (100 U/mL) and streptomycin (100 µg/mL) in a humidified atmosphere as described above. Final differentiation was carried out for 7 days in supplemented RPMI-1640 containing 0.5 mM n-butyric acid (Badewa and Heiman 2003). Viability of cells harvested for experiments was assessed by trypan blue exclusion, and only populations of cells with viability >95% were used for experiments.

A549 alveolar type II epithelial cell CCL26-siRNA treatment protocol

Trypsin-dispersed A549 cells were washed, resuspended in antibiotic free medium supplemented with 2.5% fetal calf serum, seeded at 1.0×10^5 cells/mL in 12-well cluster plates (1 mL/well), incubated and allowed to attach overnight. Dharmacon (Lafayette, CO) transfection reagent DharmaFECT 1 and ON-TARGETplus *SMART*pool designed siRNAs (product #L-007838, human CCL26, NM_006072) consisting of duplexes 5, 6, 7, and 8 alone and equal amounts of the duplexes combined as the *SMART*pool were used to inhibit CCL26. Dharmacon siCONTROL Nontargeting siRNA pool was used as the control. Sequences of the CCL26-targeted duplex sense sequences provided by Dharmacon are as follows: duplex 5 - G.A.A.A.G.U.C.U.G.U.A.C.C.C.A.U.C.C.A.U.U, duplex 6 - G.C.U.A.U.G.A.A.U.U.C.A.C.C.A.G.U.A.A.U.U, duplex 7 - C.C.G.A.A.A.C.A.A.U.U.G.U.G.A.C.U.C.A.U.U, duplex 8 - G.A.U.A.U.U.C.A.C.U.A.C.C.A.A.A.A.G.A.U.U. Antisense sequences for the duplexes are: duplex 5 - 5′-P.U.G.G.A.U.G.G.G.U.A.C.A.G.A.C.U.U.U.C.U.U, duplex 6 - 5′-P.U.U.A.C.U.G.G.U.G.A.A.U.U.C.A.U.A.G.C.U.U, duplex 7 - 5′-P.U.G.A.G.U.C.A.C.A.A.U.U.G.U.U.U.C.G.G.U.U, and duplex 8 - 5′-P.U.C.U.U.U.U.G.G.U.A.G.U.G.A.A.U.A.U.C.U.U. Dharma*FECT* 1 and siRNA products were prepared according to the manufacturer's instructions. In a series of preliminary studies it was determined that maximal siRNA effects on CCL26 protein were seen at 72 h after transfection with 120 nM siRNAs. Transfection efficiency was qualitatively assessed at >95% following treatment of cells with 120 nM si*CONTROL* Tox for 48 h. Following transfection with CCL26 siRNAs, monolayers were washed, then treated with or without IL-4 (100 ng/mL) in serum-free medium for 24 h. Cell culture medium was collected, cells detached with PBS/EDTA, pelleted and cell lysates prepared.

Detection of CCR3 by dot immunoblotting

To assess effects of CCL26-siRNA treatments on CCR3, A549 airway epithelial cells were treated as described above. Cell suspensions were centrifuged at 80*g*, supernatants aspirated and cell pellets resuspended in lysis buffer (20 mM Tris (pH 7.4), 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, and one tablet/10 mL of protease inhibitor cocktail) (Sigma-Aldrich Chemical Co., St. Louis, MO). Samples were incubated in ice for 30 min, sonicated for 3 s, centrifuged (5 min, 4ºC at 16,000*g*), supernatants collected for total protein content. Lysate protein concentrations were quantified with a Power Wave$_X$340-I microplate reader equipped with KC4 v3.0 PowerReports software (Bio-Tek Instruments, Winooski, VT, USA). To detect CCR3, 10 µg lysate proteins in 200–500 µL water were introduced into a 96-well filtration/incubation manifold I system (Schleichter and Schuell, Germany) under vacuum. Nitrocellulose membranes were blocked in 3% bovine serum albumin (BSA) in PBS containing 0.05% Tween-20 (PBS-T) overnight at 4ºC with agitation then washed three times with PBS-T followed by incubation for 1 h at room temperature with 0.6 µg/mL rabbit antihuman CCR3 antibody (Imgenex, San Diego, CA). Following three washes, membranes were incubated with 1:2000 goat anti-IgG-horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS-T containing 0.5% BSA. After the final four washes, membranes were incubated for 5 min with SuperSignal Pierce western substrate working solution and immunoblot images obtained with a Fluor-S Max MultiImaging system. Blot densities were assessed with Quantity One 1-D Analysis Software Version 4.6.0.

Chemokine and cytokine detection by specific ELISA

Synthesized and secreted CCL24 and CCL26 proteins were detected using specific enzyme-linked immunosorbent assays (ELISAs) (R&D Systems, Minneapolis, Minnesota). The secreted chemokines IL-8 (CXCL8), RANTES (CCL5), MCP-1(CCL2), and MCP-2 (CCL8) were also quantified by specific ELISA (Ray Biotech, Norcross, Georgia). ELISA results were analyzed with the Power Wave$_X$340-I microplate reader equipped with KC4 v3.0 PowerReports software.

RNA extraction and PCR

A549 airway epithelial cells were plated (0.2×10^6 cells/2 mL) in 6-well plates, allowed to attach overnight and transfected for 48 h as described above. Treated cell monolayers were washed, then treated with or without IL-4 (100 ng/mL) in fresh serum-free medium for 24 h. Total RNA was isolated using an Easy-spin™ (DNA free) total RNA extraction kit following the manufacturer's protocol (iNtRON Biotechnology, distributed by Boca Scientific Inc., Boca Raton, FL). First-strand complementary DNA (cDNA) was synthesized from 5 µg of total RNA in a 100 µL reaction volume using an iScript cDNA synthesis kit as recommended by the manufacturer (Bio-Rad Laboratories). The cDNA synthesis thermal-cycling program included three steps: 25°C for 5 min, 42°C for 30 min, then 85°C for 5 min. Polymerase chain reaction (PCR) amplification with GAPDH (R&D Systems) serving as the internal control was performed as follows. From each sample, 2.5 µL cDNA was amplified in 50 µL PCR reaction mixture (10× iTaq buffer, 50 mM $MnCl_2$), 10 mM dNTP, 5 U/µL iTaq DNA polymerase) containing 0.3 µM CCL26 primers (forward primer: GCC TGA TTT GCA GCA TCA TGA TGG and reverse primer: CGG ATG ACA ATT CAG CTG AGT CAC). The PCR thermal-cycling programs consisted of first

predenaturation at 94°C for 4 min, followed by 34 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 45 s, and finally extension at 72°C for 10 min. Aliquots of the PCR products (20 µL) were visualized with ethidium bromide staining after separation by electrophoresis in a 1.2% agarose gel in Tris borate ethylenediamine tetra-acetic acid buffer pH 8.3 at 100V for 2 h. Images were captured by a Fluor-S Max MultiImaging system and band densities were assessed with Quantity One 1-D Analysis Software Version 4.6.0.

Cytokine antibody array

Supernatants (300 µL) of CCL26-siRNA-treated cells were assayed for the presence of cytokines using the RayBio® Human Cytokine Antibody Array C series 1000 according to the manufacturer's instructions (Ray Biotech, Norcross, GA). Images were obtained with a Flour-s Max Multi-Imaging system. Densities of cytokine protein spots were quantified with Quantity One 1-D Analysis Software Version 4.6.0.

Clone 15 HL-60 eosinophilic cell migration and superoxide generation

Migration was assessed in BD Falcon HTS FluoroBlok™ 96-well insert systems with 3.0-µm pore fluorescence blocking PET membranes. Differentiated cells were centrifuged (80*g*, 10 min) washed two times in Hank's balanced salts solution containing 0.1% BSA (HBSS) and 3×10^6 cells/mL incubated in RPMI-1640 containing 0.1% BSA and 5 µM calcein AM for 30 min. Labeled cells were washed, resuspended in RPMI-1640 with 0.1% BSA at 2×10^6 cells/mL and 50 µL added to inserts. Supernatants (120 µL) collected from treated A549 airway epithelial cells were delivered to the bottom wells and plates incubated for 3 h in a humidified atmosphere of 5% carbon dioxide at 37°C. Migration of EOSs was assessed with an FL*x*800TBI fluorescence microplate reader (Bio-Tek Instrument, Inc, Winooski, VT. USA) reading with the bottom probe (excitation 494 nm and emission 517 nm). A standard curve of 0–5×10^4 calcein-loaded EOS was included to quantify numbers of EOSs migrating in each experiment and KC4 v3.0 PowerReports software used to analyze data.

Superoxide anion generation was assessed in microtiter plates with 1×10^5 cells/well in a total volume of 0.1 mL Hank's balanced salts solution containing 0.2% BSA, 80 µg ferricytochrome C with and without 30 µg superoxide dismutase and 70 µL A549-treated cell culture supernatants. Microtiter plates were incubated at 35°C in an atmosphere of 5% CO_2 for 2 h, and absorbances read at 550 nm to determine superoxide dismutase–inhibitable reduction of ferricytochrome C.

Data handling and analysis

All experiments were conducted in duplicate or triplicate as indicated in figure legends and repeated on at least three to four separate occasions. Unless otherwise stated, all data are expressed as the mean ± SEM with the mean of triplicates from one experiment serving as one observation. When indicated, one-way analysis of variance (ANOVA) followed by either the Bonferroni multiple comparisons or Dunnett's post-test, as appropriate, was applied to experimental results to determine statistical significance ($P < 0.05$) between indicated groups.

Results

CCL26-targeted siRNA duplexes decrease CCL26 mRNA and protein expression in A549 alveolar type II cells

Experiments were carried out to verify that the CCL26-targeted siRNA duplexes were blocking IL-4 stimulated CCL26 mRNA and protein expression. In preliminary PCR investigations it was found that treatment of A549 alveolar type II cells with ON-TARGETplus CCL26-siRNA duplexes 6 and 8 resulted in greater suppression of CCL26 mRNA than duplexes 5, 7 or the combination of equal amounts of the four duplexes (*SMART*pool). Specificity of CCL26 mRNA inhibition was then studied with reverse transcriptase PCR using RNA purified from A549 cells treated with siRNA duplexes 6 and 8 and a resulting typical gel image is shown in Figure 1A along with densitometric analyses of several gels. Results demonstrate that CCL26-targeted siRNA duplexes 6 and 8 significantly decreased IL-4-stimulated CCL26 mRNA by 94 and 100%, respectively. To demonstrate effects of the siRNA treatments on CCL26 protein expression, cells were treated with a nontargeting siRNA control, ON-TARGETplus siRNA duplexes or the combined duplexes (*SMART*pool). Interleukin-4-stimulated CCL26 was quantified by ELISAs and results depicted in Figure 1B. Consistent with previous work (Abonyo and others 2005), negligible CCL26 was released from unstimulated cells while IL-4 stimulated cells released 790 pg/mL CCL26. Treatment of A549 cells with CCL26-targeted siRNAs significantly reduced IL-4-stimulated CCL26 release for all duplexes and the combined *SMART*pool. While duplexes 5 or 7 reduced CCL26 release by 73 and 84%, respectively, a complete suppression of CCL26 release was measured following treatments with duplexes 6 and 8. Results indicate that the CCL26-directed siRNAs specifically and significantly suppressed CCL26 mRNA and protein expression in IL-4 stimulated A549 alveolar type II cells.

CCL26-siRNA treatments decrease CCR3 expression and CCL24 release in A549 cells

Having shown that CCL26 suppression was feasible, it was of interest to explore its effects on the CCR3–ligand system in alveolar type II cells. In our previous work, we demonstrated that IL-4 upregulated CCR3 receptors, CCL26, and CCL24, and that treatment of A549 cells with CCL26 plus IL-4 suppressed CCR3 and CCL26 but not CCL24 expression (Abonyo and others 2005). Thus, in the first series of experiments, the effect of CCL26-targeted siRNA treatments on the eotaxin CCR3 receptor was investigated. CCR3 dot blot analyses on total cell lysates prepared from CCL26-siRNA-treated A549 airway epithelial cells were carried out and a typical image is shown in Figure 2. Results indicate constitutive presence of CCR3, its upregulation following IL-4 stimulation and suppression by CCL26-targeted siRNA duplexes. Results also indicate that treatment with a nontargeting siRNA and IL-4 did not alter CCR3 receptor to GAPDH ratios. Pixel density analyses of 3 experiments are also shown in Figure 2, where inhibitory effects of the siRNA treatments on CCR3 protein expression are compared to the nontargeting siRNA/IL-4 treatment group. In contrast to duplex 7, which exerted no significant suppression, treatment with the remaining siRNA duplexes resulted in the following

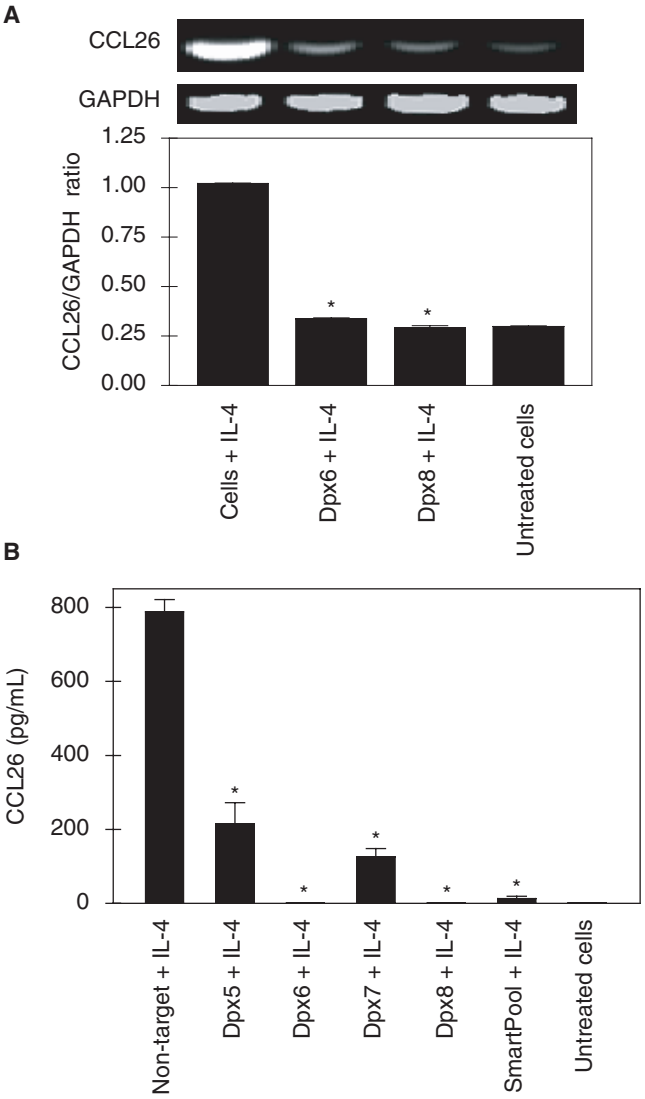

FIG. 1. CCL26-siRNA individual duplexes and the *SMART*pool combination decrease CCL26 mRNA expression and protein in IL-4–stimulated A549 airway epithelial cells. A549 airway epithelial cells were treated with indicated CCL26-siRNA (120 nM) duplexes or the *SMART*pool combination of equal amounts of the duplexes (120 nM) in the presence of 3 µl Dharma*FECT* 1 transfection reagent then stimulated with IL-4 (100 ng/mL) for 24 h. (**A**) Treated cells were washed, lysed, and RNA extracted. First-strand cDNA was synthesized from total RNA by reverse transcription (RT) and cDNA amplified by PCR using human CCR3 and GAPDH primers. PCR products were separated by electrophoresis in 1.2% agarose gels, stained with ethidium bromide, and mRNA expression captured by a Fluor-S Max MultiImager. Data (mean ± SD) from experiments are expressed as the relative density of CCL26-mRNA/GAPDH ratios. Treatments that differed significantly ($P < 0.05$) from IL-4–stimulated cells are indicated by an asterisk. (**B**) Specific sandwich ELISA was used to quantify CCL26 in culture supernatants of siRNA-treated and IL-4 stimulated cells. Data are the mean ± SEM of 3 experiments each performed in duplicate. Asterisks indicate the treatments that differed significantly from the nontargeting siRNA control with $P < 0.05$.

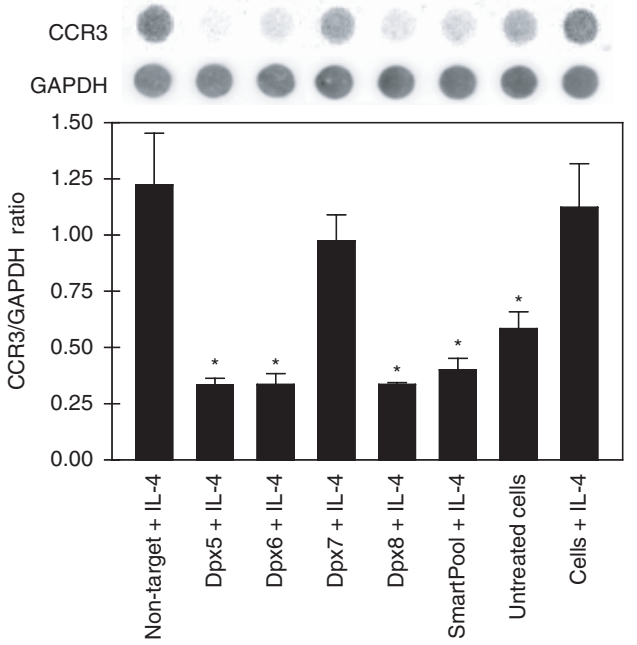

FIG. 2. CCL26-targeted siRNA treatments downregulate CCR3 expression in A549 airway epithelial cells. Cells were cultured, treated with indicated siRNA duplexes, then stimulated with IL-4 (100 ng/mL) as described in Figure 1. A typical dot blot image using 10 µg/well of total cell lysate proteins to probe for human CCR3 and GAPDH is shown above indicated treatment CCR3/GAPDH ratios calculated from 3 separate dot blot experiments with Quantity One 1-D Analysis Software Version 4.6.0. Ratios are expressed as the mean ± SEM. Asterisks indicate those treatments that differed significantly ($P < 0.05$) from cells treated with nontargeting siRNA followed by stimulation with IL-4.

suppression of CCR3 protein expression: duplexes 5, 6, and 8—72%–75% and the *SMART*pool combination—67%.

Following stimulation with Th2-type cytokines such as IL-4, alveolar type II cells increase release of CCL24 which also signals through the CCR3 receptor. It was of interest to determine the effect of CCL26 knockdown on IL-4-stimulated release of CCL24. ELISA results, depicted in Figure 3, indicate significant suppression of CCL24 release from IL-4 stimulated cells. Taken together, these results suggest that treatment of alveolar type II epithelial cells with CCL26-targeted siRNA does significantly suppress its own expression and that of CCL24 when cells are exposed to the Th2 cytokine IL-4.

CCL26-targeted siRNA treatments alter levels of other CCR3 agonist/antagonist cytokines and chemokines released from A549 airway epithelial cells

Chemokines typically bind to receptors in their subclasses; that is, the 28 known CC chemokines bind to one or more of the 10 known CC receptors. Activities of the individual chemokines at their receptors have an order delineated by their affinity, potency, and efficacy. Within this context, it was of interest to explore the effects of inhibition of the

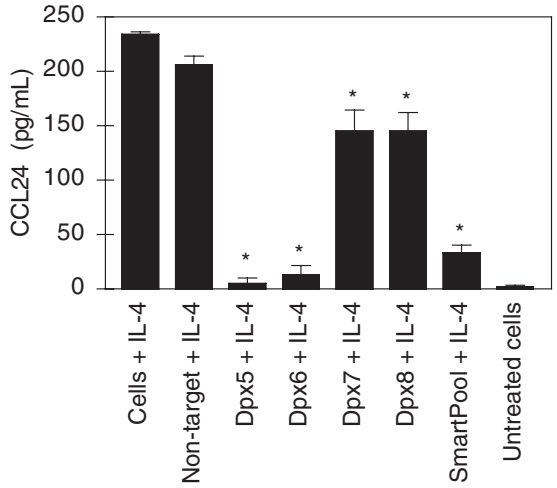

FIG. 3. CCL26-targeted siRNA treatments significantly decrease CCL24 release from IL-4–stimulated A549 airway epithelial cells. Cells were cultured and treated as described in Figure 1. CCL24 was assessed in cell culture supernatants by specific ELISA. Data are the mean ± SEM of 3 experiments, each performed in duplicate. Asterisks indicate those treatments that differed significantly from the nontargeting siRNA control as assessed by Dunnett's post-test, with $P < 0.05$.

high-affinity ligand CCL26 on the other known CC chemokines that bind to CCR3. Human antibody membrane arrays were used to determine chemokine changes in supernatants from CCL26-targeted siRNA–treated and IL-4 stimulated A549 airway epithelial cells. Results of CC chemokines, including the three eotaxins, capable of binding to CCR3 are depicted in Figure 4. Results with CCL24 and CCL26 corroborate CCL26-targeted-siRNA effects demonstrated in previous figures and thus validate the cytokine array results. In addition to CCL24 and CCL26, four additional chemokines capable of binding to CCR3 were significantly increased by IL-4 and include CCL5 (RANTES), MIP-1δ (CCL15), CCL8 (MCP-2), and CCL13 (MCP-4). Pretreatment of A549 cells with CCL26-siRNA duplexes 6 or 8 significantly decreased release of CCL5, CCL15, CCL8, and CCL13. Low levels of the CCR3 agonists eotaxin (CCL11), MEC (CCL28), and MCP-3 (CCL7) were also noted but changes were not statistically significant. MIG (CXCL9) and I-TAC (CXCL11) members of the CXC chemokine subgroup are known CCR3 antagonists and were also assessed. Results, shown in Figure 5, indicate that levels of both antagonists were suppressed in cells treated with CCL26-targeted siRNAs while only CXCL9 was significantly increased by IL-4 treatment.

We also quantified three cytokines that are constitutively released from both primary alveolar type II cells and A549 cells. Results are shown in Figure 6A and include MCP-1 (CCL2), IL-8 (CXCL8), and GRO-α (CXCL1). Interestingly, CCL26-siRNA treatment with duplex 6 was particularly effective at suppressing these chemokines in IL-4 stimulated cells. To further explore the effects of CCL26-targeted siRNA treatments on CCL2 and CXCL8, A549 cells were treated, stimulated with IL-4, and resultant supernatants assayed by specific ELISAs. Results for CCL2 are depicted in Figure 6B and demonstrate that the *SMART*pool combination of four siRNAs and the CCL26-targeted duplex 6 siRNA significantly reduced amounts of CCL2 released from A549 alveolar type II epithelial cells. The significant increases in CCL2 release from cells pretreated with duplex 8 remain unexplained. In Figure 6C results for similar experiments with assessment of released CXCL8 indicate significant reductions from cells treated with *SMART*pool and the CCL26-targeted duplex 6 siRNA preparations. These results suggest that post-transcriptional silencing of CCL26 downregulates several chemokines capable of signaling as agonists or antagonists through the CCR3 receptor. In addition, post-transcriptional suppression of CCL26 decreased release of CCL2 that signals through the CCR2 receptor and CXCL8 that uses CXCR1 to initiate bioactivities.

CCL26-targeted siRNA treatment of A549 airway epithelial cells decreases EOS migration and activation

Infiltration of EOS is one hallmark of allergic and inflammatory airway diseases such as asthma. Because EOS migration and activation is guided by CCR3-agonist pathways, the next experiments were designed to test whether treatment of the A549 airway epithelial cells with CCL26-targeted siRNA would alter EOS migration. In the first series of experiments, human clone 15 HL-60 EOSs were tested for their ability to migrate toward human recombinant CCL26. Results shown in Figure 7A indicate that CCL26 in the range of 0.3–30 ng/mL, stimulates EOS migration in a concentration-dependent manner. To demonstrate that migration was due to a CCR3-CCL26 pathway, EOSs were pretreated with human anti-CCR3-neutralizing antibody. Inhibition of migration indicates that the EOSs were using the CCR3-CCL26-signaling pathway. In the next investigations, we explored the effects of CCL26-targeted siRNA treatment followed by IL-4 stimulation on EOS migration. Supernatants were used as the chemoattractant in quantitative assays of EOS migration and results are depicted in Figure 7B. IL-4 stimulation of the siRNA controls, treated with a nontargeting siRNA, resulted in a total migration of 28,000 EOSs. The results of ELISAs indicated nontargeting siRNA plus IL-4-treated cells released 1.44 ± 0.15 ng/mL CCL26 that did not differ significantly from the 1.13 ± 0.14 ng/mL CCL26 released from cells treated with IL-4 alone. It was similarly determined that supernatants collected from *SMART*pool, duplex 6–, or duplex 8–treated cells after stimulation with IL-4 contained averages of 128, 26, and 16 pg/mL CCL26, respectively. Inhibition of CCL26 release paralleled significant reductions in EOS migration of 40%, 55%, and 80%, respectively.

Inflammatory processes generate toxic levels of reactive oxygen species (ROS) including superoxide ($O_2\bullet-$) by activated EOSs, alveolar macrophages, and neutrophils. Stimulated A549 cells release CCL26 that activates EOSs and further compounds inflammation. Therefore, EOSs were exposed to medium of IL-4-stimulated and CCL26-siRNA-treated airway epithelial cells and $O_2\bullet-$ generation by EOSs assessed. Results with supernatants from CCL26-targeted siRNA-treated A549 cells are depicted in Figure 8. Eosinophils generated 3.59 ± 0.33 and 4.89 ± 1.13 nmol $O_2\bullet-/10^6$ cells when incubated in medium with added recombinant CCL26 or CCL24 at 100 ng/mL, respectively. Nontargeting siRNA-treated cell supernatant results did not

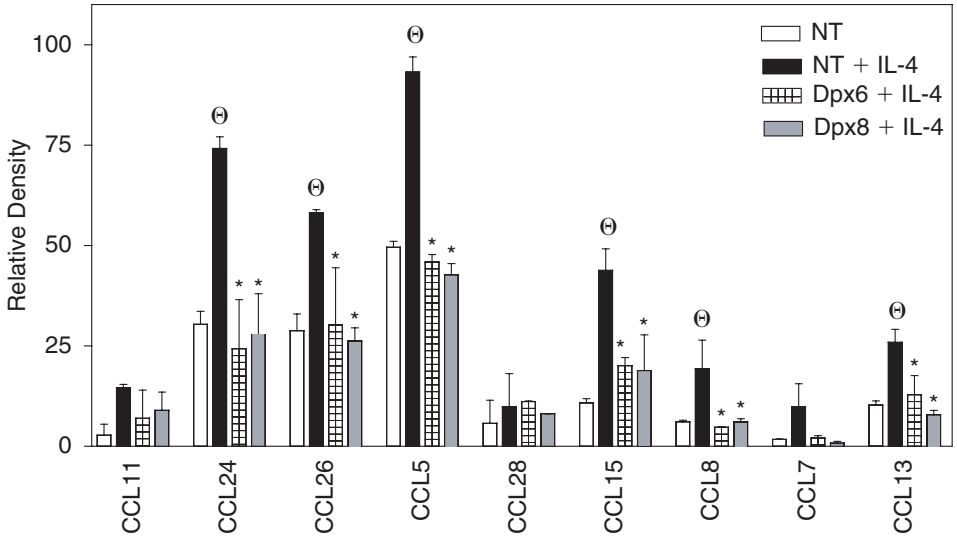

FIG. 4. CCL26-siRNA treatments of A549 alveolar type II cells modulate additional chemokines that bind to CCR3. A549 cells were treated with nontargeting or *SMART*pool CCR3 siRNA followed by IL-4 (100 ng/mL). Supernatants were assayed for the presence of cytokines using the RayBio Human Cytokine Antibody Array C series 1000. Images were obtained with a Flour-s Max MultiImager and densities of cytokine protein spots were quantified with Quantity One 1-D Analysis Software Version 4.6.0. Cytokine nomenclature: eotaxin (CCL11), eotaxin-2 (CCL24), eotaxin-3 (CCL26), RANTES (CCL5), MEC (CCL28), MIP-1δ (CCL15), MCP-2 (CCL8), MCP-3 (CCL7), and MCP-4 (CCL13). Data were normalized to the membrane positive control and are expressed as relative pixel densities of the mean ± SD of 2 experiments, each done in duplicate. Nontargeting (NT) siRNA ± IL-4 served as the unstimulated and Th2-stimulated controls, respectively. Asterisks (*) indicate treatments that differed significantly ($P < 0.05$) from nontargeting (NT) + IL-4 siRNA control. The symbol Θ was used to indicate significant differences between NT and NT+IL-4, with $P < 0.05$.

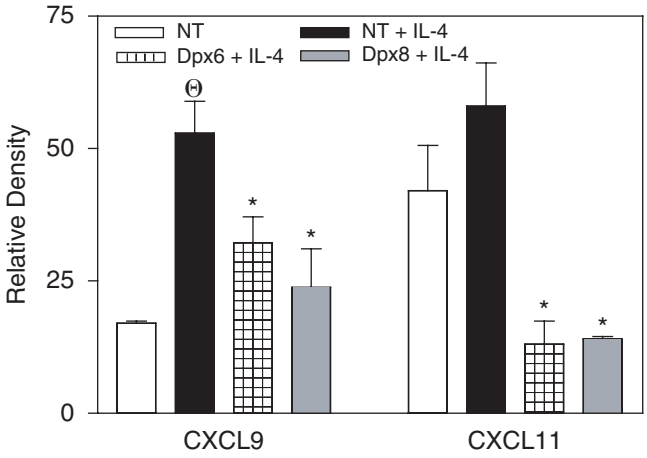

FIG. 5. CCL26-siRNA treatments of A549 alveolar type II cells modulate additional chemokines including CCR3 antagonists. Experiments were carried out and data analyzed as described in Figure 4. Cytokine nomenclature of CCR3 antagonists: MIG (CXCL9) and I-TAC (CXCL11). Nontargeting (NT) siRNA ± IL-4 served as the unstimulated and Th2-stimulated controls, respectively. Asterisks (*) indicate treatments that differed significantly ($P < 0.05$) from nontargeting (NT) + IL-4 siRNA control. The symbol Θ was used to indicate significant differences between NT and NT+IL-4, with $P < 0.05$.

differ from those of cells treated only with IL-4. Untreated A549 cells constitutively released products that stimulated EOSs to generate 1.56 nmol O_2•−/10^6. Significant reductions in stimulation of O_2•− by EOSs were noted for all CCL26-targeted treatments and ranged from 60% to 95% inhibition. Together these data demonstrate that treating airway epithelial cells with CCL26-targeted siRNA has a favorable inhibitory effect on EOS migration and activation.

Discussion

Mitigation of the underlying inflammation present in airway diseases including asthma, chronic bronchitis, COPD, and emphysema requires an understanding of the complex chemokine ligand/receptor–mediated signaling between resident lung cells and emigrating leukocytes. Compelling evidence suggests that bronchial and alveolar type II epithelial cells have impressive potentials to orchestrate leukocyte recruitment, priming, and activation at sites of allergy and/or inflammation. In these present investigations we have focused on the alveolar epithelium and the most recently discovered human eotaxin, CCL26. In the results presented and discussed herein, we demonstrate for the first time that in alveolar type II cells, CCL26-directed siRNA treatments significantly inhibit CCL26 and CCL24 expression. Of the other agonist agents capable of binding to CCR3, suppression of IL-4-induced release was noted for CCL5, CCL15, CCL8,

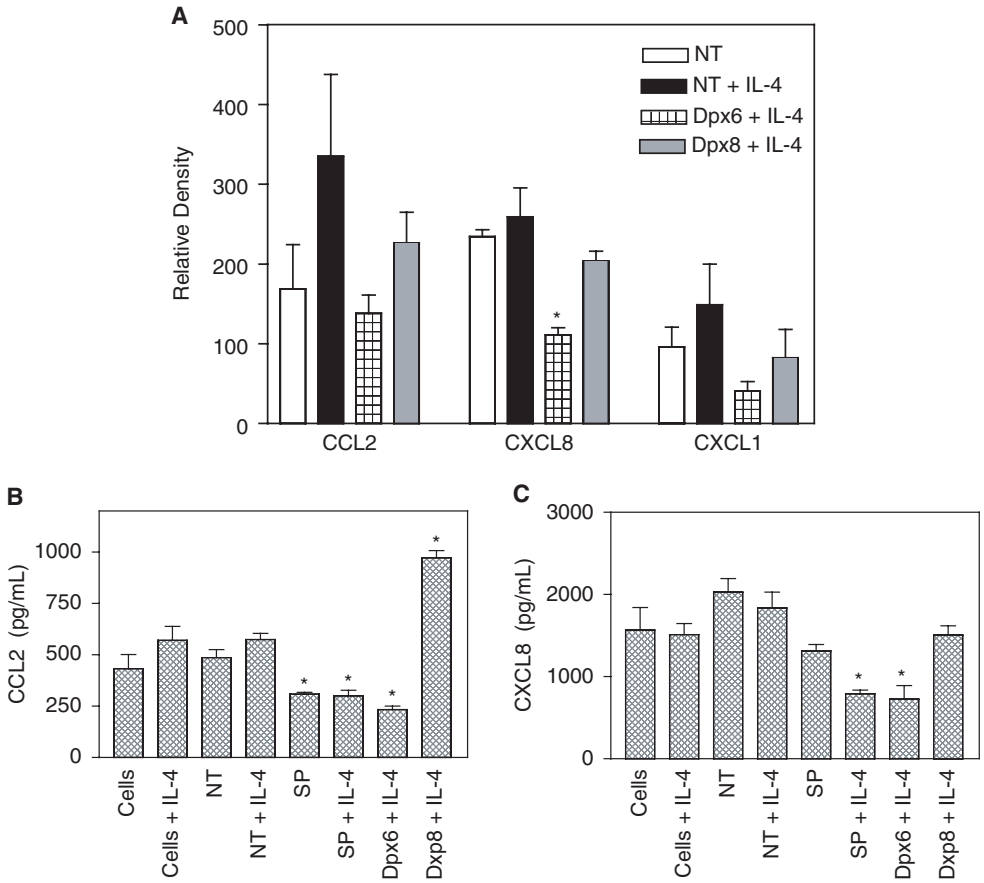

FIG. 6. CCL26-siRNA treatments decrease release of CCL2 and CXCL8 from IL-4-stimulated A549 alveolar type II cells. CCL26-siRNA treatments were carried out as described in Figure 1. (**A**) supernatants were subjected to antibody array membranes as described in Figure 4. Cytokine nomenclature: MCP-1 (CCL2), IL-8 (CXCL8), and GRO-α (CXCL1). Asterisks (*) indicate treatments that differed significantly ($P < 0.05$) from nontargeting (NT) + IL-4 siRNA control. (**B** and **C**) Specific sandwich ELISA was used to quantify CCL2 and CXCL8 in culture supernatants of siRNA-treated and IL-4-stimulated cells. Data are the mean ± SEM of three experiments each performed in duplicate. Asterisks indicate those treatments which differed significantly from the nontargeting siRNA control, with $P < 0.05$.

and CCL13. The two chemokine members of the CXC family, CXCL9 and CXCL11, which exert antagonist effects following binding to CCR3, were also suppressed. In addition, post-transcriptional silencing of CCL26 decreased release of CCL2 and CXCL8 that signal through chemokine receptors CCR2 and CXCR1, respectively. Because an active role for the alveolar epithelium in eosinophilic recruitment and activation has been supported, *in vitro* migration and assessment of O_2•− generation were studied in EOSs exposed to supernatants of CCL26-targeted siRNA–treated alveolar type II cells. Significant suppression of both EOS migration and activation was noted. These studies suggest that CCL26-targeted siRNA treatment of the airway epithelium may be a novel approach to reduce levels of Th2-responsive chemokines and leukocyte chemoattraction that are critical events in the perpetuation of underlying pulmonary inflammation.

The eotaxin(s)-CCR3-receptor system is expressed and functional as sites of allergy, sites of eosinophilic inflammation, and in specific tissue development and repair (Beck and others 2006; Haley and others 2008). In airway disease dominated by Th2 cytokines such as IL-4 and IL-13, the airway epithelium is also a major site for expression of the eotaxins-CCR3 system. The bronchial epithelium responds to IL-4 stimulation by upregulation of CCR3 and release of CCL11 and CCL26. In contrast, alveolar type II cells respond to IL-4 by upregulation of CCR3 and concomitant release of CCL24 and CCL26 with similar kinetics (Komiya and others 2003; Heiman and others 2005). Blocking the A549 cell surface CCR3 with neutralizing antibody or small molecule nonpeptide antagonists before IL-4 stimulation significantly decreased CCL24 release and CCL26 expression and secretion (Abonyo and others 2005). Using the A549 alveolar type II cell/IL-4 experimental paradigm in the present investigations, we have now shown that CCL26-targeted siRNA treatment very significantly decreased both CCL26 mRNA and protein expression. Concomitant decreases in CCR3 protein and CCL24 release were also observed. In recently reported results, we have shown that transfection of A549 cells with CCR3-targeted siRNA resulted in suppression of the targeted mRNA and protein as well as significant suppression of IL-4-stimulated CCL26 release and constitutive release of CCL24 (Taka and others 2008). Together these data suggest that condition-specific cytokine stimulation of the airway epithelium results in predictable upregulation

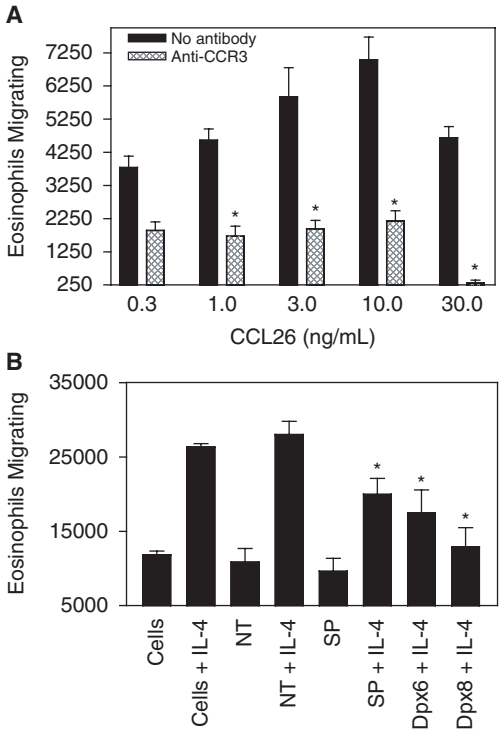

FIG. 7. CCL26-siRNA treatment of A549 airway epithelial cells decreases eosinophil (EOS) migration. A549 airway epithelial cells were treated with CCL26-siRNA as described in Figure 1. Supernatants were used to stimulate EOS migration in BD Falcon HTS FluoroBlok 96-well insert systems. Eosinophils were labeled by incubation with calcein AM (5 μM) for 30 min, washed, resuspended in RPMI-1640 with 0.1% BSA and 0.1×10^6 cells added to the upper chambers. Supernatants or medium (120 μL) containing CCL26 (0.3–30 ng/mL) were delivered to the bottom wells and migration allowed to proceed for 3 h in a humidified atmosphere of 5% carbon dioxide at 37°C. Migration was quantified from an EOS standard curve using a fluorescence microplate reader (excitation: 494 nm; emission: 517 nm) and KC4 v3.0 PowerReports software. (**A**) Calcein-loaded EOSs were pretreated without or with anti-CCR3 (50 μg/mL) for 30 min. Data are the mean ± SEM of 3 experiments each performed in duplicate. Asterisks indicate the anti-CCR3 treatments that significantly differed from their concentration-matched controls at $P < 0.05$. (**B**) There was no significant difference between cells + IL-4 and nontargeting siRNA (NT) + IL-4 groups. Data are the mean ± SEM of 3 experiments each performed in duplicate. Asterisks indicate those CCL26 siRNA + IL-4 treatments that differed significantly from the NT + IL-4 controls at $P < 0.05$.

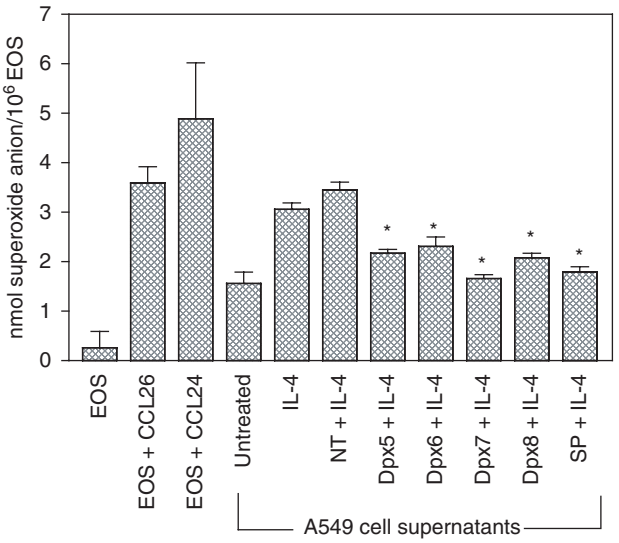

FIG. 8. Superoxide anion generation is suppressed in clone 15 HL-60 eosinophilic cells exposed to supernatants from CCL26-siRNA treated A549 alveolar type II cells. A549 alveolar type II cells were treated as described in Figure 1 and supernatants used to stimulate EOSs. Superoxide anion generation was assessed in microtiter plates (1×10^5 EOSs/well) in a total volume of 0.1 mL containing 80 μg ferricytochrome C with and without 30 μg superoxide dismutase and 70 μL A549-treated cell culture supernatants. Microtiter plates were incubated at 35°C in an atmosphere of 5% CO_2 for 2 h, and absorbances read at 550 nm to determine superoxide dismutase–inhibitable reduction of ferricytochrome C. Additional controls included unstimulated EOSs in medium, and medium to which 100 ng/mL recombinant CCL26 or CCL24 had been added. There was no significant difference between IL-4 and nontargeting (NT) siRNA + IL-4 supernatants. Asterisks indicate those treatments which differed significantly from the nontargeting siRNA control as assessed by Dunnett's post-test, with $P < 0.05$.

of the eotaxins and their predominant receptor CCR3, and that targeting the CCL26 overexpressed component with siRNA results in a favorable decrease in the eotaxins-CCR3 receptor–ligand-signaling system.

Human CCR3 binds multiple chemokine ligands that act with varying potencies and efficacies. High-affinity ligands include CCL11, CCL24, CCL26, CCL28, and CCL13 while CCL5, CCL7, CCL8, and CCL15 may be categorized as lower affinity CCR3 ligands (Pan and others 2000; Morokata and others 2006). In A549 cells, the EOS and T-cell chemoattractant CCL28 is constitutively expressed at low levels with increased expression following IL-1β and tumor necrosis factor-α (TNF-α) stimulation (John and others 2005). The present results corroborate these findings by demonstrating low constitutive levels of CCL28 and no significant increases following stimulation with the Th2 cytokine IL-4. Decreasing CCR3 levels by treatment of A549 cells with CCL26-targeted siRNA did not significantly alter CCL28 levels thus supporting its regulation by a Th1-type environment. The lower affinity CCR3 ligands CCL5, CCL7, and CCL13 have also been demonstrated in airway biopsies of atopic individuals (Elsner and others 2005). Herein, treatments of A549 cells with CCL26-siRNA duplexes significantly decreased release of CCL5 and CCL13. Low levels of CCL7 were observed and though there were reductions in release of CCL26-siRNA-treated cells, these were not significant. The data implies a CCR3-agonist regulatory mechanism because treatment with the siRNAs is known to downregulate CCR3. The regulation/autoregulation of CCR3 remains enigmatic though CCR3–ligand intercellular signaling has been the subject of numerous studies. However, of interest

is the transcription factor lung Krüppel-like factor-2 (KLF2) that has recently emerged as a central transcriptional switch point between the quiescent and activated states of endothelial cells and leukocytes. Chemokine and chemokine receptor genes repressed by KLF2 include CCR3, CCR5, CCL2, and IL-8 (Dekker and others 2006; Sebzda and others 2008). The roles of lung KLF2 as a transcriptional switch point between homeostatic and Th2-type cytokine-driven proinflammatory phenotype of alveolar type II cells await investigation.

Chemokines directly influence and modify cell bioactivities through very complex networks that include antagonist actions. Of importance to these investigations is the report that CCR3 functional responses are regulated by both CXCR3 and its high-affinity ligands CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC). Although CCL11 will bind to CXCR3, the eotaxins are inactive at this receptor (Xanthou and others 2003). Conversely, the CXCR3 ligands are known to antagonize effects of all three eotaxins at CCR3 (Loetscher and others 2001; Smitt and Lukacs 2006). In these present investigations, significant decreases in the release of both CXCL9 and CXCL11 were noted in CCL26-targeted siRNA treated A549 cells. These results imply that CXCR3 ligands may also be modulated by CCR3.

In a recent report, basal release chemokines upregulated by treatment of primary human alveolar type II cells with LPS and a Th1 cytokine mix [TNF-α, IL-1β, and interferon-γ (IFN-γ)] included IL-8 (CXCL8), MCP-1 (CCL2), RANTES (CCL5), MIP-1α (CCL3), and GRO-α (CXCL1) (Witherden and others 2004). Human cytokine antibody membrane array studies in these present investigations revealed basal release of these same chemokines from alveolar type II cells. Constitutive release of CXCL1 and CXCL8 serves as constant trafficking signals for neutrophils which are present with EOSs in cases of more severe asthma. Prior to stimulation with either CXCL8 or CXCL1, EOSs migrate toward either of these chemokines when co-incubated with neutrophils (Kikuchi and others 2006). In the present investigations, results suggest that CCL26-targeted siRNA treatment of alveolar type II cells may favorably reduce the neutrophil-recruiting signals by reducing both CXCL1 and CXCL8 release. These results imply that in response to CXCL1 and/or CXCL8, neutrophils migrate, activate, and release products that enhance the co-migration of EOSs and that, because neutrophils are refractory to anti-inflammatory steroid treatment, an siRNA therapeutic approach may be of benefit in ameliorating the inflammatory component of chronic airway diseases such as asthma.

Functionally, CCL2-CCR2B binding stimulates mononuclear cell chemotaxis (Wang and others 2007). Recent data suggest that pulmonary CCL2 targets recruitment of peripheral blood monocytes while exerting little effect on resident alveolar macrophages (Opalek and others 2007). Upregulation of CCL2 in alveolar type II cells occurs in response to Th1 stimuli including combinations of IL-1β, TNF-α, and IFN-γ (Pechkovsky and others 2005; Wang and others 2007). Results of the present studies with A549 cells corroborate these results in that no Th2-driven increases in CCL2 were observed. In contrast, a significant suppression of CCL2 was noted for CCL26-siRNA-treated and IL-4-stimulated A549 cells. Interestingly, CCL26 is a known natural antagonist for CCR2. CCL26 promotes active movement of monocytes away from a CCL26 gradient and the effect is synergistic in the presence of CCL2 (Ogilvie and others 2003). Data from the present investigations suggest that CCL26 may also modulate CCL2 levels. Further investigation is needed to delineate the mechanism by which CCL26 subtly regulates monocyte responses through the CCL2-CCR2B network.

All three eotaxins activate human EOSs with similar efficacies for chemotaxis, cytoskeletal rearrangements, activation of G_i proteins and transients of $[Ca^{2+}]_i$ but have distinct profiles for CCR3 binding and stimulation of reactive oxygen species generation (Sanders and others 1995; Sanders 1999; Dulkys and others 2001). Compelling evidence suggests that airway epithelium-derived eotaxins direct EOS migration into the airway mucosa. Eosinophils then signal to the airway epithelium through release of their reactive oxygen species and secondary granule proteins. Within this new paradigm, these EOS–epithelial cell interactions are considered a central regulatory mechanism modulating local immune responses (Jacobsen and others 2007). With respect to EOS activation, we have recently reported that each of the three eotaxins directly stimulates EOS $O_2\bullet-$–generation which may be inhibited by pretreatment of cells with specific neutralizing antibodies or bioactivity neutralizing anti-CCR3 antibodies (Badewa and others 2002). Investigations with supernatants from Th2-type cytokine–stimulated A549 cells then indicated that released proinflammatory mediators were stimulating EOS $O_2\bullet-$–generation that could be inhibited by the CCR3 antagonist SB-328437 suggesting that CCR3 agonists were activating the EOSs (Abonyo and others 2005). Results of the present investigations now demonstrate that airway epithelial cells constitutively release products that stimulate EOSs. Significant reductions in stimulation of EOSs were noted for the CCL26-targeted siRNA treatments. These results indicate that the CCR3-agonist pathway is crucial for activation of EOSs. Results suggest that decreasing these signals through targeted siRNA treatment of the airway epithelium may reduce proinflammatory signaling to leukocytes, in particular the EOSs.

With respect to EOS recruitment, two unique features of CCL26 that contribute to its critical role in transepithelial leukocyte migration are attachment of the biologically active form to the epithelium (Yuan and others 2006) and its storage in and rapid release from Weibel-Palade bodies. This is in support of the clinical data which has shown dramatic upregulation of CCL26 in asthmatics 24 h after challenge and correlation with the magnitude of the late asthmatic response (Berkman and others 2001; Ravensberg and others 2005). Results of the present investigations demonstrate that human EOSs migrate toward CCL26 in a concentration-dependent manner and that migration may be inhibited by pretreating EOSs with neutralizing anti-CCR3 antibody. Eosinophil migration toward supernatants from CCL26-siRNA-treated and IL-4-stimulated A549 cells was significantly reduced. A complete inhibition of EOS migration was not expected because in addition to the eotaxins, other chemokines including CCL5 (RANTES), CCL7 (MCP-1), and CCL13 (MCP-4) have been implicated in EOS recruitment and activation in the asthmatic airway (Holgate and others 1997). Results of the present investigations bear this out in that EOSs migrate to unstimulated airway epithelial cells. This suggests that other constitutively expressed cytokines/chemokines are also influencing EOS movement. In chemokine comparison studies, the rank order of EOS chemotaxis toward CC chemokines was reported as CCL26 = CCL11 = CCL24 >

CCL5 (RANTES) > CCL13 (MCP-4) (Dulkys and others 2001). In addition, CCL28 that is released from A549 cells is chemotactic for EOSs by signaling through CCR3 (John and others 2005). Interestingly, in addition to CCR3, the EOSs of ~20% of human donors also possess CCR1, and are over-represented among atopic individuals. Their EOSs are chemotactic toward CCR3 ligands and toward CCR1 high-affinity ligands (CCL3, CCL5, and CCL7). It has been suggested that this be taken into consideration when designing novel therapeutic agents for the amelioration of eosinophilic inflammation. CCR1 mRNA has been demonstrated in the clone 15 HL-60 eosinophilic cells used in these present investigations, and these cells do respond to the CCR1 ligand CCL3 (Tiffany and others 1998; Phillips and others 2003). These additional chemokines, shown to be released by A549 cells in these present studies, may also have contributed to EOSs migration. Taken together, these results suggest that responses of the airway epithelial cells to treatment with CCL26-targeted siRNA do result in a reduction of CCR3-agonist-induced proinflammatory mediators with subsequent reductions in EOS migration and activation.

In summary, results of these present investigations and cited studies suggest an airway epithelial chemokine receptor/ligand scheme that may be envisioned as follows. At sites of chronic inflammation there is selective upregulation of CC chemokines which then orchestrate recruitment and proinflammatory bioactivities of specific leukocytes. In the case of lung diseases such as asthma, a Th2-driven environment involving IL-4/IL-13 activates airway epithelial cells. Signal transduction induced by cytokine stimulation of the epithelium activates transcription factors including NF-κB, AP-1, and STATs that amplify and perpetuate the inflammatory response by increasing expression of multiple cytokines/chemokines (reviewed in depth by Barnes 2003). Recruitment is partially in response to increased expression of a subset of CC chemokines including CCL11, CCL24, CCL26, CCL2, CCL5, and CCL13 that act as chemoattractants and/or activating agents for EOSs, basophils, dendritic cells, lung parenchyma mast cells, and subsets of Th2 lymphocytes. Following the chemotactic events, bidirectional activating interactions between the emigrating leukocytes and the lung resident cells engage the self-sustaining proinflammatory cycle (reviewed in depth by Hogan and others 2008). The CCR3-agonist chemokine network is an integral component of this cycle (reviewed by Elsner and others 2004) and becomes an attractive target for design for novel therapeutic interventions. The use of siRNA-based therapeutics that specifically interfere with disease-promoting genes has been used in these investigations. *In vitro* designed CCL26-siRNA is delivered to cells where it is incorporated into the RNA interference–induced silencing complex (RISC). With perfect sequence complementarity of the CCL26-siRNA duplex, the sense strand is cleaved. The activated RISC containing the antisense guide strand recognizes target sites to direct cleavage of airway epithelial cell CCL26 mRNA (reviewed in depth by De Fougerolles and others 2007 and Grimm and others 2007). With degradation of the cleaved mRNA, post-transcriptional silencing of gene expression is achieved. This circumvents the CCL26-directed trafficking of proinflammatory EOSs and other leukocytes into the airways and CCR3-CCL26 modulation of other airway cytokines/chemokines. Results of these investigations suggest that CCL26-targeted siRNA treatment of the airway epithelium may be a novel approach to reduce levels of Th2-responsive chemokines and leukocyte chemoattraction and activation that are critical events in the perpetuation of underlying pulmonary inflammation. Regulation of CCL26 synthesis and release and CCR3–ligand signaling may be a viable, safe, and beneficial approach toward control of the inflammatory component of chronic airway diseases.

Acknowledgments

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References

Abonyo B, Alexander MS, Heiman AS. 2005. 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:L478–488.

Bacon K, Baggiolini M, Broxmeyer H, Horuk R, Lindley I, Mantovani A, Maysushima K, Murphy P, Nomiyama H, Oppenheim J, Rot A, Schall T, Tsang M, Thorpe R, van Damme J, Wadhwa M, Yoshie O, Zlotnik A, Zook K, IUIS/WHO subcommittee on chemokine nomenclature. 2002. Chemokine/chemokine receptor nomenclature. J Interferon Cytokine Res 22:1067–1068.

Badewa AP and Heiman AS. 2003. Inhibition of CCL11, CCL24, and CCL26-induced degranulation in HL-60 eosinophilic cells by specific inhibitors of MEK1/MEK2, p38 MAP kinase and PI 3-kinase. Immunopharm Immunotoxicol 25:145–157.

Badewa AP, Hudson CE, Heiman AS. 2002. Regulatory effects of eotaxin, eotaxin-2 and eotaxin-3 on eosinophil degranulation and superoxide anion generation. Exp Biol Med 227(8):645–651.

Banwell ME, Tolley NS, Williams TJ, and Mitchell TJ. 2002. Regulation of human eotaxin-3/CCL26 expression: modulation by cytokines and glucocorticoids. Cytokine 21:317–323.

Barnes PJ. 2003. Pathophysiology of asthma. Eur Respir Mon 23:84–113.

Beck LA, Tancowny B, Brummet ME, Asaki SY, Curry SL, Penno MB, Foster M, Bahl A, Stellato C. 2006. Functional analysis of the chemokine receptor CCR3 on airway epithelial cells. J Immunol 177:3344–3354.

Berkman N, Ohnona S, Chung FK, Breuer R. 2001. Eotaxin-3 but not eotaxin gene expression is upregulated in asthmatics 24 hours after allergen challenge. Am J Respir Cell Mol Biol 24:682–687.

Charo IF, Ransohoff RM. 2006. The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med 354:610–621.

De Fougerolles A, Vornlocher H-P, Maraganore J, Lieberman J. 2007. Interfering with disease: a progress report on siRNA-based therapeutics. Nature Rev 6:443–453.

Dekker RJ, Boon RA, Rondaij MG, Kragt A, Volger OL, Elderkamp YW, Meijers JCM, Voorberg J, Pannekoek H, Horrevoets JG. 2006. KLF2 provokes a gene expression pattern that establishes functional quiescent differentiation of the endothelium. Blood 107:4354–4363.

De Lucca CV. Recent developments in CCR3 antagonists. 2006. Curr Opin Drug Discov Devel 9 (4):516–524.

Dulkys Y, Schramm G, Kimmig D, Knöb S, Weyergraf A, Kapp A, Elsner J. 2001. Detection of mRNA for eotaxin-2 and eotaxin-3 in human dermal fibroblasts and their distinct activation profile on human eosinophils. J Invest Dermatol 116:498–505.

Elsner J, Escher SE, Forssmann U. 2004. Chemokine receptor antagonists: a novel therapeutic approach in allergic diseases. Allergy 59:1243–1258.

Elsner J, Dulkys Y, Gupta S, Escher SE, Forssmann WB, Kapp A, Forssmann U. 2005. Differential pattern of CCR1 internalization in human eosinophils:prolonged internalization by CCL5 in contrast to CCL3. Allergy 60(11):1386–1393.

Fan J, Heller NM, Gorospe M, Atasoy U, Stellato C. 2005. The role of post-transcriptional regulation in chemokine gene expression in inflammation and allergy. Eur Respir J 26:933–947.

Fulkerson PC, Fischetti CA, McBride ML, Hassman LM, Hogan SP, Rothenberg ME. 2006. A central regulatory role for eosinophils and the eotaxin/CCR3 axis in chronic experimental allergic airway inflammation. Proc Natl Acad Sci 103:16418–16423.

Garcia G, Godot V, Humbert M. 2005. New chemokine targets for asthma therapy. Curr Allergy Asthma Rep 5:155–160.

Green RH, Brighting CE, Mckenna S, Hargadon B, Parker D, Bradding P, Wardlaw AJ, Pavord ID. 2002. Asthma exacerbations and sputum eosinophil counts: a randomized controlled trial. Lancet 360:1715–1721.

Grimm D, Kay MA. 2007. Therapeutic application of RNAi: is mRNA targeting finally ready for prime time? J Clin Invest 117:3633–3641.

Haley KJ, Sunday ME, Porrata Y, Kelley C, Twomey A, Shahsafaei A, Galper B, Sonna LA, Lilly CM. 2008. Ontogeny of the eotaxins in human lung. Am J Physiol Lung Cell Mol Physiol 294:L214–L224.

Heiman AS, Abonyo BO, Darling-Reed SF, and Alexander MS. 2005. Cytokine-stimulated human lung alveolar epithelial cells release eotaxin-2 (CCL24) and eotaxin-3 (CCL26). J Interferon Cytokine Res 25:20–29.

Hogan SP, Rosenberg HF, Moqbel R, Phipps S, Foster PS, Lacy P, Kay AB, Rothenberg ME. 2008. Eosinophils: biological properties and role in health and disease. Clin Exp Allergy 38:709–750.

Holgate ST. 2007. Epithelium dysfunction in asthma. J Allergy Clin Immunol 120:1233–1244.

Holgate ST, Body KS, Janezic A, Frew AJ, Kaplin AP, Teran LM. 1997. Release of RANTES, MIP-1α and MCP-1 into asthmatic airways following endobronchial allergen challenge. Am J Respir Crit Care Med 156:1377–1383.

Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, Ghiran S, Gerard NP, Yu C, Orkin SH, Gerard C. 2004. A critical role for eosinophils in allergic airways remodeling. Science 305:1726–1729.

Jacobsen EA, Taranova AG, Lee NA, Lee JJ. 2007. Eosinophils: singularly destructive effector cells or purveyors of immunoregulation? J. Allergy Clin Immunol 119:1313–1320.

John AE, Thomas MS, Berlin AA, Lukacs NW. 2005. Temporal production of CCL28 corresponds to eosinophil accumulation and airway hyperreactivity in allergic airway inflammation. Amer J Pathol 166:345–353.

Joubert P, Lajoie-Kadoch S, Labonte I, Gounni AS, Maghni K, Wellemans V, Chakir J, Laviolette M, Hamid Q, Lamkhioued B. 2005. CCR3 expression and function in asthmatic airway smooth muscle cells. J Immunol 175:2702–2708.

Kikuchi I, Kikuchi S, Kobayashi T, Hagiwarar K, Sakamoto Y, Kanazawa M, Nagata M. 2006. Eosinophil trans-basement membrane migration induced by interleukin-8 and neutrophils. Am J Respir Cell Mol Biol 34:760–766.

Kitauri M, Suzuki N, Imai T, Takagi S, Suzuki R, Nakajima T, Hirai K, Nomiyama H, Yoshie O. 1999. Molecular cloning of a novel human CC chemokine (eotaxin-3) that is a functional ligand of CC chemokine receptor 3. J Bio Chem 274:27975–27980.

Kobayashi I, Yamamoto S, Nishi N, Tsuji K, Imayosh IM, Inada S. 2004. Regulatory mechanisms of Th2 cytokine-induced eotaxin-3 production in bronchial epithelial cells: possible role of interleukin 4 receptor and nuclear factor-kappaB. Ann Allergy Asthma Immunol 93:390–397.

Komiya A, Nagase H, Yamada H, Sekiya T, Yamaguchi M, Sano Y, Hanai N, Furuya A, Ohta K, Matsushima K, Yoshie O, Yamamoto K, and Hirai K. 2003. Concerted expression of eotaxin-1, eotaxin-2 and eotaxin-3 in human bronchial epithelial cells. Cell Immunol 225:91–100.

Lee JJ, Dimina D, Macias MP, Ochkur SI, McGarry MP, O'Neill KR, Protheroe C, Pero R, Nguyen T, Cormier SA, Lenkiewicz E, Colbert D, Rinaldi L, Ackerman SJ, Irvin CG, and Lee NA. 2004. Defining a link with asthma in mice congenitally deficient in eosinophils. Science 305:1776–1779.

Loetscher P, Pellegrino A, Gong JH, Mattioli I, Loetscher M, Bardi G, Baggiolini M, Clark-Lewis I. 2001. The ligands of CXC chemokine receptor 3, I-TAC, MIG and IP-10 are natural antagonists for CCR3. J Biol Chem 276:2986–2991.

Moon KA, Kim SY, Kim TB, Yun ES, Park CS, Cho YS, Moon HB, Lee KY. 2007. Allergen-induced CD11b+ CD11c(int) CCR3+ macrophages in the lung promote eosinophilic airway inflammation in a mouse asthma model. Int Immunol 19:1371–1381.

Morokata T, Suzuki K, Masunaga Y, Taguchi K, Morihira K, Sato I, Fujii M, Takizawa S, Toril Y, Yamamoto N, Kaneko M, Yamada T, Takahashi K, Shimizu Y. 2006. A novel, selective and orally available antagonist for CC chemokine receptor 3. J Pharamcol Exp Therap 317:244–250.

Murphy PM. 2002. International Union of Pharmacology. Update on chemokine receptor nomenclature. Pharmacol Rev. 54:227–229.

Nakamura H, Luster AD, Tateno H, Jedrzkiewicz S, Tamura G, Jaley KJ, Garcia-Zepeda EA, Yamaguchi K, and Lilly CM. 2001. IL-4 differentially regulates eotaxins and MCP-4 in lung epithelium and circulating mononuclear cells. Am J Physiol Lung Cell Mol Physiol 281:L1288–L130237.

Odaka M, Matsukura S, Kuga H, Kokubu F, Kasama T, Kurokawa M, Kawaguchi M, Icki K, Suzuki S, Watanabe S, Homma T, Takeuchi H, Nohtomi K, Schleimer RP, Adachi M. 2007. Differential regulation of chemokine expression by Th1 and Th2 cytokines and mechanisms of eotaxin/CCL-11 expression in human airway smooth muscle cells. Int Arch Allergy Immunol 143:84–88.

Ogilvie Paoletti S, Clark-Lewis I, Uguccioni M. 2003. Eotaxin-3 is a natural antagonist for CCR2 and exerts a repulsive effect on human monocytes. Blood 102:789–794.

Opalek JM, Ali NA, Lobb JM, Hunter MG and Marsh CB. 2007. Alveolar macrophages lack CCR2 expression and do not migrate to CCL2. J Inflamm 4:19–29.

Pan J, Kunkel EJ, Gosslar U, Lazarus N, Langdon P, Broadwell K, Vierra MA, Genovese MC, Butcher EC, Soler D. 2000. A novel chemokine ligand for CCR10 and CCR3 expressed by epithelial cells in mucosal tissues. J Immunol 165:2943–2949.

Pease JE, Williams TJ. 2006. Chemokines and their receptors in allergic disease. J Allergy Clin Immunol 118; 305–318.

Pechkovsky D, Goldmann T, Ludwig C, Prasse A, Vollmer E, Müller-Quernheim J, Zissel G. 2005. CCR2 and CXCR3 agonistic chemokines are differently expressed and regulated in human alveolar epithelial cells type II. Respiratory Res 6:75–92.

Phillips RM, Stubbs VEL, Henson MR, Williams TJ, Pease JE, Sabroe I. 2003. Variations in eosinophil chemokine responses:An investigation of CCR1 and CCR3 function, expression in atopy and identification of a functional CCR1 promoter. J Immunol 170:6190–6201.

Ponath, PD, Qin S, Post TW, Wang J, Wu L, Gerard NP, Newman W, Gerard C, Mackay CR. 1996. Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. J Exp Med 183:2437–2448.

Puxeddu I, Bader R, Piliponsky AM, Reich R, Levi-Schaffer F, Berkman N. 2005. The CC chemokine eotaxin/CCL11 has a selective profibrogenic effect on human lung fibroblasts. J Allergy Clin Immunol 117:103–110.

Ravensberg AJ, Ricciardolo FL, van Schadewijk A, Rabe KF, Sterk PJ, Hiemstra PS, Mauad T. 2005. Eotaxin-2 and eotaxin-3 expression associated with persistent eosinophilic bronchial inflammation in patients with asthma after allergen challenge. J Allergy Clin Immunol 115:779–785.

Regamey N, Obregon C, Ferrari-Lacraz S, van Leer C, Chanson M, Nicod LP, Geiser T. 2007. Airway epithelial IL-15 transforms monocytes into dendritic cells. Am J Respir Cell Mol Biol 37:75–84.

Sanders SP. 1999. Nitric oxide in asthma. Pathogenic, therapeutic, or diagnostic? Am J Respir Cell Mol Biol 21:147–149.

Sanders SP, Zweier JL, Harrison SJ, Trush MA, Rembish SJ and Liu MC. 1995. Spontaneous oxygen radical production at sites of antigen challenge in allergic subjects. Am J Respir Crit Care Med 151:1725–1733.

Sebzda E, Zou Z, Lee JS, Wang T, Kahn ML. 2008. Transcription factor KLF2 regulates the migration of naive T cells by restricting chemokine receptor expression patterns. Nat Immunol 9(3):233–235.

Shinkai A, Yoshisue H, Loike M, Shoji E, Nakagawa S, Saito A, Takeda T, Imabeppu S, kato Y, Hanai N, Anazawa H, Kuga T, Nishi T. 1999. A novel human CC chemokine, eotaxin-3, which is expressed in IL-4-stimulated vascular endothelial cells, exhibits potent activity toward eosinophils. J Immunol 163:1602–1610.

Smit JJ and Lukacs. 2006. A closer look at chemokines and their role in asthmatic responses. Eur J Pharmacol 533:277–288.

Stellato C, Brummet ME, Plitt JR, Shahabuddin S, Baroody FM, Liu MC, Ponath PD, and Beck LA. 2001. Expression of the C-C chemokine receptor CCR3 in human airway epithelial cells. J Immunol 166:1457–61.

Taka E, Errahali YJ, Heiman AS. 2008. Post-transcriptional silencing of CCR3 downregulates IL-4 stimulated release of CCL26 in alveolar type II cells. FASEB J:22(4): abstract 762.4.

Tiffany HL, Alkhatib G, Combadiere C, Berger EA, Murhpy PM. 1998. CC chemokine receptors 1 and 3 are differentially regulated by IL-5 during maturation of eosinophilic HL-60 cells. J Immuno 160:1385–1392.

Wang H, Yi T, Zheng Y, He S. 2007. Induction of monocyte chemoattractant protein-1 release from A549 cells by agonists of protease-activated receptor-1 and -2. Eur J Cell Biol 86:233–242.

Witherden IR, Vanden Bon EJ, Goldstraw P, Ratcliffe C, Pastorino U, Tetley TD. 2004. Primary human alveolar type II epithelial cell chemokine release—Effects of cigarette smoke and neutrophil elastase. Am J Respir Cell Mol Biol 30:500–509.

Xanthou G, Duchesnes CE, Williams TJ, and Pease JE. 2003. CCR3 functional responses are regulated by both CXCR3 and it s ligands CXCL9, CXCL10 and CXCL11. Eur J Immunol 33:2241–2250.

Yamamoto S, Kobayashi I, Tsuji K, Nishi N, Muro E, Miyazaki M, Zaitsu M, Inada S, Ichimaru T and Hamasaki Y. 2004. Upregulation of interleukin-4 receptor by interferon-gamma: enhanced interleukin-4-induced eotaxin-3 production in airway epithelium. Am J Respir Cell Mol Biol 31:456–462.

Ying S, Meng Z, Zeibecoglou K, Robinson DS, Macfarlane A, Humbert M, Kay AB. 1999. Eosinophil chemotactic chemokines (eotaxin, eotaxin-2, RANTES, monocytes chemoattractant protein-3 (MCP-3), and MCP-4, and C-C chemokine receptor 3 expression in bronchial biopsies from atopic and nonatopic asthmatics. J Immunol 163:6321–6329.

Yuan Q, Campanella GS, Colvin RA, Hamilos DL, Jones KJ, Mathew A, Means TK, Luster AD. 2006. Membrane-bound eotaxin-3 mediates eosinophil transepithelial migration in IL-4 stimulated epithelial cells. Eur J Immunol 36:2700–2714.

Zimmermann N, Hershey GK, Foster PS, Rothenberg ME. 2003. Chemokines in asthma: cooperative interactions between chemokines and IL-13. J Allergy Clin Immunol 111:227–242.

Address reprint requests or correspondence to:
Ann S. Heiman
Florida A&M University
College of Pharmacy and Pharmaceutical Sciences
Tallahassee, FL 32307

Tel: 850-599-3774
Fax: 850-599-3323
E-mail: heimanta@nettally.com

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2. Lin Feng, Deting Xue, Erman Chen, Wei Zhang, Xiang Gao, Jiawei Yu, Yadong Feng, Zhijun Pan. 2016. HMGB1 promotes the secretion of multiple cytokines and potentiates the osteogenic differentiation of mesenchymal stem cells through the Ras/MAPK signaling pathway. *Experimental and Therapeutic Medicine* **12**:6, 3941-3947. [Crossref]
3. Rudolf Richter, Shipra Gupta, Ulf Forssmann. Chemokine CCL15 316-323. [Crossref]
4. Younes J. Errahali, LeeShawn D. Thomas, Thomas C.S. Keller III, Henry J. Lee. 2013. Inhibition by New Glucocorticoid Antedrugs [16α, 17α-d] Isoxazoline and [16α, 17α-d]-3′-Hydroxy-Iminoformyl Isoxazoline Derivatives of Chemotaxis and CCL26, CCL11, IL-8, and RANTES Secretion. *Journal of Interferon & Cytokine Research* **33**:9, 493-507. [Abstract] [Full Text] [PDF] [PDF Plus]
5. . Eosinophil Cell–Cell Communication 329-390. [Crossref]
6. Marit Låg, Dmitrii Rodionov, Johan Øvrevik, Oddmund Bakke, Per E. Schwarze, Magne Refsnes. 2010. Cadmium-induced inflammatory responses in cells relevant for lung toxicity: Expression and release of cytokines in fibroblasts, epithelial cells and macrophages. *Toxicology Letters* **193**:3, 252-260. [Crossref]