Published in final edited form as:

Cytokine. 2008 December ; 44(3): 342–351. doi:10.1016/j.cyto.2008.09.006.

Post-transcriptional silencing of CCR3 downregulates IL-4 stimulated release of eotaxin-3 (CCL26) and other CCR3 ligands in alveolar type II cells

Equar Taka, Younes J. Errahali, Barack O. Abonyo, David M. Bauer, and Ann S. Heiman^{*}
Florida A & M University, College of Pharmacy and Pharmaceutical Sciences, Tallahassee, FL 32307

Abstract

Trafficking and inflammation in airway diseases are, in part, modulated by members of the CC chemokine family, eotaxin-1 (CCL11), eotaxin-2 (CCL24), and eotaxin-3 (CCL26), which transduce signals through their CCR3 receptor. In this context, we hypothesized that transfecting alveolar type II epithelial cells with CCR3-targeted siRNA or antisense (AS-ODN) sequences will downregulate cellular synthesis and release of the primary CCR3 ligands CCL26 and CCL24 and will modulate other CCR3 ligands. The human A549 alveolar type II epithelium-like cell culture model was used for transfection and subsequent effects on CCR3 agonists. siRNAs were particularly effective. PCR showed a 60-80% decrease in mRNA and immunoblots showed up to 75-84% reduction of CCR3 in siRNA treated cells. CCR3-siRNA treatments reduced IL-4 stimulated CCL26 release and constitutive CCL24 release by 65% and 80%, respectively. Release of four additional CCR3 agonists RANTES, MCP-2, MCP-3 and MCP-4 was also significantly reduced by CCR3-siRNA treatments of the alveolar type II cells. Activation of eosinophils, assessed as superoxide anion generation, was reduced when eosinophils were treated with supernatants of A549 cells pretreated with CCR3-targeted siRNAs or AS-ODNs. Collectively, the data suggest that post-transcriptional regulation of CCR3 receptors may be a potential therapeutic approach for interrupting proinflammatory signaling.

Keywords

alveolar type II cells; CCR3 receptor; eotaxins; siRNA; antisense oligonucleotides

1. Introduction

Global Initiative for Asthma (GINA) scholars have operationally defined asthma as a “chronic inflammatory disorder of the airways in which cells and cellular elements play a role. The chronic inflammation causes an associated increase in airway hyperresponsiveness and leads

Corresponding author: Ann S. Heiman, Florida A & M University, College of Pharmacy and Pharmaceutical Sciences, Tallahassee, FL 32307. Telephone: 850-599-3774; FAX: 850-599-3323; e-mail: heimanta@netally.com, Equar Taka, Florida A & M University, College of Pharmacy and Pharmaceutical Sciences, Tallahassee, FL 32307. Telephone: 850-561-2786; FAX: 850-599-3323; e-mail: equar.taka@fam.u.edu, Younes Errahali, Florida A & M University, College of Pharmacy and Pharmaceutical Sciences, Tallahassee, FL 32307. Telephone: 850-599-3661; FAX: 850-599-3323; e-mail: youneserrahali3@yahoo.com, Barack Abonyo, Florida A & M University, College of Pharmacy and Pharmaceutical Sciences, Tallahassee, FL 32307. Telephone: 850-561-2553; FAX: 850-599-3323; e-mail: abonyo.barack@fam.u.edu, David Bauer, Florida A & M University, College of Pharmacy and Pharmaceutical Sciences, Tallahassee, FL 32307. Telephone: 850-561-2786; FAX: 850-599-3323; e-mail: dreuab@yahoo.com.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

to episodic wheezing, breathlessness and coughing” (1). While the episodic symptoms may often be successfully treated, the chronic airway inflammation is more insidious. Bronchial biopsy studies have revealed that asthmatics in clinical remission do show ongoing airway inflammation and remodeling of the pulmonary epithelium (2). The emerging paradigm of asthma depicts an incompletely repaired damaged epithelium that displays altered permeability and releases increased amounts of numerous growth factors, cytokines and chemokines (3).

Chemokines orchestrate leukocyte trafficking during states of homeostasis, immune responses and inflammation, and are implicated in the pathogenesis of chronic diseases including asthma, chronic obstructive pulmonary disease (COPD), emphysema and chronic bronchitis. Chemokines directly modulate cell bioactivities through complex networks wherein a particular chemokine may bind multiple chemokine receptors with varying affinities and subsequently direct migration/activation of specific types of inflammatory leukocytes (4). For example, asthma is characterized by Th2-type cytokine-driven elevation of CC chemokines including the three eotaxins CCL11 (eotaxin-1), CCL24 (eotaxin-2) and CCL26 (eotaxin-3) (2). The eotaxins serve as recruitment signals for eosinophils (EOS) with concomitant increases in Th2 lymphocytes, mast cells and basophils. Bidirectional interactions between the emigrating leukocytes and the resident cells, in particular the epithelium, engage the self-sustaining proinflammatory cycle (3). The three eotaxins, as well as CCL28, exert effector actions by binding to their high affinity G-protein-coupled receptor designated CCR3 (7-9) which is expressed by EOS, basophils, mast cells, subsets of Th2 lymphocytes and dendritic cells (10-12). Of particular interest to the present studies are the findings that the constitutively expressed CCR3 of airway epithelial cells is increased following stimulation with IL-4 or IL-13. This stimulation also increases synthesis and release of CCL11, CCL24 and large amounts of CCL26. CCR3-CCL26 receptor-agonist autocrine regulatory mechanisms result in downregulation of both CCR3 and CCL26. Thus, the eotaxins may contribute to pathogenesis at sites of Th2 phenotypic inflammation not only through leukocyte recruitment but ligand-CCR3 receptor autoregulatory mechanisms (12).

In this context, CCR3 antagonism has emerged as a therapeutic target for the treatment of airway diseases and the associated underlying inflammation (13-16). Reported approaches to inhibiting CCR3-ligand transduction signaling have focused on synthesis of low molecular weight antagonists and eotaxin-neutralizing antibodies (17-20). Attractive alternatives include antisense oligonucleotides (AS-ODNs) and short-interfering RNAs (siRNAs) directed at post-transcriptional inhibition of gene expression rather than blocking gene products (21,22). Of importance here is the recent report indicating that AS-ODN-induced topical inhibition of CCR3 expression may represent a novel and efficacious approach for the treatment of airway disease (23,24). The therapeutic potential of siRNA for treatment of human disease is being explored at the levels of target identification, product specificity, potency, delivery and clinical effects (25). Studies involving siRNA-based treatment of respiratory diseases have focused predominantly on viral infections and acute lung injury (26-28). Reports involving the eotaxins have been limited to delineating factors which modulate their synthesis (29,30). To date, effects of CCR3-targeted siRNA delivered to human airway epithelial cells have not been reported.

Thus, experiments were designed to delineate the effects of CCR3-targeted siRNAs and AS-ODNs on post-transcriptional inhibition of CCR3 gene expression in resting and IL-4-stimulated cells. Studies were carried out to test the hypothesis that transfection of alveolar type II cells with CCR3-targeted AS-ODNs or siRNAs will downregulate alveolar type II epithelial cell CCR3, synthesis and release of the agonists CCL26 and CCL24 and will also modulate other chemokines that bind to CCR3. Briefly, the data confirm that CCR3-targeted AS-ODNs and siRNAs significantly inhibited CCR3 expression which then concomitantly decreased CCL26 and CCL24 synthesis and release as well as release of other CCR3 ligands. Supernatants from CCR3 AS-ODN or siRNA treated A549 cells significantly reduced

eosinophil superoxide anion release suggesting that inhibition of CCR3 expression at the alveolar epithelium level may be a key approach to limiting eosinophil activation within the airway.

2. Methods

2.1. Culture of airway epithelial cells

Human A549 alveolar type II epithelial-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 $0.25 \times 10^6 - 1 \times 10^6$ cells/ml. Experiments were performed after subcultured cells had reached approximately 80% confluence (10,12).

2.2. A549 alveolar type II epithelial cell siRNA treatment protocol

Trypsin-dispersed A549 cells were washed, resuspended in antibiotic free medium supplemented with 2.5% fetal calf serum and seeded at 1.0×10^5 cells/ml in 12-well cluster plates (1 ml/well). Cells were incubated and allowed to attach overnight. Dharmacon (Lafayette CO) transfection reagent DharmaFECT 1 and ON-TARGETplus SMARTpool designed siRNAs (product #L-005450, human CCR3, NM_178329) consisting of duplexes 7, 8, 9 and 10 alone and combined as the SMARTpool were used to silence CCR3. Dharmacon siCONTROL Non-Targeting siRNA pool was used as the control. DharmaFECT 1 and siRNA products were prepared according to the manufacturer's instructions. In preliminary studies it was determined that maximal siRNA effects on CCR3 protein were seen at 96 hours post transfection with 120 nM siRNAs. Transfection efficiency was qualitatively assessed by treatment of cells with 120 nM siCONTROL Tox which led to >95% cell death within 48 hrs of transfection. Following transfection, monolayers were washed, then treated with or without IL-4 (100 ng/ml) in serum free medium for 24 hours. Cell culture medium was collected, cells detached with PBS/EDTA, pelleted and cell lysates prepared.

2.3. A549 alveolar type II epithelial cell antisense oligonucleotide treatment protocol

Prior to chemokine and/or cytokine stimulation, A549 cells, subcultured into 6-well cluster plates ($0.25 \times 10^6 - 0.5 \times 10^6$ cells/ml), were washed, then incubated in serum-free RPMI 1640/F12K (50:50, v/v) medium containing indicated concentrations of human CCR3 predesigned Biognostik (Göttingen, Germany) antisense 1 (sequence ID: 1.04967) and antisense 2 (sequence ID: 2.04968) concentrations with 6 µl FuGENE 6 transfection reagent. Biognostik sense controls (reverse complement of target sequences 1.04967 and 2.04968) with no known human cross homologies, were included in all experiments. Cells were incubated for 30 hours and an additional 1 ml complete medium added and cells cultured an additional 12 hours followed by IL-4 (100 ng/ml) (Atlanta Biologicals, Atlanta, GA) for 4 or 24 hours, as indicated. Cell culture medium was collected, centrifuged (4°C at $100 \times g$) for 5 minutes and supernatants stored at -20°C until analyses were carried out.

2.4. Detection of alveolar epithelial cell surface CCR3 receptors by flow cytometry

Treated cells were detached with 0.5 mM EDTA in PBS, centrifuged at $100 \times g$, 4°C for 5 min, washed twice in cold FACSflow buffer (BD Biosciences, San Jose, CA) and resuspended to a final concentration of 5×10^6 cells/ml. Aliquots of cells were stained with 500 ng/ml biotinylated human recombinant eotaxin or 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 minutes in the dark at 4°C (12). After two

washes in cold FACSflow buffer, stained cells were maintained at 4°C then subjected to flow cytometry in a FACSCalibur and data analyzed using CellQuest software (BD Biosciences, San Jose, CA)

2.5. Detection of CCR3 by Western Immunoblotting

To assess effects of CCR3-siRNA or AS-ODN treatments on CCR3 receptor expression, A549 airway epithelial cells (1×10^5 cells/well in 12-well cluster plates) were treated, stimulated and detached as described above. Cell suspensions were centrifuged at $100 \times g$ 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). Samples were incubated in ice for 30 min, sonicated for 3 seconds, centrifuged (5 min, 4°C at $16,000 \times g$), supernatants collected for total protein content. Lysate protein concentrations were quantified with a Power WaveX340-I microplate reader equipped with KC4 v3.0 PowerReports software (Bio-Tek Instruments, Winooski, VT, USA). Cell lysates were separated by electrophoresis on 10% SDS-polyacrylamide gels (10 µg protein/lane) and then transferred to Immobilon-P PVDF 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 PBS containing 0.05% Tween 20 (PBST) and then incubated overnight at 4°C with 3 µg/ml rabbit anti-human CCR3 affinity purified antibody (Imgenex, San Diego, CA). Membranes were washed three times with PBST, incubated with 1:1500 goat anti-rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology, CA) in PBST for three hours then washed three times in PBST. Immunoblot images were obtained using a Fluor-s Max Multimager (Bio-Rad Laboratories, Hercules, CA). Protein loading was monitored by probing membranes with anti-GAPDH antibodies (R & D Systems, Minneapolis, MN). Lane density data were acquired with Quantity One 1-D Analysis Software Version 4.6.0 (Bio-Rad Laboratory, Hercules, CA).

2.6. Detection of CCR3 by Dot-Blot

Cells were treated as described above and CCR3 detected using a minifold I (Schleier and Schuell, Germany) 96-well filtration/incubation unit fitted with a PBS presoaked nitrocellulose membrane and blot paper support sheets. Ten µg of lysate proteins in 200-500 µl water were introduced into manifold wells under vacuum. The nitrocellulose membrane was blocked in PBS containing 3% BSA and 0.05% Tween-20 (PBS-T) overnight at 4°C, washed three times with PBS-T and incubated for 1 hr with 0.6 µg/ml rabbit anti-human CCR3 antibody (Imgenex., San Diego, CA). Following three washes with PBS-T, the membranes were incubated with 1:2000 goat anti-IgG-horseradish peroxidase (Santa Cruz Biotechnology, CA) in PBS-T containing 0.5% BSA. Membranes were then washed four times with PBS-T and incubated for five minutes with SuperSignal Pierce western substrate working solution. Images were obtained and dot blot densities assessed as described above.

2.7. CCL24 and CCL26 detection by specific ELISA

Both synthesized and secreted CCL24 and CCL26 proteins were detected using specific eotaxin ELISA kits (R&D Systems, Minneapolis, MN) These ELISAs recognize both natural and recombinant human CCL24 and CCL26 (31). Eotaxins were quantified with the Power WaveX340-I microplate reader equipped with KC4 v3.0 PowerReports software.

2.8. RNA extraction and PCR

A549 airway epithelial cells were plated (1×10^5 cells/ml, 2ml in each well) in 6-well plates and allowed to attach over night. Cells were transfected for 48 hrs as described above. Treated cell monolayers were washed then incubated in serum-free medium for 6 hrs followed by treatment with or without IL-4 (100 ng/ml) in fresh serum free medium for 24 hrs. Total RNA was isolated using an Easy-spin™ (DNA free) total RNA extraction kit following the protocol

of the manufacturer (iNtRON Biotechnology, distributed by Boca Scientific Inc., Boca Raton, FL). First-strand 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. PCR amplification was performed according to the CCR3 primer manufacturer's protocol (R&D Systems) with GAPDH (R&D Systems) serving as the internal control. From each sample, 2.5 µL cDNA was amplified in 50 µL PCR reaction mixture (10X iTaq buffer, 50 mM MnCl₂), 10 mM dNTP, 5 units/µL iTaq DNA polymerase) containing 0.3 µM CCR3 primers. The PCR thermal cycling programs were first pre-denaturation 94°C for 4 min, followed by 33 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 hr. Images were captured and densities assessed as described above.

2.9. Cytokine Antibody Arrays

Supernatants of CCR3 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 (Raybiotech, Norcross, GA). Very briefly, membranes were blocked, 300 µL of A549 cell supernatants added and incubated at 4°C overnight. Membranes were then washed and incubated in biotin-conjugated primary antibodies overnight at 4°C. Following three washes, membranes were incubated in 1,000-fold diluted HRP-conjugated streptavidin for 2 hours, washed again and detection buffer added. Images were obtained with a Flour-s Max Multi-imager. Densities of cytokine protein spots were quantified with Quantity One 1-D Analysis Software Version 4.6.0.

2.10. 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, Inc., Herndon, VA) supplemented with 10% fetal calf serum, 10 ng/ml IL-5 (Atlanta Biologicals, Atlanta, GA), penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 5% carbon dioxide at 35°C as previously described (12). 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) and washed two times in Hank's balanced salts solution (HBSS). Superoxide anion generation was assessed in microtiter plates with 1 × 10⁵ 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 and 70 µL A549 treated cell culture supernatants. Microtiter plates were incubated at 35°C in an atmosphere of 5% CO₂ for two hours, and absorbances read at 550 nm to determine superoxide dismutase-inhibitable reduction of ferricytochrome C (12).

2.11. 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 ± S.E.M with the mean of triplicates from one experiment serving as one observation. When indicated, one-way analysis of variance (ANOVA) followed by either the Tukey multiple comparisons or Dunnett post-test, as appropriate, was applied to experimental results to determine statistical significance ($p < 0.05$) between indicated groups.

3. Results

3.1. CCR3 siRNA duplexes and their combination treatments decrease CCR3 mRNA, protein and receptor surface expression in A549 alveolar type II cells

Silencing of the A549 alveolar type II epithelial cell CCR3 receptors was accomplished by treating cells with four ON-TARGETplus siRNA duplexes individually or together (*SMARTpool*). A non-targeting siRNA control (a pool of four siRNAs confirmed to have minimal targeting of known genes in human cells) was included in all experiments to distinguish sequence-specific silencing from non-specific effects. Results of flow cytometry studies are depicted in figure 1A. When compared with the non-targeting siRNA treated cells it is evident that treatment with duplex 8, duplex 10 or the *SMARTpool* combination greatly reduced CCR3 cell surface expression. Reductions in CCR3 mRNA resulting from siRNA treatments were demonstrated with PCR using RNA purified from treated A549 cells. Results, depicted in figures 1B and 1C show a typical image and densitometric analyses of several gels, respectively. Results indicate that all CCR3-siRNA treatments significantly suppressed CCR3 mRNA in IL-4 stimulated A549 cells.

Immunoblot experiments were then carried out to demonstrate that the siRNA treatments were altering total CCR3 protein expression. Dot blot analyses for CCR3 on total cell lysates are shown in figure 2A. Results indicate the constitutive presence of CCR3 in untreated cells and upregulation of the receptor following IL-4 stimulation. Suppression of CCR3 protein expression by the *SMARTpool* and the individual duplexes is evident in the dot blot image. Pixel density analyses of three experiments are shown in figure 2B where CCR3 protein expression is compared to expression in cells treated with non-targeting siRNA followed by IL-4 stimulation. Duplex 9 treatments did not have an effect on CCR3 protein expression when compared to the non-targeting siRNA control. In contrast, treatment with the remaining siRNA duplexes resulted in the following effects on CCR3 protein expression: duplex 7 – 33%, duplex 8 – 70% and duplex 10 – 62% inhibition. When cells were treated with the combined siRNA *SMARTpool*, CCR3 expression decreased by 75%. As shown in figure 2C, western blot analyses corroborated those reported for dot blot studies. Results of several experiments were analyzed by densitometry and the outcome depicted in figure 2D. When compared to the non-targeting siRNA treated cells, the densitometry data confirm that duplexes 7, 8, 10 and the combined *SMARTpool* induced CCR3 mRNA degradation which resulted in decreased CCR3 protein expression by 40%, 85%, 50% and 84%, respectively. Collectively, these data demonstrate CCR3-targeted siRNA-induced post-transcriptional silencing of CCR3.

To further validate specific post-transcriptional CCR3 silencing, parallel studies were carried out with two CCR3 AS-ODN sequences and their scrambled controls. Results with antisense 1 concentrations from 100 – 3000 nM indicated a concentration dependent reduction in CCR3 cell surface receptors. Selected concentrations are depicted in figure 3A. CellQuest histogram statistical analyses indicated the following reductions in fluorescence intensity of antisense 1 treated cells versus IL-4 stimulated cells in absence of antisense 1: 500 nM – 40% (not shown), 1000 nM – 60% and 3000 nM – 88%. There was not alteration in surface CCR3 receptors in cells treated with 100-3000 nM CCR3 sense ODNs (data not shown). Western immunoblotting experiments were carried out on cell lysates prepared from A549 alveolar type II cells treated with CCR3 AS-ODNs or their sense ODN controls. Results, shown in figure 3B, demonstrate a concentration dependent decrease of CCR3 in cells treated with 100 nM – 6 μ M AS-ODN sequence 1 in IL-4 stimulated cells. The sense sequence had no effect on CCR3. Collectively these results demonstrate that the CCR3-targeted siRNAs and AS-ODNs effectively suppressed CCR3 gene expression.

3.2. CCR3 antisense 1 or 2 treatments decrease expression of IL-4 stimulated CCL26

We had previously reported that the CCR3 antagonist SB-328437 decreased IL-4 stimulated release of CCL26 from A549 airway epithelial cells (12). It was of interest to explore the effect of CCR3 antisense treatment on expression of CCL26. Results are shown in figure 4. Treatment of cells with antisense 1 or 2 did not significantly decrease amounts of CCL26 measured in whole cell lysates. In contrast, both antisense 1 and 2 significantly decreased IL-4 stimulated release of CCL26. Treatment with sense AS-ODN sequences 1 or 2 had no significant effect. These results suggest that regulation of CCL26 may be partly controlled by blockade of the CCR3-ligand pathway through targeted antisense treatment.

3.3. CCR3 siRNA treatments decrease IL-4 stimulated CCL26 release and constitutively released CCL24

Since siRNA is considered a novel tool to inhibit gene function in human diseases, it was of interest to explore the possibility that CCR3 siRNA delivered to airway epithelial cells would decrease the release of CCR3 ligands, specifically CCL26 and CCL24. Results for constitutive release and IL-4-stimulated release of CCL26 are depicted in figure 5A and 5B, respectively. As expected, very little constitutive release of CCL26 was noted. Cells stimulated with IL-4 in the absence of transfection mix released 1564 ± 112 pg/ml CCL26 (data not shown). Cells treated with the non-targeting siRNA transfection mix released 959 ± 201 pg/ml CCL26 while treatment of cells with duplex 7 reduced CCL26 by only 13%. In contrast, significant inhibition of IL-4-stimulated release of CCL26 was noted for duplexes 8, 9, 10 and the combined *SMART*pool. The percents inhibition of CCL26 release were 63%, 53%, 65% and 64%, respectively. Alveolar type II cells constitutively release CCL24 which increases following treatment of cells with IL-4. Results of the present experiments indicate that cells constitutively released 176 ± 61 pg/ml CCL24. Effects of constitutive release of CCL24 in cells treated with CCR3-siRNA sequences are shown in figure 6A. Very significant inhibition of CCL24 release was noted following treatment with CCR3-siRNA transfection mixes. Inhibitions were as follows: duplex 8 – 77%, duplex 9 – 59%, duplex 10 – 74% and *SMART*pool – 95%. Interestingly, when siRNA treated cells were stimulated with IL-4, less inhibition of CCL24 was measured, as shown in figure 6B. Although the percent inhibition of stimulated CCL24 release was 53% in duplex 8 treated cells and 55% in *SMART*pool treated cells, these were not significantly different than the non-targeting siRNA treated cells. Taken together, these results suggest that treatment of alveolar type II epithelial cells with CCR3-targeted siRNAs significantly influences the release of eotaxins under basal and stimulated conditions.

3.4. CCR3 siRNA treatment of A549 cells modulates levels of additional cytokines/chemokines

We had recently reported that human membrane array studies revealed that unstimulated A549 alveolar type II cells released a number of cytokines (10). Thus, it was of interest to explore effects of CCR3 siRNA treatments on other cytokines/chemokines capable of binding to CCR3. Cells were treated with either non-targeting siRNA or CCR3 *SMART*pool siRNA with or without subsequent stimulation with IL-4. Results of selected cytokines capable of binding to CCR3 are depicted in figure 7. Results with CCL24 and CCL26 corroborate CCR3 siRNA effects demonstrated in previous figures and thus validate the cytokine array results. Four additional chemokines capable of binding to CCR3 were significantly increased by treatment with IL-4 and include CCL5 (RANTES), CCL8 (MCP-2), CCL13 (MCP-4), and CXCL11 (I-TAC). Comparisons of the NT + IL-4 versus SP + IL-4 treatments indicated significant decreases for CCL5, CCL8, CCL7 (MCP-3) and CCL13. Other ligands of interest included the CCR3 antagonists CXCL9 (MIG) and CXCL11 (I-TAC). CXCL9 (MIG) was not altered by IL-4 or CCR3 siRNA treatments, in contrast, data indicate that CXCL11 may be upregulated

by IL-4. These results suggest that post-transcriptional silencing of CCR3 modulates chemokines capable of signaling through the receptor as either agonists or antagonists.

3.5. Supernatants from A549 cells treated with CCR3 AS-ODN or siRNA induce decreased superoxide anion generation by EOS

In asthma, inflammatory processes generate toxic levels of reactive oxygen species (ROS) including superoxide ($O_2^{\bullet-}$) by activated EOS, alveolar macrophages, and neutrophils. Stimulated A549 cells release CCL26 which activates EOS and further compounds inflammation. Therefore, EOS were exposed to medium of IL-4 stimulated and AS-ODN or CCR3-siRNA treated airway epithelial cells and $O_2^{\bullet-}$ generation by EOS assessed and depicted in table 1 and figure 8, respectively. Significant decreases in $O_2^{\bullet-}$ generation were measured from EOS exposed to supernatants collected from cells pretreated with antisense 1 or 2. Results with supernatants from CCR3-targeted siRNA treated A549 cells are depicted in figure 8. EOS generated 5.8 and 5.3 nmol $O_2^{\bullet-}$ / 10^6 cells when incubated in medium with exogenous CCL26 or CCL24 at 100 ng/ml, respectively. Non-targeting siRNA treated cell supernatant results did not differ from those of cells treated with IL-4 alone. All CCR3-siRNA duplex treatments of A549 cells yielded supernatants which decreased $O_2^{\bullet-}$ generation by EOS. These data suggest that products released from stimulated A549 cells contained proinflammatory mediators including the CC chemokines CCL26 and CCL24, that act to stimulate $O_2^{\bullet-}$ generation by EOS, and CCR3 targeted AS-ODN or siRNA treatment may lead to desirable decreases in EOS activation.

Discussion

Chemokine ligand/receptor pathways orchestrate critical events in the underlying inflammatory conditions in airway diseases. Thus, chemokine ligands and receptors are important candidates in the development of novel anti-inflammatory strategies. In these present studies we have focused on the airway epithelial cell CCR3/eotaxin axis as a viable target for development of novel post-transcription-based therapies to interrupt the underlying proinflammatory cycle of chronic airway diseases. CCR3 is expressed on EOS, basophils, airway epithelial cells and subpopulations of mast cells, activated Th1 and Th2 cells, macrophages and dendritic cells. The present experiments were designed to test the hypothesis that transfection of alveolar type II cells with CCR3-targeted AS-ODNs or siRNAs will result in downregulation of surface CCR3, decrease synthesis and release of CCL24 and CCL26, and modulate other CCR3 ligands. Results demonstrate that: 1 - both CCR3-targeted AS-ODNs and siRNAs decreased CCR3 mRNA which, in turn, caused a decrease in CCR3 protein, 2 - release of IL-4-stimulated CCL26 was significantly decreased, 3 - constitutive release of CCL24 was likewise significantly reduced, 4 - downregulation of chemokines was extended to non-eotaxin CCR3 ligands and, 5 - EOS bioactivity was reduced in response to products released from the siRNA treated alveolar type II cells.

There is intense interest in development of post-transcriptional modulation of gene expression as a therapeutic approach for treatment of pulmonary infections, lung cancer and chronic airway diseases. Cell culture systems, including the A549 alveolar type II epithelial cell system, have been valuable tools for delineating potential targets (29,32-34). In these present investigations, the A549 cell culture system was employed to demonstrate that CCR3-targeted siRNA resulted in specific reduction in the target gene mRNA and subsequently its protein. Use of several effective siRNA duplexes suggested specificity of the effects. Absence of "off-target" effects is also supported by the lack of effect of the non-targeting duplexes designed as scramble sequences not present in the human genome. This is the first report of the efficacy of CCR3-targeted siRNAs for downregulation of CCL24 and CCL26 as well as other known CCR3 ligands, namely CCL5, CCL8, CCL7 and CCL13. Collectively, the data suggest that post-

transcriptional regulation of this chemokine receptor may be a potential therapeutic approach for interrupting proinflammatory signaling.

Humans possess all three eotaxins, CCL11, CCL24 and CCL26, which transduce signals predominantly, though not exclusively, via the CCR3 receptor. Results of previous investigations from this laboratory have demonstrated that alveolar type II cells constitutively express CCR3, and the Th2 cytokines IL-4 and IL-13 increase both CCR3 expression and synthesis and release of CCL24 and large, sustained amounts of CCL26 (31,12). Elevated levels of both IL-4 and IL-13 have been reported in asthmatic lungs where they act as chemotactic agents and activate effector pathways of both resident cells and emigrated EOS and other leukocytes (35-39). Importantly, these present investigations demonstrate that post-transcriptional silencing of CCR3 decreased release of the receptor's predominant ligands CCL26 and CCL24. Since several other resident pulmonary cells express CCR3 and produce eotaxins (40-45), these results suggest that CCR3-targeted siRNAs may downregulate cognate CCR3 receptors on resident cells of the lung with subsequent downregulation of the leukocyte-attracting chemokines and a favorable interruption of persistent underlying inflammation.

Chemokines modulate immune system responses by coordinating leukocyte recruitment and activation during both innate and adaptive inflammatory responses (46). Complexity of the chemokine network adds to the challenge of designing inflammation-targeted therapies. The CC and CXC chemokine networks, categorized by receptors, principal agonists, antagonists and functional activities, have been implicated in human allergy/inflammation. In addition to the three eotaxins, known agonists for CCR3 include CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL13 (MCP-4), CCL15 (MIP-1 δ) and CCL28 (MEC) (5,47). Conversely, the CXCR3 high affinity ligands CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC) are known antagonists at CCR3 (47-49). In the case of asthma, increased release of several of these CC chemokines namely CCL2 (MCP-1), CCL13, CCL5, the three eotaxins, CCL17 and CCL22, is firmly established (50,51). Herein, cytokine array results demonstrate that, in addition to the eotaxins, several other CCR3 agonists were increased during IL-4 stimulation of untreated alveolar type II cells. These included CCL5, CCL8 and CCL13. In IL-4 stimulated cells pretreated with CCR3-targeted siRNA, there was a significant decrease in these same agonists as well as CCL7. Interestingly, the MCPs and RANTES (CCL5), specifically chemotactic for monocytes and lymphocytes, were also affected. The CCR3 antagonists CXCL9 and CXCL11, chemotactic for T lymphocytes and mast cells (52), were not significantly altered by CCR3 siRNA and IL-4 treatments. Collectively, these results suggest that CCR3 gene-specific silencing may extend beyond recruitment of EOS and may also include mononuclear cells.

Prominent airway mucosa infiltration of activated EOS is considered a central pathologic feature of chronic airway diseases such as asthma (53). Airway epithelial cells are an important source of cytokines/chemokines which stimulate EOS bioactivities (54) including generation of reactive oxygen species such as O₂^{•-}, release of leukotrienes, degranulation of EOS-specific cationic proteins and release of cytokines. The compelling evidence which indicates that CCL11 and CCL24 function with IL-5 to promote EOS emigration and activation and CCL26 supports prolonged recruitment and activation (55,39,56) may require expansion to include additional CCR3 ligands, in particular CCL7, CCL8 and CCL13 (47). Thus, exploring the effects of CCR3-siRNA and CCR3-AS-ODN treatments of alveolar type II cells on bioactivity of EOS was of interest since EOS are known to generate O₂^{•-} generation stimulated by occupation of their CCR3 receptors (57). Significantly reduced levels of O₂^{•-} were generated by EOS exposed to CCR3-targeted siRNAs or AS-ODN treated/IL-4 stimulated A549 cell supernatants. These results imply that depletion of CCR3 and resulting modulation of its ligands would result in an effective reduction of activated EOS in the airway.

In summary, although some symptoms of airway diseases may be controlled, there is a need for therapies that provide control of chemokine-driven chronic airway inflammation (58). These investigations have focused on the CCR3/ligand network and demonstrated that alveolar type II cell release of CCL24 and CCL26 may be down-regulated by post-transcriptional silencing of CCR3. Concomitant decreases in additional CCR3 ligands (CCL5, CCL8, CCL13 and CCL7) also occurred and together reduced EOS bioactivity in response to products released from the treated cell. These findings establish the appropriateness of CCR3 as a target for development of siRNA or AS-ODN post-transcriptional gene inhibition therapies for the control of alveolar inflammation in airway diseases.

Acknowledgements

Support for this research was provided in part by NIH grants RR08111 and RR03020.

References

1. Global Strategy for Asthma Management and Prevention. NIH publication No 02-3659. www.ginaasthma.org
2. van den Toorn LM, Prins JB, de Jongste JC, Leman K, Mulder PGH, Hoogsteden HC, Overbeek SE. Benefit from anti-inflammatory treatment during clinical remission of atopic asthma. *Respir Med* 2005;99:779–787. [PubMed: 15878496]
3. Holgate ST. Epithelium dysfunction in asthma. *J Allergy Clin Immunol* 2007;120:1233–1244. [PubMed: 18073119]
4. Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol* 2005;6:1182–1190. [PubMed: 16369557]
5. Fan J, Heller NM, Gorospe M, Atasoy U, Stellato C. The role of post-transcriptional regulation in chemokine gene expression in inflammation and allergy. *Eur Respir J* 2005;25:933–947. [PubMed: 16264057]
6. Luster AD. The role of chemokines in linking innate and adaptive immunity. *Curr Opin Immunol* 2002;14:129–135. [PubMed: 11790543]
7. Beck LA, Tancoway B, Brummet ME, Asaki SY, Curry SL, Penno MB, Foster M, Bahl A, Stellato C. Functional analysis of the chemokine receptor CCR3 on airway epithelial cells. *J Immunol* 2006;177:3344–3354. [PubMed: 16920975]
8. Johns AE, Thomas MS, Berlin AA, Lukacs NW. Temporal production of CCL28 corresponds to eosinophil accumulation and airway hyperreactivity in allergic airway inflammation. *Am J Pathol* 2005;166:345–353. [PubMed: 15681819]
9. O'Gorman MT, Jatoi NA, Lane SJ, Mahon BP. IL-1 β and TNF- γ induce increased expression of CCL28 by airway epithelial cells via an NF- κ B-dependent pathway. *Cellular Immunology* 2005;238:87–96. [PubMed: 16581045]
10. Abonyo BO, Lebby KD, Tonry JH, Ahmad M, Heiman AS. Modulation of eotaxin-3 (CCL26) in alveolar type II epithelial cells. *Cytokine* 2006;36:237–244. [PubMed: 17350279]
11. Stellato C, Brummet ME, Plitt JR, Chahabuddin S, Baroody FM, Liu MC, Ponath PD, Beck LA. Expression of the C-C chemokine receptor CCR3 in human airway epithelial cells. *J Immunol* 2001;166:1457–1461. [PubMed: 11160184]
12. Abonyo BO, Alexander MS, Heiman AS. Autoregulation of CCL26 synthesis and secretion in A549 cells: a possible mechanism by which alveolar epithelial cells modulate airway inflammation. *Amer J Physiol Lung Cell Molec Physiol* 2005;289:L478–L488. [PubMed: 15863444]
13. De Lucca GV. Recent developments in CCR3 antagonists. *Curr Opin Drug Discov Devel* 2006;9:516–24.
14. Das AM, Vaddi KG, Solomon KA, Drauthauser C, Jiang X, McIntyre KW, Yang XX, Wadman E, Welch P, Covington M, Graden D, Weleswaram K, Trzaslos JM, Newton RC, Mandlekar S, Ko SS, Carter PH, Davies P. Selective inhibition of eosinophil influx into the lung by small molecule CC chemokine receptor 3 antagonists in mouse models of allergic inflammation. *J Pharmacol Exp Ther* 2006;318:411–417. [PubMed: 16614169]

15. Pease JE. Asthma, allergy and chemokines. *Curr Drug Targets* 2006;7:3–12. [PubMed: 16454696]
16. Garcia G, Godot V, Lambert M. New chemokine targets for asthma therapy. *Curr Allergy Asthma Rep* 2005;5:155–160. [PubMed: 15683617]
17. Wegmann M, Goggel R, Sel S, Erb K, Kalkbrenner F, Renz H, Garn H. Effects of a low-molecular-weight CCR3 antagonist on chronic experimental asthma. *Am J Respir Cell Mol Biol* 2007;36:61–67. [PubMed: 16917075]
18. Mori A, Ogawa K, Someya K, Kunori Y, Nagakubo D, Yoshie O, Kitamura F, Hiroi T, Kaminuma O. Selective suppression of Th2-mediated airway eosinophil infiltration by low-molecular weight CCR3 antagonists. *Int Immunol* 2007;19:913–921. [PubMed: 17804691]
19. Main S, Handy R, Wilton J, Smith S, Williams L, Fou LD, Andrews J, Conroy LA, May R, Anderson I, Waughan TJ. A potent human anti-eotaxin 1 antibody, CAT-213: isolation by phage display and *in vitro* and *in vivo* efficacy. *J Pharmacol Exp Ther* 2006;319:1395–1404. [PubMed: 16973884]
20. Wise EL, Duchesnes C, da Fonseca PCA, Allen RA, Williams JG, Pease JE. Small molecule receptor agonists and antagonists of CCR3 provide insight into mechanisms of chemokine receptor activation. *J Biol Chem* 2007;282:27935–27943. [PubMed: 17635911]
21. Popescu FD. Antisense- and RNA interference-based therapeutic strategies in allergy. *J Cell Mol Med* 2005;9:840–53. [PubMed: 16364194]
22. Wang LC, Lee JH, Yang YH, Lin YT, Chiang BL. New biological approaches in asthma: DNA-based therapy. *Curr Med Chem* 2007;14:1607–1618. [PubMed: 17584068]
23. Fortin M, Ferrari N, Higgins ME, Séquin S, Allam M, Allakhverdi Z, Piaget-Rodriguez C, Paquet L, Renzi PM. Effects of antisense oligodeoxynucleotides targeting CCR3 on the airway response to antigen in rats. *Oligonucleotides* 2006;16:203–212. [PubMed: 16978084]
24. Allakhverdi Z, Allam M, Guimond A, Ferrari N, Zemzoumi K, Séquin R, Paquet L, Renzi PM. Multitargeted approach using antisense oligonucleotides for the treatment of asthma. *Ann N Y Acad Sci* 2006;1082:62–73. [PubMed: 17145927]
25. de Fougerolles A, Vornlocher HP, Maragonore J, Lieberman J. Interfering with disease: a progress report on siRNA-based therapeutics. *Nature Rev* 2007;6:443–453.
26. Bitko V, Musiyenko A, Shulyayeva O, Barik S. Inhibition of respiratory viruses by nasally administered siRNA. *Nature Med* 2005;11:50–55. [PubMed: 15619632]
27. Li BJ, Tang Q, Cheng D, Qin C, Xie FY, Wei Q, Xu J, Liu Y, Zheng BJ, Woodle MC, Ghong N, Lu PY. Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nature Med* 2005;11:944–951. [PubMed: 16116432]
28. Lomas-Neira JL, Chung CS, Wesche DE, Perl M, Ayala A. In vivo gene silencing (with siRNA) of pulmonary expression of MIP-2 versus KC results in divergent effects on hemorrhage-induced, neutrophil-mediated septic acute lung injury. *J Leuk Biol* 2005;77:846–853.
29. Rippmann JF, Schnapp A, Weith A, Hobbie S. Gene silencing with STAT6 specific siRNAs blocks eotaxin release in IL-4/TNF- α stimulated human epithelial cells. *FEBS Letters* 2004;579:173–178. [PubMed: 15620709]
30. Nimi K, Asano K, Shiraishi Y, Nakajima T, Wakaki M, Kagyo J, Takihara T, Suzuki Y, Fukunaga K, Shiomi T, Oguma T, Sayama K, Yamaguchi K, Natori Y, Matsumoto M, Seya T, Yamaya M, Ishizaka A. TLR3-mediated synthesis and release of *eotaxin-1*/CCL11 from human bronchial smooth muscle cells stimulated with double-stranded RNA. *J Immunol* 2007;17:489–495.
31. Heiman AS, Abonyo BO, Darling-Reed SF, Alexander MS. Cytokine-stimulated human lung alveolar epithelial cells release eotaxin-2 (CCL24) and eotaxin-3 (CCL26). *J Interferon Cytokine Res* 2005;25:82–91. [PubMed: 15695929]
32. Sweeney FJ, Wachtmann TS, Eskra JD, Verdries KA, Lambalot RH, Carty TJ, Rerez JR, Audoly LP. Inhibition of IL-1 β -dependent prostaglandin E2 release by antisense microsomal prostaglandin E synthase I oligonucleotides in A549 cells. *Molec and Cellular Endocrinol* 2003;205:151–157. [PubMed: 12890577]
33. Platz J, Pinkenburg L, Beisswenger C, Püchner, Damm T, Bals R. Application of small interfering RNA (siRNA) for modulation of airway epithelial gene expression. *Oligonucleotides* 2005;15:132–138. [PubMed: 15989427]
34. Billich A, Bornancin F, Mechtcheriakova D, Natt F, Huesken D, Baumruker T. Basal and induced sphingosine kinase 1 activity in A549 carcinoma cells: function in cell survival and IL-1 β and TNF-

- α induced production of inflammatory mediators. *Cellular Signalling* 2005;17:1203–1217. [PubMed: 16038795]
35. Zimmermann N, Hershey GK, Foster PS, Rothenberg ME. Chemokines in asthma: cooperative interaction between chemokines and IL-13. *J Allergy Clin Immunol* 2003;111:227–242. [PubMed: 12589338]
 36. Komiya A, Nagase H, Yamada H, Sekiya T, Yamaguchi M, Sano Y, Hanai N, Furuya A, Ohta K, Matsushima K, Yoshie O, Yamamoto K, Hirai K. Concerted expression of eotaxin-1, eotaxin-2, and eotaxin-3 in human bronchial epithelial cells. *Cellular Immunology* 2003;225:91–100. [PubMed: 14698143]
 37. Zimmermann N, Daugherty BL, Stark JM, Rothenberg ME. Molecular analysis of CCR3 events in eosinophilic cells. *J Immunol* 2000;164:1055–1064. [PubMed: 10623856]
 38. Ravensberg AJ, Ricciardolo FL, van Schadewijk A, Rabe KF, Sterk PJ, Hiemstra PS, Mauad T. Eotaxin-2 and eotaxin-3 expression associated with persistent eosinophilic bronchial inflammation in patients with asthma after allergen challenge. *J Allergy Clin Immunol* 2005;115:779–785. [PubMed: 15805998]
 39. Berkman N, Ohnana S, Chung FK, Breuer R. Eotaxin-3 but not eotaxin gene expression is upregulated in asthmatics 24 hours after allergen challenge. *Am J Respir Cell Mol Biol* 2001;24:682–687. [PubMed: 11415932]
 40. Puxeddu I, Bader R, Piliponsky AM, Reich R, Levi-Schaffer F, Berkman N. The CC chemokine eotaxin/CCL11 has a selective profibrogenic effect on human lung fibroblasts. *J Allergy Clin Immunol* 2005;117:103–110. [PubMed: 16387592]
 41. Regamey N, Obregon C, Ferrari-Lacraz S, van Leer C, Chanson M, Nicod LP, Geiser T. Airway epithelial IL-15 transforms monocytes into dendritic cells. *Am J Respir Cell Mol Biol* 2007;37:75–84. [PubMed: 17363780]
 42. Moon KA, Kim SY, Kim TB, Yun ES, Park CS, Cho YS, Moon HB, Lee KY. Allergen-induced CD11b⁺ CD11(int) CCR3⁺ macrophages in the lung promote eosinophilic airway inflammation in a mouse asthma model. *Int Immunol* 2007;19:1371–1381. [PubMed: 17977814]
 43. 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. 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* 2007;143:84–88. [PubMed: 17541284]
 44. Joubert P, Lajoie-kadoch S, Labonte I, Gounni AS, Maghni K, Wellemans V, Chakir J, Laviolette M, Hamid Q, Lamkhieoued B. CCR3 expression and function in asthmatic airway smooth muscle cells. *J Immunol* 2005;175:2702–2708. [PubMed: 16081847]
 45. 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. A novel human CC chemokine, eotaxin-3, which is expressed in IL-4-stimulated vascular endothelial cells, exhibits potent activity toward eosinophils. *J Immunol* 1999;163:1602–1610. [PubMed: 10415065]
 46. Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokine grammar for immune cells. *Annu Rev Immunol* 2004;22:891–928. [PubMed: 15032599]
 47. Smit JJ, Lukacs. A closer look at chemokines and their role in asthmatic responses. *Eur J Pharmacol* 2006;533:277–288. [PubMed: 16464446]
 48. Loetscher P, Pellegrino A, Gong JH, Mattioli I, Loetscher M, Bardi G, Baggiolini M, Clark-Lewis I. The ligands of CXC chemokine receptor 3, I-TAC, MIG and IP-10 are natural antagonists for CCR3. *J Biol Chem* 2001;276:2986–2991. [PubMed: 11110785]
 49. Ying S, Meng Q, Zeibecoglou K, Robnson DS, Macfarlane A, Humbert M, Kay AB. Eosinophil chemotactic chemokines (eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein (MCP-3 and MCP-4) and C-C chemokine receptor-3 expression in bronchial biopsies from atopic and nonatopic (intrinsic) asthmatics. *J Immunol* 1999;163:6321–6326. [PubMed: 10570327]
 50. Lukacs NW. Role of chemokines in the pathogenesis of asthma. *Nat Rev Immunol* 2001;1:108–116. [PubMed: 11905818]
 51. Nickel R, Beck LA, Stellato C, Schleimer RP. Chemokines and allergic disease. *J Allergy Clin Immunol* 1999;104:723–742. [PubMed: 10518815]

52. Bradding P, Walls AF, Holgate ST. The role of the mast cell in the pathophysiology of asthma. *J Allergy Clin Immunol* 2006;117:1277–1284. [PubMed: 16750987]
53. Foley SC, Préfontaine D, Hamid Q. Role of eosinophils in airway remodeling. *J Allergy Clin Immunol* 2007;119:1563–1566. [PubMed: 17481718]
54. Sexton DW, Walsh GM. Eosinophil-epithelial cell interactions: an important facet of asthmatic inflammation. *Clin Exp Allergy* 2002;32:811–813. [PubMed: 12047423]
55. Pope SM, Brandt EB, Mishra A, Hogan SP, Zimmermann N, Matthaei KI, Foster PS, Rothenberg ME. IL-13 induces eosinophil recruitment into the lung by an IL-5- and eotaxin-dependent mechanism. *J Allergy Clin Immunol* 2001;108:594–601. [PubMed: 11590387]
56. Yang M, Hogan SP, Mahalingam S, Pope SM, Zimmermann N, Fulkerson P, Dent LA, Young IG, Matthaei KI, Rothenberg ME, Foster PS. Eotaxin-2 and IL-5 cooperate in the lung to regulate IL-13 production and airway eosinophilia and hyperreactivity. *J Allergy Clin Immunol* 2003;112:935–943. [PubMed: 14610483]
57. Badewa AP, Hudson CE, Heiman AS. Regulatory effects of eotaxin, eotaxin-2 and eotaxin-3 on eosinophil degranulation and superoxide anion generation. *Exp Biol Med* 2002;227:645–651.
58. Gerard C, Rollins BJ. Chemokines and disease. *Nature Immunol* 2001;2:108–115. [PubMed: 11175802]

Figure 1. CCR3 siRNA individual duplexes and the SMARTpool combination decrease surface CCR3 receptors and mRNA in IL-4 stimulated A549 airway epithelial cells
A549 airway epithelial cells were treated with indicated CCR3-siRNA (120 nM) in the presence of 3 μ l DharmaFECT 1 transfection reagent then stimulated with IL-4 (100 ng/ml) for 24 hrs. Panel A - Cells were detached with 0.5M EDTA in PBS, washed and stained with biotinylated eotaxin followed by avidin-fluorescein. Cell CCR3 fluorescence intensity was assessed by flow cytometry. Labels: Gray filled histogram - unstimulated control; light gray line histogram - CCR3-FITC stained non-targeting siRNA treated cells; black line histogram - indicated duplex or SMARTpool combination. Histograms are representative of experiments performed in duplicate on three separate occasions. Panel B - 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. Panel C - Data from experiments are expressed as the relative CCR3/GAPDH ratio mean $\pm$ SD. Treatments which differed significantly ($p < 0.05$) from the non-targeting siRNA followed by IL-4 treatment group are indicated by an asterisk.

Figure 2. Immunoblotting of lysates from A549 airway epithelial cells treated with CCR3 siRNA individual duplexes 7-10 and the SMARTpool combination demonstrates inhibition of CCR3 protein expression

Cells were cultured, treated with siRNA then stimulated with IL-4 (100 ng/ml) as described in figure 1. Panel A depicts a typical dot blot of 10 μ g/well of lysate proteins probed for human CCR3. Panel B summarizes dot blot densities of CCR3 from three separate experiments acquired with Quantity One analysis software. Data are expressed as mean pixel density $\pm$ SEM. Panel C shows similar experiments where cell lysate proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels (10 μ g lysates protein/lane), transferred to Immobilon-P PVDF membranes and probed for human CCR3 and GAPDH. Panel D depicts

the western blot band density ratios of CCR3/GAPDH in three separate experiments expressed as relative mean CCR3/GAPDH ratio $\pm$ SEM. In panels B and D asterisks indicate those treatments which differed significantly ($p < 0.05$) from cells treated with non-targeting siRNA followed by stimulation with IL-4.

Figure 3. CCR3 antisense oligonucleotide 1 treatment decreases surface receptors and gene expression in a concentration dependent manner

A549 airway epithelial cells (0.3×10^6 cells/well in 6-well plates) were treated with indicated antisense/sense concentrations in the presence of FuGENE 6 transfection reagent for 30 hr. Cells were rested for 12 hr then stimulated for 4 hr with IL-4 (30 ng/ml). An aliquot of cells was stained for flow cytometry as described in figure 1. Panel A: Histograms are labeled as follows: isotype antibody control is the gray filled histogram, CCR3-FITC stained untreated cells are represented by the light gray dotted line and the thick black line represents cells stimulated with IL-4 for four hours but without antisense 1. The light black lines indicate CCR3-FITC staining following treatments with CCR3 AS-ODN sequence 1 at 1 μ M or 3 μ M. Histograms are representative of experiments performed in duplicate on three separate occasions. Panel B: The remaining cells were used for western immunoblotting. Cell lysates proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels (10 μ g protein/lane), transferred to Immobilon-P PVDF membranes and probed for human CCR3.

Figure 4. CCR3 antisense 1 or 2 treatments decrease expression of IL-4 stimulated CCL26

Cells were treated with 3 μ M antisense/sense 1 or 2 as described in figure 3 followed without (panel A) or with IL-4 stimulation (30 ng/ml) for 18 hr (panel B). Cell lysates were prepared, total protein assessed and 50 μ g protein used for CCL26 sandwich ELISAs. Asterisks indicate treatments that differed significantly ($p < 0.05$) from cells stimulated with IL-4 in the absence of oligonucleotides.

Figure 5. Treatment of A549 alveolar type II epithelial cells with CCR3 siRNA significantly decreases IL-4 stimulated CCL26 release

Cells were treated for 96 hr with 120 nM siRNA (non-targeting control, duplex-7, -8, -9, 10 alone and the SMARTpool combination in the presence of DharmaFECT 1. Treated cell monolayers were washed with serum-free medium then incubated for an additional 24 hr in the absence (panel A) or presence of IL-4 (100 ng/ml) (panel B). CCL26 was quantified in culture supernatants by specific sandwich ELISA. Data are the mean $\pm$ SEM of three experiments each performed in duplicate. Asterisks indicate those treatments which differed significantly from the non-targeting siRNA control as assessed by Dunnetts post test with $p < 0.05$.

Figure 6. Treatment of A549 alveolar type II epithelial cells with CCR3 siRNA significantly decreases constitutively released CCL24

Cells were treated as described in figure 5. CCL24 was assessed in cell culture supernatants by specific ELISA following incubation without (panel A) or with IL-4 (100 ng/ml) (panel B). Data are the mean $\pm$ SEM of three experiments each performed in duplicate. Asterisks indicate those treatments which differed significantly from the non-targeting siRNA control as assessed by Dunnetts post test with $p < 0.05$.

Figure 7. CCR3 siRNA treatment of A549 alveolar type II cells modulates additional cytokines/chemokines

A549 cells were treated with non-targeting or SMARTpool 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 Fluor-s Max Multi-imager. Densities of cytokine protein spots were quantified with Quantity One 1-D Analysis Software Version 4.6.0. Data were normalized to the membrane positive control and are expressed as relative pixel densities of the mean $\pm$ SD of two samples. The ANOVA Tukey post test was used with $p < 0.05$ to determine significant differences between non-targeting (NT) and NT + IL-4 (◆) or between NT + IL-4 and SP + IL-4 (*).

Figure 8. Superoxide anion generation is suppressed in clone 15 HL-60 eosinophilic cells exposed to supernatants from CCR3-siRNA treated A549 alveolar type II cells

A549 alveolar type II cells were treated as described in figure 1 and supernatants used to stimulate EOS as described in table 1. Additional controls included EOS in medium (control), and medium to which 100 ng/ml recombinant CCL26 or CCL24 had been added. There was no significant difference between IL-4 and non-targeting (NT) siRNA + IL-4 supernatants. Asterisks indicate those treatments which differed significantly from the non-targeting siRNA control as assessed by Dunnetts post test with $p < 0.05$.

Table 1

Superoxide anion generation by clone 15 HL-60 eosinophilic cells is suppressed by incubation with supernatants from CCR3-AS-ODN treated A549 alveolar type II cells

A549 Cell Treatments ^a	nmol Superoxide Anion/10 ⁶ Eosinophils ^b	
	No IL-4	IL-4 (100 ng/ml)
No treatment	4.88 ± 0.25 ^c	9.16 ± 0.44
CCR3 antisense #1	4.61 ± 0.23	4.65 ± 0.58 *
Sense #1	5.83 ± 0.84	8.48 ± 0.42
CCR3 antisense #2	4.76 ± 0.35	4.81 ± 0.37 *
Sense #2	6.46 ± 1.11	8.05 ± 0.57

^a A549 cells were cultured and treated with CCR3 sense and antisense 1 and 2 as described in figure 3 followed by 24 hr without or with IL-4. Supernatants were collected, centrifuged and immediately used to stimulate eosinophils.

^b 70 µl supernatants were used to stimulate 1 × 10⁵ eosinophils in a total volume of 100 µl containing 80 µg ferricytochrome C with and without 30 µg superoxide dismutase.

^c Data are represented as the mean ± SEM of three experiments each carried out in triplicate. Asterisks indicate values which differed significantly from untreated supernatants at p < 0.05.