Transcriptional Analyses before and after Suppression of Immediate Hypersensitivity Reactions by CCR3 Blockade in Eyes with Experimental Allergic Conjunctivitis

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PURPOSE. To characterize the transcriptome of allergic conjunctivitis mediated by eosinophil-related chemokine receptor CCR3 and to identify a candidate for possible therapeutic intervention in eosinophilic inflammation of the eye.

METHODS. Mice were sensitized to ragweed pollen, and allergic conjunctivitis was induced by an allergen challenge. The induction of allergic conjunctivitis was used to determine whether an inhibition of CCR3 would suppress eosinophilic inflammation and the allergen-induced immediate hypersensitivity reaction. In addition, sensitized mice were treated with a CCR3 antagonist or an anti–CCR3 antibody before the allergen challenge. Eosinophilic inflammation was evaluated histologically at 24 hours after the allergen challenge. Transcriptional changes with or without a blockade of CCR3 were determined by microarray analyses.

RESULTS. Blockade of CCR3 significantly suppressed allergen-induced clinical signs, mast cell degranulation, and eosinophilic inflammation. Clustering analysis of the transcriptome during the early phase identified clusters of genes associated with distinct biological processes. A CCR2 ligand, monocytic chemoattractant protein (MCP)-1, was identified in the cluster of genes related to mast cell activation. MCP-1, an attractant of monocytes but not eosinophils, was in the top 10 transcripts among the genome and was suppressed by CCR3 blockade. Importantly, antibody blockade of MCP-1 suppressed the eosinophilic inflammation significantly.

CONCLUSIONS. CCR3 regulates not only the eosinophilic inflammation but also the clinical signs and mast cell degranulation. The CCR3-mediated transcriptome is characterized by many biological processes associated with mast cell activation. Among these CCR3-mediated processes, MCP-1 was found to be significantly involved in eosinophilic inflammation probably by an indirect pathway. (Invest Ophthalmol Vis Sci. 2008;49:5307–5313) DOI:10.1167/iovs.08-2154

Allergic diseases affect approximately one-third of the population and constitute one of the major healthcare problems in the Western world.1 Ocular allergic reactions, ranging from mild itching to sight-threatening shield ulcers, are initiated as IgE-mediated immediate hypersensitivity reactions.

Clinical symptoms and signs, such as itching, chemosis, and congestion, are manifested quickly by the cross-linking of IgE by an allergen, and cross-linking is mediated by the release of degranulation products of mast cells. Early events culminate in eosinophilic inflammation with the infiltration of neutrophils, macrophages, lymphocytes, and mast cells. In especially severe forms of ocular allergy, numerous inflammatory cells infiltrate the conjunctiva, leading to the formation of giant papillae.2 The products released from activated eosinophils and mast cells include eosinophil cationic proteins and major basic proteins that damage the corneal epithelia, resulting in shield ulcers. Inflammatory cytokines, including IL-4, IL-6, TNF-α, and eotaxin-1, can stimulate conjunctival fibroblasts, keratocytes, and recruited inflammatory cells during the remodeling process.3

Although detailed analyses of these complex processes have been conducted, it remains unclear how the initial events orchestrate the different inflammatory processes. To understand the aggravating processes and to search for a therapeutic target, a global view of these processes is necessary. In general, the processes involved in acute inflammation triggered by allergen exposure involve mast cell activation, vascular endothelial activation, and rapid recruitment of neutrophils.4 Activation cascades can be divided into rapid responses that are independent of new gene expression and slower responses that require new gene expression. The rapid response is mediated by the released mediators that bind to G-protein–coupled receptors (GPCRs), including the histamine H1 receptor. This leads to complex molecular cascades, including activation of phospholipase C, intracellular release of phosphatidylinositol-1,4,5-trisphosphate, elevation in cytosolic Ca2+,5 activation of small G protein and RHO, activation of nitric oxide synthase 3 to produce NO, and activation of cellular phospholipase A2, which generates arachidonic acid. Free arachidonic acid is converted to a potent vasodilator, prostaglandin I2, by cyclooxygenase-1, and prostacyclin synthase sequentially.

CCR3, an eotaxin receptor, is a signature GPCR of eosinophils5 and is required not only for eosinophil activation but also for mast cell activation. We hypothesized that examination of the transcriptomes before and after a blockade of CCR3 would allow us to construct a transcriptional model of the early events of eosinophilic inflammation. We show here that a transcriptional model of the early events of eosinophilic inflammation was obtained with this approach and that it allowed us to identify other genes that may play a critical role in eosinophilic inflammation of ocular allergy. By using bioinformatics analysis of the transcriptional snapshot, we found a possible mediator of eosinophilic inflammation, monocyte chemoattractant protein (MCP)-1, and evaluated its role using the experimental allergic conjunctivitis model.

MATERIALS AND METHODS

Animals

The procedures used in this study conformed to all regulations for laboratory animal research outlined by the Animal Welfare Act, Na-
Induction of Allergic Inflammation of Conjunctiva

SWR/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Ragweed pollen-induced murine experimental allergic conjunctivitis was used to evaluate the allergen-specific eosinophilic inflammation. A 50-μg suspension of ragweed pollen (ICN, Aurora, OH) combined with 1 mg aluminum hydroxide (Sigma, St. Louis, MO) was injected into the left hind footpad of anesthetized mice. On day 22 after injection, conjunctivitis was induced by topical application of 1.5 mg ragweed suspended in 10 μL phosphate-buffered saline (PBS). Control mice were mock sensitized with 1 mg aluminum hydroxide and challenged identically with a ragweed suspension.

To block the CCR3-mediated eosinophilic inflammation, the specific CCR3 antagonist W-56750. 4-(3-aminophenyl)thiazol-2-ylthio)-N-[1-(3,4-dichlorobenzyl)piperidin-4-yl] acetamide (Mitsubishi Tanabe Pharma Co., Tokyo, Japan), or anti-CCR3 antibody (clone 83103; R&D Systems, Minneapolis, MN) was used.

W-56750, a benzylpiperidine compound, inhibits the binding of [125I]-eotaxin to human eosinophils with IC50 values at nanomolar concentrations. W-56750 also inhibits the eotaxin-induced intracellular calcium influx in human eosinophils with IC50 values of 3.8 nM. W-56750 antagonizes not only human CCR3 but also murine CCR3 with IC50 values of approximately 235 nM (unpublished data from our laboratory, 2008). However, W-56750 has no affinity for other GPCRs, including CCR1, CCR2, CCR4, CCR5, CXCR1, CXCR2, PAF, and LTD4 (unpublished data from our laboratory, 2008).

To block MCP-1, an anti-MCP-1 antibody (clone 123616; R&D Systems, Minneapolis, MN) was used. W-56750 was orally administered on days 20 (2 days before), 21, and 22 before the allergen challenge. A total of 120 μg/mouse anti-CCR3 antibody or anti-MCP-1 antibody was injected intravenously through the tail vein on days 21 and 22 just before the allergen challenge.

For histologic evaluation of conjunctival eosinophilic inflammation, mice were killed 24 hours after allergen exposure. Tissues were collected, fixed in 4% paraformaldehyde, and embedded (Historesin; Leica Instruments GmbH, Wetzlar, Germany). Serial sagittal sections (3-μm thick) were stained with toluidine blue, Giemsa, or hematoxylin and eosin. Three consecutive conjunctival sections from each eye were examined, and mast cells and other inflammatory cells were counted under a 200× field microscope by an observer who was masked to the treatment received by the mouse.

Clinical reactions were graded 15 to 20 minutes after the challenge using a modified version of our published methods. Conjunctival edema, lid edema, tear/discharge, and conjunctival redness were graded from 0 to 4 based on the grading criteria by one observer who was masked to the treatment protocol (Supplementary Table S1, online at http://www.iovs.org/cgi/content/full/49/12/5307/DC1). The clinical score, ranging from 0 to 16, was calculated as the sum of the scores of each of the four parameters.

Measurement of Vascular Permeability

Vascular permeability was assessed by the Evans blue dye extravasation method performed during the immediate hypersensitivity period.7 For this, mice were injected with 1% Evans blue in PBS (6.7 μg/kg body weight) into the tail vein and were challenged with the allergen. Mice were killed 90 minutes after allergen exposure, and the eyelids and conjunctival tissues were collected. The tissues were weighed, and the extravasated Evans blue was extracted in 500 μL formamide for 24 hours. The extravasated Evans blue concentration was determined by spectrophotometry at 620 nm based on a standard curve of Evans blue in formamide.

Measurement of Allergen-Specific IgE and IgG1 Antibodies

Sera were collected by cardiac puncture 24 hours after allergen challenge. Ragweed pollen-specific IgE and IgG1 levels were measured by ELISA.7 Sera were pipetted onto ragweed pollen extract-coated microtiter plates (Maxisorp; Nunc, Naperville, IL), incubated with biotin-conjugated anti-IgE (Pharmingen, San Diego, CA) or anti-IgG1 (Southern Biotechnology Associates, Birmingham, AL) antibodies, and developed for peroxidase-based substrate detection.

Microarray Procedures

Mice were killed 90 minutes after allergen challenge, and the conjunctival tissues were collected. Total RNA was isolated from the tissue samples using RNA reagent (STAT-60; Tel Test, Friendswood, TX) and purified with a purification kit according to the manufacturer’s protocol (RNeasy Mini Kit; Qiagen, Hilden, Germany). Total RNA was reverse transcribed and amplified using an aRNA kit (Amino Allyl MessageAmp; Ambion, Austin, TX) for Cy5 and Cy3 dye labeling. Cy5 dye was used to generate the experimental cRNA probe from the allergen-challenged conjunctiva, and Cy3 dye was used to generate the reference cRNA probe from the control conjunctiva. Labeled cRNA probes were hybridized on oligo-microarrays (AceGene; Hitachi, Yokohama, Japan) corresponding to 29,568 genes and were scanned (FLA-8000; Fuji Film, Tokyo, Japan). The intensities of the fluorescent signals were quantified with an array program (DNASIS Array; Hitachi). After subtraction of the background levels, the signal from the gene spots was adjusted to compensate for excitation differences between the two dyes. Then the fluorescent signal was corrected for image intensity, background/spatial artifacts, and chip-to-chip comparisons using a custom database constructed by the array program (DNASIS Array; Hitachi).

Cluster Analyses

Before clustering and display, the logarithm of the ratio of the measured fluorescence for each gene was centered by subtracting the arithmetic mean of all ratios measured for that gene (DNASIS STAT [Hitachi] and GeneSpring [Agilent, Santa Clara, CA]). We applied a hierarchical clustering algorithm of the genes using the matching coefficient of Sokal and Michener as a measure closely linked to the average clustering. For visual display of the rows and columns of the initial data, tables were reordered to conform to the structures of the dendrogram. The data in the table were represented graphically by coloring each gene by the measured fluorescence ratio. Each gene in the cluster-ordered data table was replaced by a graded color, pure red through black to pure green, representing the mean-adjusted ratio for each gene.

Statistical Analyses

Data are presented as the mean ± SEM. Statistical analyses were performed using ANOVA.

RESULTS

Suppression of Mast Cell–Mediated Immediate Hypersensitivity Reactions by CCR3 Blockade

Allergen-sensitized mice were given oral W-56750 (10 mg/kg or 30 mg/kg) for 3 consecutive days before the allergen challenge, and the allergic signs were compared with those in the vehicle-treated group or in the prednisolone-treated group. W-56750 treatment significantly decreased the clinical score in a dose-dependent way compared with the vehicle-treated group (Fig. 1A). The marked suppressive effect of W-56750 (30 mg/kg) was almost equivalent to that induced by prednisolone.

To test whether W-56750 suppressed the mast cell–mediated increase in vascular permeability, W-56750–treated mice were evaluated by the Evans dye extravasation method (Fig. 1B). Vehicle-treated mice showed allergen-specific extravasa-
tion. In contrast, the Evans blue dye extravasation was significantly suppressed in the W-56750–treated group and the prednisolone-treated group.

To examine the inhibitory effect of W-56750 on mast cell degranulation, the degranulation of conjunctival mast cells after an allergen challenge was evaluated. Histologic evaluation of the allergen-challenged mice showed a significant suppression of mast cell degranulation in the W-56750–treated group and the prednisolone-treated group (Figs. 1C, 1D). These results were consistent with the clinical scores and Evans blue dye extravasation results. This suppressive effect of W-56750 on the clinical signs and mast cell degranulation was independent of allergen-specific IgE because the W-56750 treatment did not reduce the serum levels of ragweed-specific IgE or IgG1 levels (Fig. 2).

To confirm that the suppression of the mast cell–mediated clinical signs is indeed the result of the CCR3 blockade, a monoclonal anti–CCR3 antibody was administered the day before allergen challenge and was evaluated. Again, the clinical scores were significantly depressed by CCR3 (IgG-treated mock-immunized group, 2.6 ± 0.5; anti–CCR3–treated mock-immunized group, 2.8 ± 0.8; IgG-treated ragweed-immunized group, 8.4 ± 0.6; anti–CCR3–treated ragweed-immunized group, 4 ± 1.0; n = 10; P < 0.01).

### Suppression of Allergen-Induced Late-Phase Eosinophilic Inflammation by CCR3 Blockade

We also evaluated the effects of CCR3 blockade on the late-phase eosinophilic response of experimental allergic conjunctivitis. Ragweed-sensitized mice were given W-56750 (10 mg/kg or 30 mg/kg) orally for 3 consecutive days before the allergen challenge and were assessed for inflammatory cell recruitment 24 hours later. As expected from our findings of CCR3 on the eosinophil activation, W-56750 treatment significantly suppressed eosinophil recruitment in a dose-dependent manner (Fig. 3A; P < 0.05). W-56750 treatment also reduced neutrophil recruitment in a dose-dependent manner. The resident mast cell count was not altered after W-56750 treatment (data not shown).

To confirm that the CCR3 blockade suppressed the late-phase eosinophilic response, monoclonal anti–CCR3 antibody-treated mice were similarly analyzed. Consistent with the outcomes of W-56750 treatment, CCR3 antibody treatment significantly suppressed eosinophilic and neutrophilic inflammation (Fig. 3B).

### Transcriptional Scanning of Immediate Hypersensitivity Reactions in Experimental Allergic Conjunctivitis

The findings indicated that CCR3 blockade may be a pivotal point for allergic reactions culminating in eosinophilic inflammation in the conjunctiva. Therefore, we next analyzed how the pathognomonic eosinophilic inflammatory responses are regulated or linked to the initial events. Because the CCR3-mediated orchestration of the immediate hypersensitivity reactions appeared to regulate mast cell activation, we analyzed the very early events after allergen challenge by transcriptional profiling. For this, allergen-sensitized mice, with or without W-56750 treatment, were challenged with the allergen, and the conjunctival RNA collected 90 minutes after challenge was analyzed.

The transcriptional snapshot obtained by microarray (AceGene; Hitachi) of the acute-phase reaction detected a differential expression of 834 genes (>1.4-fold change; ANOVA; P < 0.05). There were 379 genes that were up-regulated and 455 genes that were downregulated after the allergen challenge. To determine whether the differential expressions were treatment or sensitization dependent, clustering analysis using a dendrogram was applied on the chip direction of 170 genes (Supplementary Table S2, online at http://www iovs org/cgi/content/ full/49/12/5307/DC1; ANOVA, P < 0.005). This unsupervised approach successfully classified all the samples into three groups (allergen-sensitized without W-56750 treatment, allergen-sensitized with W-56750 treatment, naive; ANOVA, P < 0.005; Fig. 4). This indicated that the transcriptional information was sufficient to explain differential physiological responses after the W-56750 treatment. When clustering analysis using the dendrogram was applied in the gene direction, the 170 genes were summarized into 29 categories that may play...
distinctive roles in the CCR3-mediated early events of allergic inflammation.

To understand the molecular events activated by the allergen exposure, pathway analysis using GenMAPP (free download available at www.genmapp.org) was applied on the extracted genes after gating to >1.4-fold changes after W-56750 treatment (834 genes; ANOVA; \( P < 0.05 \)). The assigned pathway of these genes, in descending order, was mRNA processing (23 genes), regulation of actin skeleton (19 genes), TGF-\( \beta \) signaling (19 genes), focal adhesion (16 genes), IGF receptor 1 signaling (16 genes), integrin-mediated cell adhesion (15 genes), IL-3 receptor signaling (12 genes), and T-cell receptor signaling (12 genes).

Physiologically, allergen exposure to the conjunctiva induces mast cell degranulation and activation and immediate vascular endothelial cell responses. The summarized transcriptional profiles into the pathways may well describe the early molecular phenomena. We assumed that genes that were highly activated by allergen exposure and suppressed by the W-56750 treatment may serve as therapeutic targets. Such genes, previously annotated and at the highest expression levels, were olfactory receptor MOR38–2, Kruppel-like factor 16, protein tyrosine phosphatase, non-receptor type 13 (Ptpn13), syntaxin-binding protein 4 (Stxbp4), glycoprotein 49B (Gp49b), zinc finger protein 398 (Zfp398), x-ray repair complementing defective repair in Chinese hamster cells 3 (Xrcc3), MCP-1 (Ccl2), dihydrofolate reductase, and kinesin family member 20A (Kif20a). Of the 10 most highly expressed genes in the conjunctival transcriptome, Ptpn13, Gp49b, Xrcc3, Ccl2, and Kif 20a were classified in the same category by gene tree analysis. Because Gp49b is expressed mainly on mast cells and plays pivotal roles in mast cell regulation,\(^9\) we assumed that the genes in this category might be related to CCR3-mediated mast cell activation and other related phenomena.

**Suppression of Eosinophilic Inflammation by Anti–MCP-1 Treatment**

CCR1, -2, -3, and CXCR3 are highly expressed on mature mast cells in the conjunctiva.\(^9\) MCP-1 is the main ligand of CCR2 but is not directly involved in eosinophilic attraction. Therefore, we hypothesized that MCP-1 may have an indirect link to eosinophilic inflammation from CCR3-mediated mast cell activation. To test this hypothesis, anti–MCP-1 antibody was injected intravenously into allergen-immunized mice and challenged with allergen. Eosinophilic inflammation in the conjunctiva was evaluated 24 hours later. Anti–MCP-1 treatment almost completely suppressed the eosinophilic inflammation (Fig. 5). This indicated that MCP-1 was also involved in the CCR3-mediated immediate hypersensitivity reaction in the eye, probably in an indirect manner. However, MCP-1 with eotaxin-1/CCR3 axis may serve as a therapeutic target in ocular allergy.

**DISCUSSION**

Our observations demonstrated the importance of CCR3 signaling in the early and late events of the immediate hypersensitivity reactions in the mouse eye. First, mast cells that are activated by CCR3 orchestrate late-phase eosinophilic responses in vivo. Second, transcriptional snapshots of the whole genome indicated that these early events are related to numerous activities, such as mast cell activation, vascular endothelial cell responses, and mRNA processing. Third, of the numerous mast cell regulation–related transcripts, MCP-1, though not an eosinophil attractant, is an important link to late-phase eosinophilic inflammation.

Eosinophils are characterized by their strong expression of CCR3 and are attracted by its ligands.\(^10\) Our results, obtained after the use of a CCR3 antagonist and an anti–CCR3 antibody, showed similar suppression of eosinophil recruitment into the conjunctiva. More important, early-phase clinical responses...
and mast cell degranulation were significantly depressed. Because late-phase eosinophil inflammation is orchestrated by early-phase mast cell degranulation, depression of the late-phase eosinophil recruitment may also be caused by reduced mast cell activation by direct CCR3 blockade of mast cells.

**FIGURE 3.** Suppression of inflammatory cell recruitment by CCR3 blockade. Administration of W-56750 (A) or anti-CCR3 antibody (B) significantly depressed the allergen-induced recruitment of eosinophils and neutrophils. *P < 0.05.

**FIGURE 4.** Clustering analysis of allergen-induced transcriptome in experimental allergic conjunctivitis. Chip direction analysis by dendrogram correctly classified each sample into three experimental groups (RW/Vehicle, ragweed-sensitized vehicle treated; RW/W-56750, ragweed-sensitized W-56750 treated; naive/vehicle, nonsensitized vehicle treated) in an unsupervised manner.
tival mast cells by gp49b was shown using the allergic conjunctivitis model.8

The possibility that MCP-1 is an eosinophilic mediator also comes from genomic findings from the clinic. When the increased eosinophil levels of patients with asthma were studied, single-nucleotide polymorphisms (SNPs) of the MCP-1 regulatory region were shown to be associated.16 The −2518 G/G genotype is significantly correlated with the severity of asthma. Another CCR3 ligand, RANTES, failed to show a detectable effect on asthma susceptibility.16

On the other hand, the roles played by the c-ckoxitin family on eosinophilic responses are controversial. Genetic analyses of the eotaxin gene family have not identified any significant correlation between eosinophils and eotaxin gene SNP in patients with asthma. A significant association of eotaxin-1 was found with serum IgE levels, whereas eotaxin-2 and eotaxin-3 were associated with asthma susceptibility.17,18 In atopic keratoconjunctivitis, a genetic analysis for the eotaxin family has not been reported, though tear eotaxin-1 levels are correlated with tear eosinophils or disease severity.19

To understand how other inflammatory mediators are possibly involved in the pathophysiology of severe allergic conjunctivitis, cytokine array profiling of the tears was recently performed.15,20,21 In the tears of eyes with vernal keratoconjunctivitis or atopic keratoconjunctivitis, numerous cytokines were indeed secreted, including IFN-γ, IL-1β, -2, -4, -6, -6sR, -12, -13, eotaxin-1, eotaxin-2, MCP-1, MIP-1ß, and M-CSF. The MCP-1 level was markedly elevated together with two CCR3 ligands, eotaxin-1/eotaxin-2, and IL-4 and IFN-γ. These observations support our view that MCP-1 may indeed be clinically involved in ocular allergy.

To summarize, we showed that blocking CCR3 depressed early-phase mast cell activation and late-phase eosinophilic inflammatory responses. Based on transcriptional profiling of the early-phase events under CCR3 blockade, MCP-1 probably serves as an eosinophilic mediator.

**References**


**Figure 5.** Suppression of allergen-induced eosinophil recruitment by MCP-1 inhibition. Anti-MCP-1 treatment significantly suppressed allergen-induced eosinophil recruitment in the conjunctiva.

Based on the observations of allergen-sensitized, mast cell-deficient W/W° mice, mast cell deficiency caused defective eosinophilic and neutrophilic responses to allergen exposure (data not shown). This is supported by earlier reports that show the contribution of mast cell mediators to such inflammatory responses.11,12 Our results have shown that neutrophils were also suppressed by the W-56750 treatment and by CCR3 antibody. Because neutrophils do not express CCR3, the suppression of neutrophil recruitment may be attributed to an indirect outcome of mast cell suppression. Indeed, mast cell activation by CCR3 was shown to induce neutrophilic responses.15 When CCR3 ligand eotaxin-1 or eotaxin-2 was injected into the skin of human volunteers, an acute wheal-and-flare response was followed by the accumulation of basophils, neutrophils, and macrophages in addition to eosinophils. Thus, suppressed neutrophil recruitment by W-56750 or anti-CCR3 antibody treatment appears consistent with impaired mast cell activation by the CCR3 blockade.

Our whole genome scanning of early-phase transcripts successfully identified MCP-1 as a candidate eosinophilic mediator. However, MCP-1 is not active on eosinophils.14 Besides being a potent chemoattractant of monocytes and macrophages, MCP-1 is a potent activator of mast cells and is secreted by mast cells, monocytes, and fibroblasts15 but not by eosinophils. These observations suggest that MCP-1 action on eosinophils is indirect. To support our view that MCP-1 is associated with mast cell activation, our clustering analysis of early-phase transcriptome classified MCP-1 into the mast cell regulation-related category (Fig. 4). Gp49b was also classified in this category. Gp49b is one of the representative regulatory receptors on murine mast cells, together with the Fcy receptor IIb, which has an immunoreceptor tyrosine-based inhibitory motif in its cytoplasmic region. Recently, the regulation of conjunctival mast cells by gp49b was shown using the allergic conjunctivitis model.8


