

Blocking Mast Cell–Mediated Type I Hypersensitivity in Experimental Allergic Conjunctivitis by Monocyte Chemoattractant Protein-1/CCR2

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PURPOSE. To characterize the roles played by monocyte chemoattractant protein-1 and its preferential receptor CCR2 (MCP-1/CCL2) in acute allergic inflammation.

METHODS. The direct effects of MCP-1 were evaluated histologically after a subconjunctival injection of recombinant MCP-1 into naïve mice. The mice were sensitized to ragweed pollen, and allergic conjunctivitis was induced by an allergen challenge. The location of the induced MCP-1 was determined by immunohistochemistry. Anti-MCP-1 antibody and CCR2-specific antagonist, RS 504393, were used to determine whether an inhibition of MCP-1 or CCR2 signals would suppress the allergen-induced immediate hypersensitivity reaction. The effect of blocking CCR2 was tested in vitro with isolated mast cells from connective tissue, to evaluate the co-stimulatory signals mediated by CCR2 in mast cells directly.

RESULTS. A subconjunctival injection of MCP-1 stimulated conjunctival mast cell degranulation and recruited monocytes/macrophages. In the allergic conjunctivitis model, the allergen-induced MCP-1 protein was located in the monocytes/macrophages in the substantia propria of the conjunctiva. Blocking MCP-1 significantly suppressed the allergen-induced clinical signs and mast cell degranulation without affecting the allergen-specific IgE, or the release of Th2 cytokine from the isolated draining lymph node cells. Inhibition of CCR2 similarly suppressed the acute inflammatory responses. Consistent with the outcome of the disease model, inhibition of CCR2 suppressed allergen-specific degranulation of IgE-primed, isolated conjunctival mast cells.

CONCLUSIONS. Stimulation of the co-stimulatory axis of CCR2 by MCP-1 is essentially required for mast cell-mediated hypersensitivity reactions in mouse eyes. (*Invest Ophthalmol Vis Sci*. 2009;50:5181–5188) DOI:10.1167/iovs.09-3637

Because of the high morbidity caused by allergic diseases throughout the Western world and the enormous burden of these diseases to health care budgets, efficacious therapeutic and prophylactic interventions for allergic diseases are needed.¹ Among the diseases, ocular allergy is the most prevalent form of mucosal allergy. Ocular allergic reactions are initiated as an IgE-

mediated immediate hypersensitivity reaction. Severe ocular allergies, including atopic keratoconjunctivitis and vernal keratoconjunctivitis, are refractory to conventional treatments, and patients can develop vision-threatening shield ulcers.²

The clinical symptoms and signs of ocular allergy are induced by the cross-linking of IgE by an allergen followed by the degranulation of mast cells. With the rapid induction of CC chemokines, the early events culminate in an eosinophilic inflammation with infiltration of neutrophils, macrophages, lymphocytes, and mast cells.³ Inflammatory cytokines, including IL-4, IL-6, TNF- α , and eotaxin-1, can stimulate conjunctival fibroblasts, keratocytes, and monocytes, and recruit inflammatory cells during the remodeling process.⁴

The very early acute inflammatory responses that are triggered by allergen exposure involve mast cell activation, vascular endothelial cell activation, and rapid recruitment of neutrophils or monocytes.⁵ However, it has still not been determined how the initial events orchestrate the different inflammatory processes. The recruitment of inflammatory cells is generally mediated by chemokines, but the roles of chemokines are not simply confined to a traffic regulator. For example, eotaxin-1, a CC chemokine, is a signature eosinophil mediator⁶ but also serves as an allergen-primed mast cell activator.^{7,8} MIP-1 α , another CC chemokine, is also necessary for mast cell activation.⁹ Thus, the CC chemokines are critically involved in mast cell-mediated acute inflammation, and the allergen-primed mast cell degranulation leads to late-phase eosinophilic inflammation.¹⁰

To understand the molecular processes of these initiating events, we recently constructed transcriptional models before and after CCR3 blockade to determine the mast cell-mediated disease processes.⁷ Comprehensive bioinformatics analyses of the transcriptome showed that another CC chemokine, MCP-1/CCL2, was a possible modulator of eosinophilic inflammation. However, how MCP-1/CCL2 modulates the eosinophilic inflammation has not been determined.

Thus, the purpose of this study was to determine the role played by MCP-1, a monocyte-activating CC-chemokine, as a signaler of mast-cell priming. We shall show that blocking MCP-1 or its receptor, CCR2, greatly decreased the clinical signs of the acute-phase allergic reactions. This depression of the immediate hypersensitivity reaction was not due to a decrease in the numbers of resident mast cells, impaired IgE/IgG₁ priming, or allergen-primed T-cell responses, but appeared to be caused by a failure of mast cell degranulation. In vitro analyses of isolated conjunctival mast cells demonstrated that the suppression of the IgE-mediated degranulation by the MCP-1/CCR2 axis was a direct effect on mast cells. We propose that the activation of CCR2 by MCP-1 is a critical component of the acute-phase reaction in ocular allergy.

MATERIALS AND METHODS

Animals

SWR/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The procedures used conformed to all regulations for laboratory

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animal research outlined in the Animal Welfare Act, National Institutes of Health, and in the ARVO Statement for the Use of Ophthalmic and Vision Research.

Induction of Allergic Inflammation in Conjunctiva

Mice were sensitized with a protocol that we have reported in detail.^{7,10,11} Briefly, a suspension of 50 μ g ragweed pollen (ICN, Aurora, OH) and 1 mg aluminum hydroxide (Sigma-Aldrich, St. Louis, MO) was injected into the left hind footpad with the subject under general anesthesia. Conjunctivitis was induced 42 days later by topical application of 1.5 mg ragweed suspended in 10 μ L of phosphate buffered saline (PBS). Control mice were mock-sensitized and challenged identically with a ragweed suspension.

The clinical responses were recorded 20 minutes after the challenge, and graded by the criteria described in our earlier report, with slight modifications.¹² The signs were evaluated by an ophthalmologist masked to the identity of each mouse and graded 0 to 4 using defined criteria (Supplementary Table S1, online at <http://www.iovs.org/cgi/content/full/50/11/5181/DC1>). The cumulative clinical score was calculated as the sum of the scores of four parameters (0–16).¹²

To block MCP-1 of the effector phase, 40 μ g/mouse of anti-MCP-1 antibody (clone 123616; R&D Systems, Minneapolis, MN) was given intravenously on days 41 and 42 (20 μ g/d) before the allergen challenge. Anti-keyhole limpet hemocyanin IgG was injected as control. For the pharmacological inhibition of CCR2, the specific CCR2 antagonist RS 504393, 6-methyl-1'-[2-(5-methyl-2-phenyl-4-oxazolyl)ethyl]-spiro[4H-3,1-benzoxazine-4,4'-piperidin]-2(1H)-one (Tocris, Bristol UK), was used. The IC₅₀ for CCR2 is 98 nM. RS 504393 inhibits the MCP-1-induced chemotaxis with an IC₅₀ of 330 nM. Sixty micrograms/mouse of RS 504393 was given orally twice daily from 3 days before the allergen challenge.

Histologic Evaluation

For histologic evaluation of the immediate hypersensitivity reactions, mice were killed, and the tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Serial sagittal sections (3 μ m in thickness) were stained with Giemsa or hematoxylin and eosin. Three consecutive sections from each eye were examined, and the number of mast cells and other inflammatory cells were counted under a 200 $\times$ microscope field by an independent scientist in a masked fashion.

Immunohistochemistry

Serial frozen sections (5 μ m in thickness) were fixed in acetone and stained for MCP-1 using hamster monoclonal anti-MCP-1 antibody (clone 2H5; Biolegend, San Diego, CA). Peroxidase-conjugated anti-hamster IgG (GeneTex, San Antonio, TX) was used as the secondary antibody. The MCP-1 signals were made visible by incubating the sections in a chromogen (HistoGreen; AbCys, Paris, France) and counterstained with hematoxylin. Anti-keyhole limpet hemocyanin IgG was used as the control.

For fluorescence immunohistochemistry, Alexa 555-labeled tyramid (Invitrogen, Carlsbad, CA) was used to label MCP-1 signal and counterstained with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI, Invitrogen). Macrophage marker, F4/80, was stained using rat anti-F4/80 antibody (Biolegend) and Dylight 488 labeled-anti-rat immunoglobulin (Rockland Immunochemicals, Gilbertsville, PA).

Enzyme-Linked Immunosorbent Assay

To measure the serum IgE levels, mice were bled and sera collected after the final antigen challenge. ELISA was performed on the sera for IgE (Opt EIA mouse IgE set; BD Biosciences, Franklin Lakes, NJ). Antigen-specific ELISA was performed using plates coated with ragweed extract (Greer Laboratory, Lenoir, NC) instead of capture antibodies.

Measurement of Cytokines in Culture Supernatants

Isolated draining lymph node cells (2×10^6 cells/well) were cultured for 72 hours with ragweed extract (50 μ g/mL; Greer Laboratory) in 96-well flat-bottomed plates in a final volume of 0.2 mL RPMI 1640 medium supplemented with 10% FCS. The levels of IL-2, -4, and -17 were measured with commercially available ELISA kits (ELISA Max Set; Biolegend, San Diego, CA), according to the manufacturer's instructions.

Subconjunctival Injection of MCP-1

Five microliters of recombinant murine MCP-1 (Peprotech, Rocky Hill, NJ) was injected subconjunctivally into naive mice with a 30-gauge needle.⁹ The mice were killed 24 hours after the injection, and conjunctival samples were prepared for morphologic analyses.

Isolation of Mast Cells from Conjunctiva

Mast cells were isolated from mouse skin and conjunctiva using an enzymatic digestion protocol described earlier.¹⁰ Briefly, the conjunctiva of 8- to 12-week-old SWR/J mice were enzymatically digested in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1.5 mg/mL collagenase (Nitta Gelatin, Osaka, Japan), 0.5 mg/mL hyaluronidase (Sigma-Aldrich, St. Louis, MO), and 0.5 mg/mL DNase I (Sigma) for 2 hours at 37°C. The dispersed cells were layered onto isotonic density medium (density 1.041; Percoll; Amersham Pharmacia Biotech, Piscataway, NJ), and centrifuged at 800g for 20 minutes. The cell pellet was collected and cultured in RPMI1640 with murine recombinant IL-3 (10 ng/mL, Peprotech), recombinant SCF (10 ng/mL, Peprotech), and 5% serum for 6 days. Lineage marker-positive cells and plasmacytoid dendritic cells were depleted after labeling with rat anti-CD4, rat anti-CD8a, rat anti-CD11b, rat anti-B220 (all from eBiosciences), and rat anti-PDCA-1 (Miltenyi Biotec, Auburn, CA) using anti-rat immunoglobulin κ chain antibody-conjugated microbeads (BD Biosciences, Franklin Lakes, NJ).

β -Hexosaminidase Assay

Isolated mast cells were sensitized by incubation with anti-DNP IgE (SPE7; Sigma) in RPMI1640 containing 10 ng/mL of murine recombinant IL-3, 10 ng/mL of recombinant SCF, and 5% murine serum. The cells were then washed with HBSS containing 10 ng/mL of murine recombinant IL-3, 10 ng/mL of recombinant SCF, 0.04% BSA, and 10 mM HEPES. Resuspended cells at a concentration of 2 to 8×10^4 cells/100 μ L were transferred into triplicate wells of a 96 well U-bottom plate and allowed to equilibrate at 37°C for 10 minutes before the addition of DNP-albumin (Sigma-Aldrich) or compound 48/80 (Sigma-Aldrich). After 45 minutes, the plate was centrifuged at 290g for 5 minutes at 4°C. The β -hexosaminidase activity of the culture supernatant was determined using a published protocol.¹³ Fifty-microliter aliquots of the supernatant were placed in wells of another 96-well plate together with 100 μ L of 2.5 mM *p*-nitrophenyl-N-acetyl β -D-glucosaminide (Sigma-Aldrich) solubilized in 0.04 M citrate buffer adjusted to pH 4.5 with disodium phosphate. After incubation at 37°C for 90 minutes, the reactions were terminated by addition of 50 μ L of 0.4 M glycine adjusted to pH 10.7 with sodium hydroxide. The colored product was measured at 405 nm with a reference filter of 570 nm. The relative release of β -hexosaminidase was defined as the activity in the supernatant of the tested cells divided by the activity in the positive control cell supernatant, multiplied by 100. Compound 48/80 stimulus was used for assay control.

Statistical Analyses

Data are presented as the mean $\pm$ SEM. Statistical analyses were performed with unpaired Student's *t*-tests (two-tailed), Mann-Whitney *U* tests, or ANOVA.

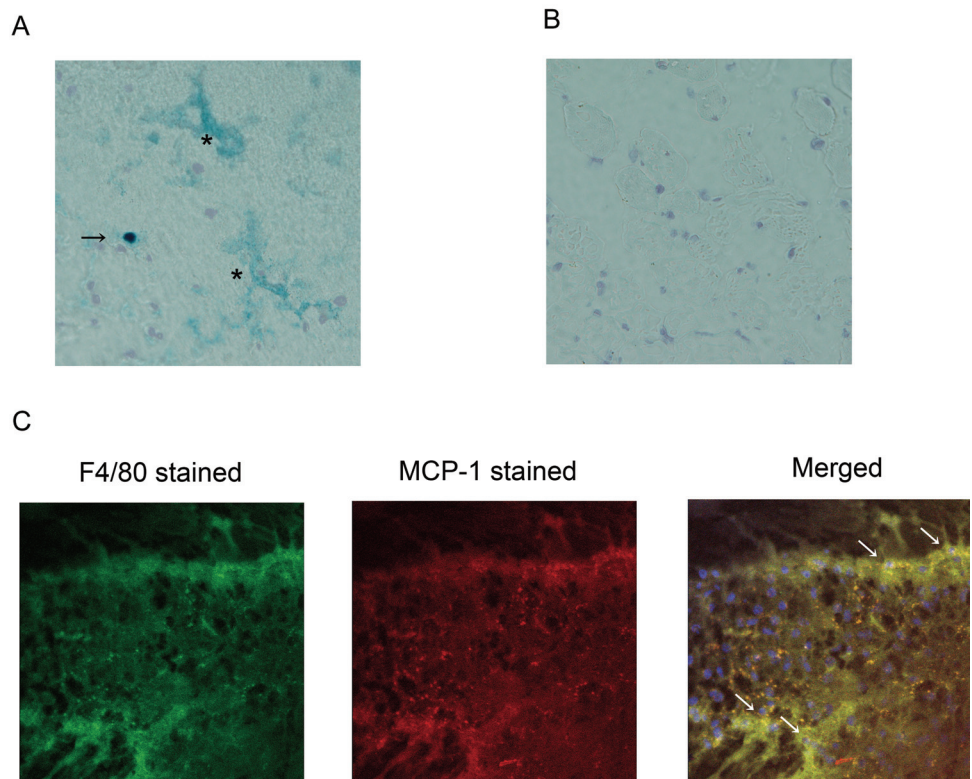


FIGURE 1. Localization of MCP-1-expressing cells in the allergen-challenged conjunctiva of sensitized mice. (A) Intense green staining of MCP-1 can be seen in the mononuclear cell population (arrow) in the subconjunctival tissue surrounded by fibroblasts and diffuse deposits (asterisk) in the extracellular matrix of the substantia propria. The section was counterstained with hematoxylin. (B) Allergen-challenged conjunctiva from naïve mice showing background level of MCP-1. (C) Intense red staining (Alexa 555) of secreted MCP-1 was mainly co-localized with green staining (Dylight 488) of macrophage marker, F4/80 (arrow). The section was counterstained with DAPI.

RESULTS

Localization of MCP-1 Expression in Conjunctiva

We first determined the location of the cells producing MCP-1 after allergen challenge and evaluated their spatial relationship to conjunctival mast cells. Conjunctival tissue is known to contain a large number of mast cells particularly in the substantia propria at the eyelid root.⁹ Immunohistochemical analysis was performed on serial sections of conjunctiva of allergen-challenged animals (Fig. 1). Monocytes are known to be a source of MCP-1,¹⁴ and strong signals for MCP-1 were located predominantly in the mononuclear cells in the substantia propria. Vascular endothelial cells and fibroblast also stained positive but weakly. MCP-1 was also detected in the extracellular matrix of the substantia propria, suggesting an allergen-induced secretion of MCP-1. Signals for MCP-1 were very weakly detected in the conjunctiva of naïve animals.

To determine the major source of MCP-1 in the allergen-challenged animals, fluorescence immunohistochemistry was used (Fig. 1C). Diffuse and intense MCP-1 signal was mainly observed in the forniceal subconjunctiva, and mostly colocalized with F4/80-positive areas. This confirms that the major source of MCP-1 is the mononuclear cells/macrophage population.

Effects of MCP-1 Injection on Conjunctival Eosinophils and Mast Cells

CCR2 receptors of MCP-1 are highly expressed on monocytes and macrophages to attract and activate these cells. Of importance, CCR2 is also expressed on mature mast cells.¹⁵ Because mast cell activation is pivotal in the pathophysiology of allergic conjunctivitis, we performed *in vivo* experiments to test whether MCP-1 can activate conjunctival mast cells. Recombinant MCP-1 was injected beneath the conjunctiva of naïve mice. This process allowed us to evaluate the effect of MCP-1

on mast cells before beginning the FcεRI priming effect. We analyzed the morphology of the resident mast cells by Giemsa and hematoxylin and eosin staining at 24 hours after the injection.

As expected, the injection of 10 ng/mL MCP-1 led to a recruitment of monocytes and macrophages (data not shown). Of interest, conjunctival mast cells were also recruited by 0.1 ng/mL MCP-1 (mast cell count after injection of MCP-1 = 34 ± 4 cells/field of view; PBS = 22 ± 2 cells/field of view; $P < 0.01$, Fig. 2A). More important, an increase in degranulation was observed (percentage of mast cell degranulation after injection of 0.1 ng/mL MCP-1 = $34\% \pm 3\%$; PBS = $22\% \pm 2\%$; $P < 0.05$, Fig. 2B). These results confirmed the functional importance of the MCP-1/CCR2 axis on the direct or indirect activation of conjunctival mast cells.

Impairment of Mast Cell Degranulation and Immediate Hypersensitivity in Experimental Allergic Conjunctivitis by MCP-1/CCR2 Blockade

From these results, we hypothesized that allergen-induced MCP-1 will activate or prime mast cell-mediated diseases. To test this hypothesis, we analyzed the effects of blocking MCP-1 on the allergen-induced mast cell-related signs and degranulation. In murine experimental allergic conjunctivitis, conjunctival mast cell degranulation can be evaluated using several clinical signs.^{9,10} In control IgG-treated mice, clear evidence was present that acute inflammation had developed, but in anti-MCP-1 antibody-treated mice, the clinical signs of immediate hypersensitivity did not develop (Fig. 3A). No effect was observed in naïve animals.

We also evaluated mast cell degranulation in the challenged conjunctivas of control IgG-treated and anti-MCP-1 antibody-treated mice. Because mast cell activation drives the acute-phase reaction,^{9,10} these data suggest that MCP-1 blockade does indeed affect mast cell activation *in vivo*. Consistent with

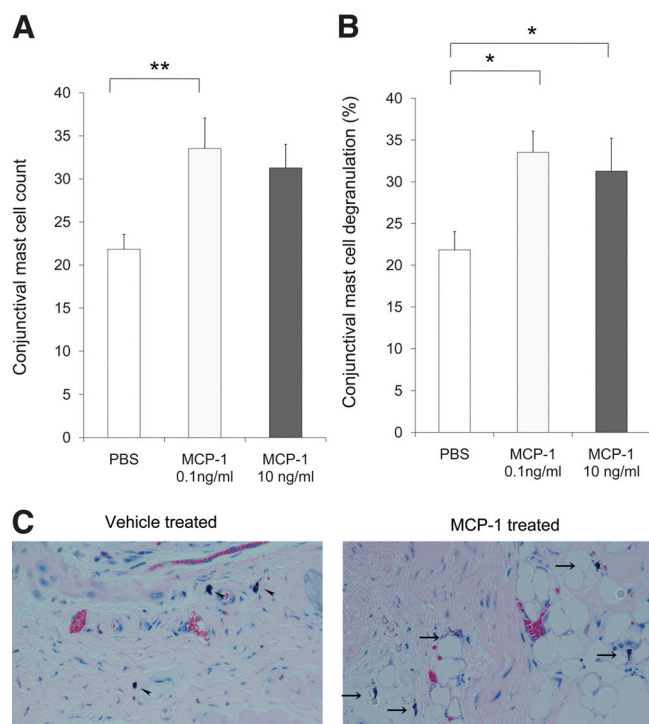


FIGURE 2. In vivo effect of recombinant MCP-1 treatment. Recombinant MCP-1 or PBS was injected subconjunctivally into naïve mice and histologically evaluated 24 hours after Giemsa staining (C). The recombinant MCP-1 significantly activated the recruitment of conjunctival mast cells (at 0.1 ng/mL of MCP-1) (A) and the degranulation (at 0.1 and 10 ng/mL of MCP-1) (B). * $P < 0.05$; ** $P < 0.01$. Arrow: degranulated mast cells; arrowhead: mast cells.

the suppression of the clinical signs, mast cell degranulation was significantly inhibited in MCP-1 antibody-treated mice but had no effect on naïve mice (Fig. 3B). Total conjunctival mast cell numbers in control treated-mice were not significantly different from that in anti-MCP-1 antibody-treated mice (control IgG-treated immunized mice = 85 ± 4 cells/field of view; anti-MCP-1 antibody-treated mice = 86 ± 4 cells/field of view).

To further examine whether blocking the immediate hypersensitivity reaction in the anti-MCP-1-treated mice was due to a direct effect on CCR2, we analyzed the effects of CCR2 blockade by using RS 504393, a specific CCR2 antagonist. Whereas vehicle-treated mice developed clear signs of acute inflammation, RS 504393-treated mice failed to show clinical signs of immediate hypersensitivity (Fig. 4A). No effect was observed in naïve animals. Consistent with the suppression of the clinical signs, the degranulation of mast cells was significantly inhibited in RS 504393-treated mice but had no effect on naïve mice (Fig. 4B). The total number of conjunctival mast cells was slightly reduced by RS 504393 treatment, although the reduction was not statistically significant (vehicle-treated immunized mice = 82 ± 3 cells/field of view; RS 504393-treated mice = 75 ± 2 cells/field of view). Thus, MCP-1/CCR2 is required for allergen-dependent mast cell degranulation and the acute phase inflammation in experimental allergic conjunctivitis but not critical for mast cell homing.

Unimpaired Induction of Allergen-Specific IgE and Cytokine Secretion by MCP-1/CCR2 Blockade

The induction of allergen-specific IgE and inflammatory cytokines results in a complex series of phenomena involving proper antigen processing and presentation, B-cell maturation,

and class switching driven by Th2 cells. Because an elevated level of allergen-specific IgE is necessary for mast cell activation, we examined whether the observed impairment of acute phase inflammation by MCP-1/CCR2 blockade is independent of priming of allergen-specific IgE. Analysis of allergen-specific IgE showed that no antibody deficit was induced by either MCP-1 antibody treatment or RS 504393 treatment (Fig. 5).

CCR2 is also expressed on antigen-presenting cells, and then contributes to allergen-specific T-lymphocyte priming. Because inflammatory cytokines are secreted by allergen stimulation and may affect mast cell reactivity and acute inflammation, we examined whether their induction in the draining lymph node cells was independent of MCP-1 or CCR2 blockade. Our analysis of the mixed lymphocyte reaction of draining lymph node cells showed an induction of a panel of cytokines that might affect mast cell reactivity. However, the outcome of cytokines secretion, including IL-2, IL-4, or IL-17, was not impaired by MCP-1 antibody treatment or RS 504393 treatment (Fig. 6).

Impaired Mast Cell Degranulation by CCR2 Inhibition

To determine whether an impairment of the immediate hypersensitivity reaction by an CCR2 blockade was due to a direct action on mast cells and independent of the inductive phase phenomena, isolated connective tissue mast cells (from the

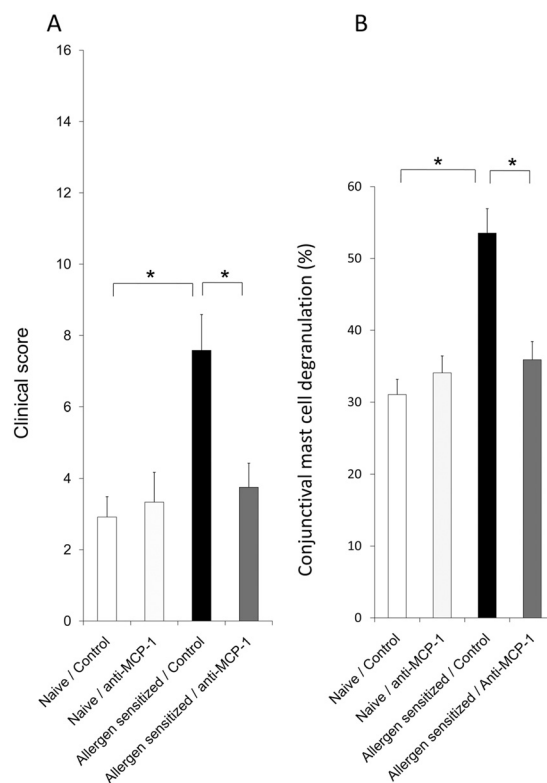


FIGURE 3. Impairment of allergen-induced immediate hypersensitivity reaction and mast cell degranulation after MCP-1 blockade. Allergen sensitized mice were intravenously treated with anti-MCP-1 antibody or control IgG on days 41 and 42, and challenged with allergen on day 42. The allergen-induced clinical signs (clinical scores) and degranulated conjunctival mast cell count were analyzed as parameters of immediate hypersensitivity. (A) The immediate hypersensitivity reaction was abolished in anti-MCP-1 antibody-treated mice. (B) Degranulation of mast cells was significantly impaired in anti-MCP-1 antibody-treated mice (right). * $P < 0.05$. $n = 10$ /group.

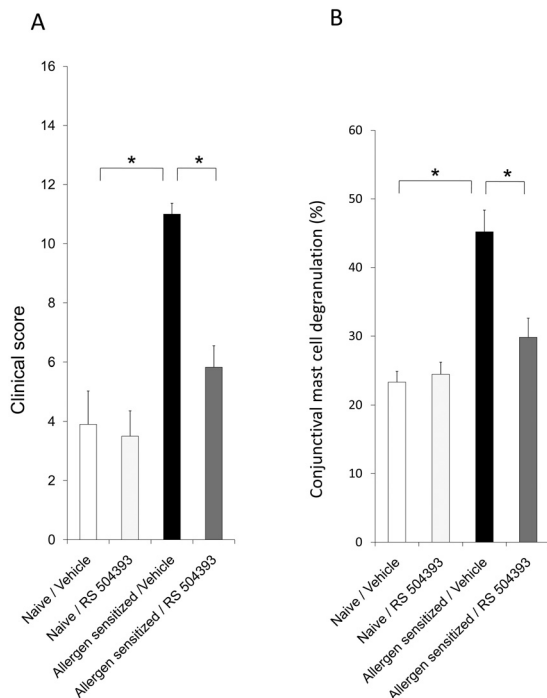


FIGURE 4. Impairment of allergen-induced immediate hypersensitivity reaction and mast cell degranulation by CCR2 blockade. Allergen-sensitized mice were orally treated with RS 504393, a specific CCR2 antagonist, or vehicle on days 40, 41, and 42 and challenged with allergen on day 42. The allergen-induced clinical signs (clinical scores) and degranulated conjunctival mast cell count were analyzed as parameters of immediate hypersensitivity. (A) The immediate hypersensitivity reaction was abolished in RS 504393 treated-mice. (B) Degranulation of mast cells was significantly impaired in RS 504393-treated mice. * $P < 0.05$. $n = 10$ /group.

skin) were first examined for allergen-specific degranulation. The reactivity of mast cells was measured by allergen-induced β -hexosaminidase release. Consistent with the outcome of the inhibitory effects of anti-MCP-1 (Fig. 3), anti-MCP-1 significantly suppressed allergen induced β -hexosaminidase release (Fig. 7A). CCR2 antagonist, RS 504393 treatment, also suppressed allergen induced β -hexosaminidase release significantly (Fig. 7B). This inhibitory effect was partially reversed by a supplementation of recombinant MCP-1 protein (100 pg/mL), confirming that the inhibitory effect on CCR2 was mainly mediated by MCP-1.

Next, isolated conjunctiva-derived connective tissue mast cells were tested for CCR2 blockade. RS 504393 exposure significantly suppressed allergen-induced β -hexosaminidase release. Without allergen priming, MCP-1 induced mast cell degranulation, which was completely suppressed by RS 504393. Again, anti-MCP-1 treatment significantly suppressed allergen-induced β -hexosaminidase release (data not shown). Thus, MCP-1/CCR2 may serve as regulators of conjunctival mast cells in ocular allergic reactions.

DISCUSSION

Taken together, these data suggest that the MCP-1/CCR2 axis is essential for mast cell-mediated acute inflammatory responses. Our observations demonstrated the important aspects of the MCP-1/CCR2 axis in the early events of the immediate hypersensitivity reactions in mouse eyes. First, MCP-1 is a crucial mediator of IgE-mediated mast cell activation and provides the

complementary signal for ocular allergic reactions. Another important aspect of our findings is the pivotal role of CCR2 in activating mature connective tissue-type mast cells in ocular tissues.

Conjunctival mast cells express various chemokine receptors, including CCR1, CCR2, CCR3, CCR5, and CXCR3.¹⁵ Their expression levels are elevated on mature mast cells together with Fc ϵ RI. We have shown that MIP-1 α , a CCR1 ligand, is required for the immediate hypersensitivity reaction and is important for mast cell activation.⁹ Eotaxin-1, a CCR3 ligand, is known to serve as the canonical activation and homing signal for eosinophils. However, the roles of eotaxin-1 and CCR3 are not confined to eosinophilic responses, but they also play critical roles in mast cell activation. We recently showed that ablation of eotaxin-1 significantly suppresses allergen-induced signs in the eyes and mast cell degranulation.⁸ Thus, both CCR1- and CCR3-mediated signals appear to be necessary for optimal mast cell activation. This implicates that the CC chemokine-receptors may serve as indispensable stimuli for inflamed conjunctiva, although their relative importance needs to be carefully evaluated.

Some of the stimulatory effects of CC-chemokines on mast cells have been generally recognized in mast cell lines in vitro; however, proof of this principle using a disease model has been scarce.¹⁶ Indeed, earlier findings suggest that their roles on mast cells are related to homing and differentiation.^{16,17} On the other hand, the results of our recent series of experiments failed to detect defective homing to the tissues by chemokine receptor signaling blockade either with or without allergen priming. Thus, chemokine receptors appear to be redundant for conjunctival homing of mast cells or progenitors.

Collectively, our findings indicate that blocking the chemokine signals significantly suppresses allergen-induced mast cell degranulation. These findings indicate that CCR2 can be added to the arsenal of stimuli that can activate the immediate hypersensitivity in ocular allergy.

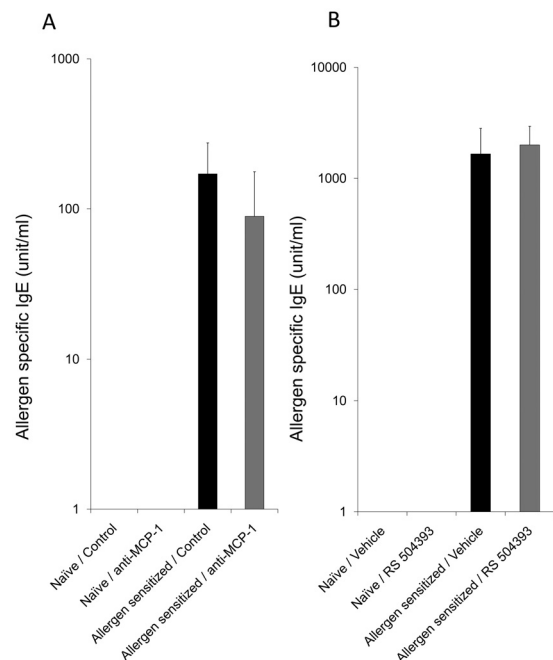


FIGURE 5. Unaffected priming of allergen-specific IgE by MCP-1 or CCR2 blockade. Allergen-specific serum IgE ($n = 7$ /group) was not affected by either anti-MCP-1 antibody (A) or by RS 504393 treatment (B). The level of allergen-specific IgE in the mock-immunized mice was below the detection limits. Values are expressed as the mean $\pm$ SEM.

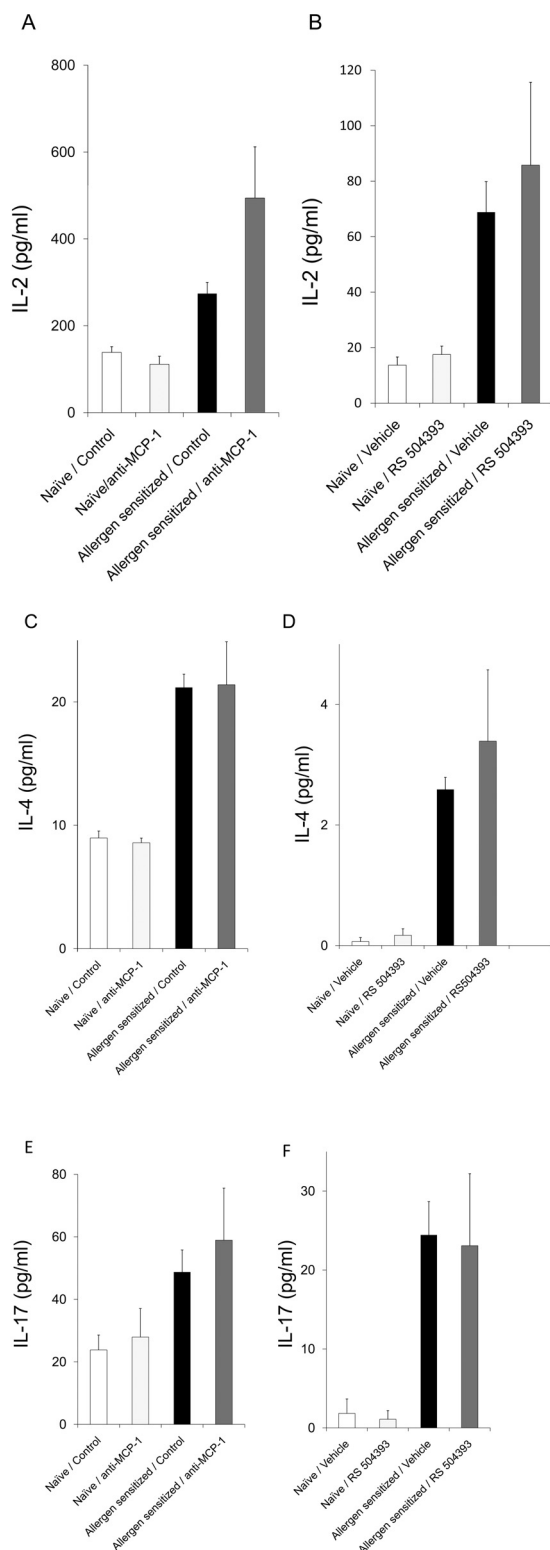


FIGURE 6. Secretion of inflammatory cytokines by allergen-stimulated draining lymph node cells. Allergen-specific secretions of IL-2 (A, B), IL-4 (C, D), and IL-17 (E, F) were not impaired by anti-MCP-1 (A, C, E) or RS 504393 (B, D, F). Data are expressed as the mean $\pm$ SEM.

Because CCR3 is a signature chemokine receptor for eosinophilic allergic responses, we conducted a whole-genome analysis of the acute phase in an experimental allergic conjunctivitis model.⁷

The outcome surprisingly detected MCP-1 as an eosinophilic mediator. Consistent with this finding, administration of RS 504393 significantly suppressed eosinophil recruitment in the late phase of experimental allergic conjunctivitis (Tominaga T, Miyazaki D, unpublished observation, 2008). Although MCP-1 is a potent stimulus for inflammatory monocytes and macrophages, it is still unclear how MCP-1 can stimulate the eosinophilic inflammation. Two major cascades contribute to the late phase of experimental allergic conjunctivitis. One is the well-known Th2-cell-mediated activation of eosinophils,¹⁸ and the other is mediated by allergen-induced

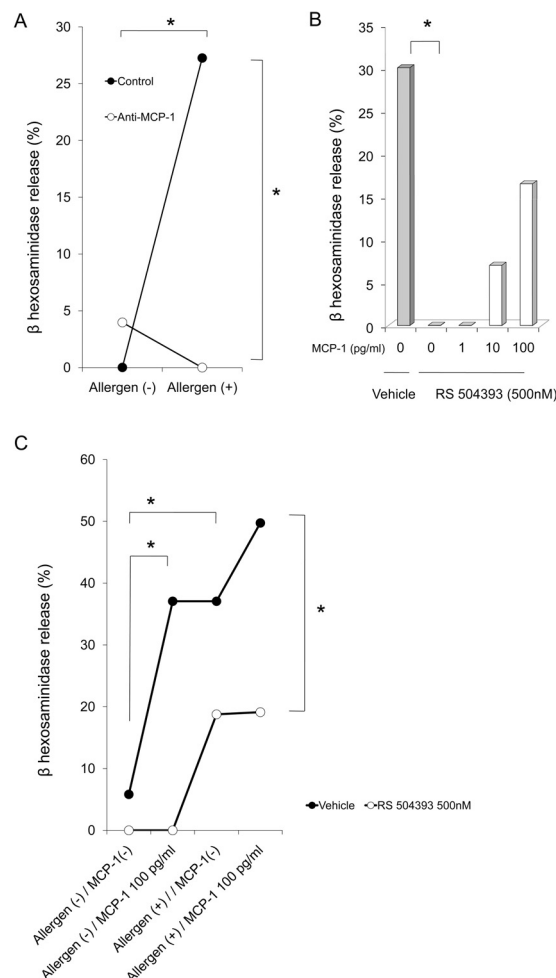


FIGURE 7. Suppression of connective tissue-type mast cell degranulation by CCR2 blockade. (A) Mature connective tissue-type mast cells from the skin were cultured for 1 week (without anti-MCP-1) and were tested for DNP-albumin (allergen)-induced degranulation by measurement of β -hexosaminidase release. The connective tissue-type mast cells were sensitized with anti-DNP-IgE and stimulated by DNP-albumin with control or anti-MCP-1. Anti-MCP-1 antibody significantly suppressed allergen specific β -hexosaminidase release. (B) Mature connective tissue-type mast cells from the skin (cultured for 1 week without RS 504393) were sensitized with anti-DNP-IgE and stimulated by DNP-albumin, with or without recombinant MCP-1 (1, 10, and 100 pg/mL) and/or RS 504393 (500 nM). CCR2 blockade by RS 504393 significantly suppressed allergen-specific β -hexosaminidase release. The impaired response was partially restored by addition of MCP-1 (100 pg/mL) in a dose-dependent manner. (C) Mature connective tissue-type mast cells from the conjunctiva (cultured for 1 week without RS 504393) were sensitized with anti-DNP-IgE and stimulated by DNP-albumin. A CCR2 blockade by RS 504393 suppressed the allergen-specific β -hexosaminidase release. * $P < 0.05$. $n = 5$ /group.

mast cell degranulation, which we recently examined in an adoptive transfer approach of conjunctival mast cells to allergen-primed mast cell-deficient mice.¹⁰ Because MCP-1 is the critical mediator of allergen-primed mast cell degranulation, we assumed that MCP-1 would indirectly provoke eosinophilia by mast cell activation. Th2-cell-mediated activation of eosinophils is less likely, since the blockade of MCP-1/CCR2 did not affect the Th2 cytokine secretion in our model.

Our findings on the importance of MCP-1/CCR2 are derived from murine mast cells, which allowed us to evaluate their roles in a disease model. Considering a therapeutic intervention to target this axis in the clinic, there still remain some possibilities of functional differences. However, the importance of the MCP-1/CCR2 axis as a therapeutic candidate has also been reported from the results of genomic analyses of patients. Analysis of patients with seasonal rhinitis and conjunctivitis by PCR restriction fragment length polymorphism identified a significant association of single nucleotide polymorphisms (SNP) in CCR2 and in CCR3 with cedar pollinosis.¹⁹ When the increased eosinophil levels of asthmatic subjects were studied, an SNP of the MCP-1 regulatory region was shown to be significantly associated.²⁰ The -2518 G/G genotype correlated significantly with the severity of the asthma.

MCP-1 also plays a key role in the pathogenesis of various inflammatory diseases.²¹ The blood and tissue levels of MCP-1 are elevated in patients with chronic obstructive diseases, rheumatoid arthritis, atherosclerosis, and multiple sclerosis.^{22,23} Moreover, the level of the induced MCP-1 generally correlates with the severity of the disease.²¹ A blockade of MCP-1 or CCR2 ameliorates the signs of the disease in animal models of arthritis, experimental autoimmune encephalomyelitis, atherosclerosis, airway hypersensitivity, nephritis, and kidney fibrosis.^{24–29} Thus, CCR2 antagonism may be an attractive therapeutic approach.

Analyses of the tear cytokines in eyes with severe allergic conjunctivitis identified numerous cytokines, including IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-7, IL-6sR, IL-12, IL-13, eotaxin-1, eotaxin-2, MIP-1 δ , and M-CSF.³⁰ The MCP-1 levels in the tears of eyes with vernal keratoconjunctivitis and atopic keratoconjunctivitis were markedly elevated together with two CCR3 ligands, eotaxin-1/eotaxin-2. These observations support our view that inhibition of MCP-1 may indeed serve as a therapeutic strategy for ocular allergy.

To summarize, we showed that blocking the MCP-1/CCR2 axis depressed the early phase mast cell activation and immediate hypersensitivity reactions of the eye. These changes then led to a suppression of the late-phase eosinophilic inflammatory responses in an indirect manner. Our findings strongly suggest that pharmacologic intervention of the MCP-1/CCR2 axis may serve as a therapeutic strategy for severe ocular allergic diseases.

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Supplementary Table S1. Grading criteria of immediate hypersensitivity reaction in experimental allergic conjunctivitis

	0	1	2	3	4
Conjunctival edema	none	focal edema	edema confined within one quadrant	edema extending to 3 quadrants	massive edema.
lid edema	none	slightly narrowed palpebral fissure (3/4 normal width) with congestion	narrowed palpebral fissure with edema (2/3 of normal width)	narrowed fissure with severe edema (1/3 of normal width)	massive edema (cornea barely visible).
tear/discharge	minimal level of tear meniscus	increased tear level with concave meniscus	increased tear level with convex meniscus	highly increased tear level with mucous secretion	excessive tearing with copious discharge
Conjunctival redness	none	barely detectable venous dilatation	three or four dilated vessels at corneal limbus	five or six dilated vessels at limbus	marked ciliary injection