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# Macrophage inflammatory protein-1 $\alpha$ as a costimulatory signal for mast cell-mediated immediate hypersensitivity reactions

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**Regulation of the immune response requires the cooperation of multiple signals in the activation of effector cells. For example, T cells require signals emanating from both the TCR for antigen (upon recognition of MHC/antigenic peptide) and receptors for costimulatory molecules (e.g., CD80 and CD60) for full activation. Here we show that IgE-mediated reactions in the conjunctiva also require multiple signals. Immediate hypersensitivity reactions in the conjunctiva were inhibited in mice deficient in macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) despite normal numbers of tissue mast cells and no decrease in the levels of allergen-specific IgE. Treatment of sensitized animals with neutralizing antibodies with specificity for MIP-1 $\alpha$  also inhibited hypersensitivity in the conjunctiva. In both cases (MIP-1 $\alpha$  deficiency and antibody treatment), the degranulation of mast cells in situ was affected. In vitro sensitization assays showed that MIP-1 $\alpha$  is indeed required for optimal mast cell degranulation, along with cross-linking of the high-affinity IgE receptor, Fc $\epsilon$ RI. The data indicate that MIP-1 $\alpha$  constitutes an important second signal for mast cell degranulation in the conjunctiva in vivo and consequently for acute-phase disease. Antagonizing the interaction of MIP-1 $\alpha$  with its receptor CC chemokine receptor 1 (CCR1) or signal transduction from CCR1 may therefore prove to be effective as an antiinflammatory therapy on the ocular surface.**

## Introduction

Allergic diseases such as asthma, rhinitis, dermatitis, urticaria, conjunctivitis, food allergy, and severe anaphylactic responses (e.g., to pharmaceuticals or insect venom) affect approximately one-third of the population in the Western world, and costs associated with them dominate public health budgets. Since current treatments are not completely effective, and result in significant adverse side-effects in patients, there is a continuing effort to better understand the molecular basis of the allergic response. It is hoped that this information will permit the design of better and safer treatments.

The course of allergic diseases can typically be divided into 2 phases: the immediate hypersensitivity reaction (the early- or acute-phase reaction) and the late-phase reaction. The immediate hypersensitivity reaction occurs within 1 hour after allergen exposure (in a sensitized individual) and is thought to be driven by cross-linking of allergen-specific IgE bound to the surface of resident mast cells via the high-affinity IgE receptor, Fc $\epsilon$ RI (1). Thus, the mast cell is the key effector cell in immediate hypersensitivity reactions, releasing histamine, mast cell proteases, inflammatory cytokines, chemokines, and lipid mediators upon antigenic stimulation.

The late-phase reaction is in many respects a sequel to these mast cell-driven events and occurs 12–24 hours after allergen challenge. The hallmark of the late-phase reaction is the recruit-

ment of inflammatory cells, including eosinophils, basophils, T cells, neutrophils, and macrophages, to the site of allergic inflammation. Chemokines such as eotaxin-1, eotaxin-2, RANTES, monocyte chemoattractant protein-3 (MCP-3), MCP-4, and macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) play a key role in driving the late-phase reaction. The chemokines both affect the expression of adhesion molecules on vascular endothelium and provide a chemotactic gradient for cells recruited in the late-phase reaction (2–11). The acute-phase reaction therefore is not only responsible for early clinical signs of allergic inflammation, but is essential for the generation of late-phase responses and chronic allergic disease.

In contrast to the wealth of information on the role of chemokines in the late-phase reaction, there is much less known about how (and in what situations) chemokines might contribute to acute disease. However, there is reason to carefully consider such a role, as mast cells and basophils express the chemokine receptors CCR1, CCR2, CCR3, CCR5, CXCR1, CXCR2, and CXCR4 (12, 13). A role for the chemokine/chemokine receptor system in the immediate hypersensitivity reaction has also been suggested in recent analyses of CCR1- and CCR3-deficient mice. In this article, we report that the immediate hypersensitivity reaction in the conjunctiva requires the  $\beta$  chemokine MIP-1 $\alpha$ . MIP-1 $\alpha$  expression is rapidly induced in specific mononuclear cells after allergen challenge, and this expression is required for optimal mast cell degranulation. Neutralization of MIP-1 $\alpha$  in sensitized animals also inhibits mast cell degranulation and the acute response in the conjunctiva. Passive sensitization experiments using ex vivo mast cells and CCR1-positive RBL-2H3 cells show directly that MIP-1 $\alpha$  serves as a costimulatory signal for mast cell degranulation.

**Nonstandard abbreviations used:** DNP, 2,4-dinitrophenol; Fel d1, *Felis domesticus* allergen 1; MCP-3, monocyte chemoattractant protein-3; MIP-1 $\alpha$ , macrophage inflammatory protein-1 $\alpha$ .

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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## Results

Our current understanding of mast cell activation stems largely from studies using in vitro-generated, bone marrow-derived mast cells (14) or mast cell lines, chiefly RBL-2H3 cells. Studies of ex vivo or native mast cells have been more limited and have been restricted to those purified from a small number of tissues, such as skin and lung (15). While these studies have provided a remarkably detailed picture of mast cell activation requirements and signal transduction, certain aspects of mast cell activation in vivo might be missing from this picture. The variance of data obtained from such ex vivo studies with those from studies of in vivo mast cells might reflect the artificial nature of in vitro-generated or cultured mast cell-like lines, or the studies may simply fail to recapitulate the terminal phenotypes and/or local influences of fixed tissue mast cells in vivo. Indeed, there is now overwhelming evidence that mast cells resident in distinct mucosal tissues are heterogeneous with respect to their molecular profiles and activation requirements. Thus, it is important that studies also be performed on mast cell activation in intact organisms.

We previously developed a murine model of allergic conjunctivitis in order to investigate the role of chemokines in mast cell activation in vivo (16). In this model, the immediate hypersensitivity reaction is observed within the first hour following allergen challenge. Since the late-phase reaction occurs many hours after the acute response, we are able to study mast cell activation and acute disease in this tissue in complete isolation from the late-phase reaction. Specifically, we have studied the kinetics of inflammatory cell recruitment in this model, and these cells do not appear in the conjunctiva until several hours after the peak of clinical symptoms in the acute-phase reaction.

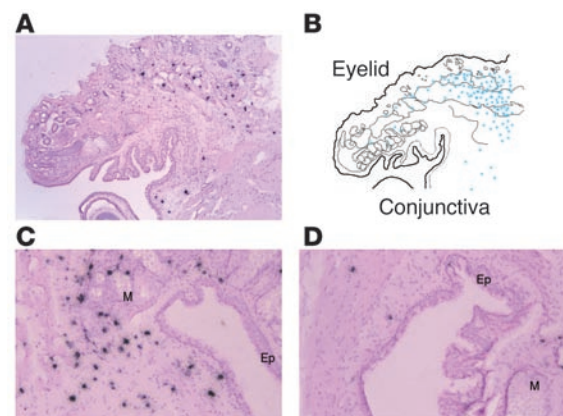
Using this system, we screened for genes expressed early after allergen challenge using gene profiling (16, 17). Numerous immediate response genes were found to be induced after allergen challenge; we focused our interest first on CC chemokines, in view of the reports of chemokine receptor expression on effector mast cells. The presence of receptors for these chemokines on mast cells immediately suggested that the induced expression of the chemokines we observed had the potential to induce physiologically relevant signaling from the receptors.

In our model, we detected rapid induction of MIP-1 $\alpha$  transcripts within the first 30 minutes after allergen exposure (Figure 1A) (16). Levels of MIP-1 $\alpha$  peaked at 3 hours after challenge and then declined at 24 hours. The enhanced levels of MIP-1 $\alpha$  mRNA after allergen challenge translated into significant increases in levels of its gene product. When we measured MIP-1 $\alpha$  protein levels in conjunctival homogenates using a specific ELISA, significant induction of total MIP-1 $\alpha$  protein was observed in the conjunctival homogenates (MIP-1 $\alpha$  after *Felis domesticus* allergen 1 [Fel d1] challenge:  $1.3 \pm 0.3$  pg/eye; after PBS challenge:  $0.2 \pm 0.1$  pg/eye;  $P < 0.05$ ). The rapid induction kinetics of chemokine expression was in agreement with that observed in the airway and skin. This induction was not dependent on specific allergens, as we observed the same induction using other allergens, such as short ragweed (17).

We then performed experiments to localize the cells producing MIP-1 $\alpha$  after allergen challenge and their spatial relationship relative to conjunctival mast cells (Figure 1A). It is already well known that conjunctival tissue harbors large numbers of mast cells, exceeding those in the lung or skin. We observed that conjunctival mast cells concentrate in the substantia propria of the eyelid root (Figure 1B). The retro-orbital area adjacent to the optic nerve also harbors significant, but much smaller, numbers of mast cells.

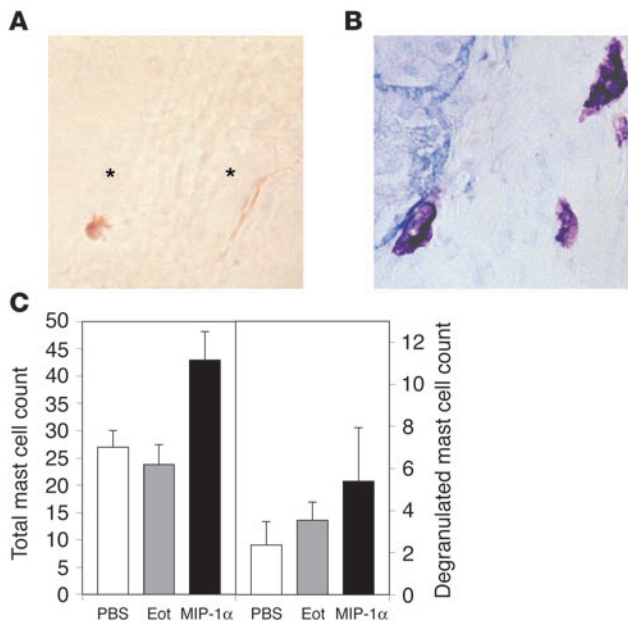
To probe the nature of the MIP-1 $\alpha$ -producing cells and their spatial relationship to mast cells, we performed in situ hybridization analysis of MIP-1 $\alpha$  gene expression on serial sections of conjunctival biopsies along with histologic/morphologic analysis of constituent cells (16, 18). The MIP-1 $\alpha$ -positive signal was localized mainly to mononuclear cells within the substantia propria of eyelid root (Figure 1A) in close proximity to the resident mast cells. Surprisingly, we did not detect MIP-1 $\alpha$  transcripts in other cells (e.g., endothelial cells) known to produce chemokines in other inflamed tissues. The cells producing chemokines in our system are likely analogous to the CD68 $^+$  cells thought to be a source of allergen-induced chemokine production in the lung. We cannot rule out the possibility that other cell types in the conjunctiva might also express lower levels of allergen-induced chemokine or that they may express chemokines later in the acute-phase reaction. However, it is clear from our experiments that the predominant source of inducible MIP-1 $\alpha$  transcripts was the mononuclear cells in the substantia propria.

The resident mononuclear cells in this region, comprising CD68 $^+$  macrophages and monocytes, were the predominant sources of MIP-1 $\alpha$  during the acute-phase reaction. PBS-treated mice showed a constitutive very low-level expression of MIP-1 $\alpha$  mRNA in resident mononuclear cells (Figure 1, A, C, and D). In the late-phase reaction (more than 6 hours after challenge), subsets of newly recruited eosinophils were identified as positive for MIP-1 $\alpha$  expression. This is consistent with the fact that human eosinophils can express MIP-1 $\alpha$  (19). Much weaker expression of MIP-1 $\alpha$  was also observed in the tarsal conjunctiva. Once again, mast cells were found in close proximity to the MIP-1 $\alpha$  $^+$  mononuclear cells in this subregion of the conjunctiva. The finding that MIP-1 $\alpha$  expression is induced following allergen exposure on immune mononuclear cells located nearby resident mast cells suggested that newly synthesized MIP-1 $\alpha$  could feasibly bind to nearby CCR1 $^+$  mast cells. Our analysis of chemokine receptor expression on conjunctival



**Figure 1**

Location of MIP-1 $\alpha$ -expressing cells in the allergen-challenged conjunctiva. (A) Localization of MIP-1 $\alpha$  mRNA-positive cells in the allergen-challenged conjunctiva. Slides were exposed for 4 days, developed, and counterstained with H&E staining. (B) Schematic description of conjunctival mast cell distribution. Note colocalization with MIP-1 $\alpha$ -positive cell distribution as shown in A. (C) MIP-1 $\alpha$  mRNA-positive cells were also shown in the tarsal conjunctival area of allergen-challenged mice (3 weeks exposure). (D) MIP-1 $\alpha$ -positive cells in PBS-challenged conjunctiva (3 weeks exposure). Ep, conjunctival epithelium; M, meibomian gland. Magnification,  $\times 40$  (A),  $\times 200$  (C and D).



**Figure 2**

Conjunctival mast cells express CCR1 and respond to recombinant MIP-1α. (A) Conjunctival mast cells from a naive mouse stain positive for CCR1. (B) Toluidine blue staining of mast cells in a serial section. CCR1+ mast cells are indicated with asterisks in A. (C) Subconjunctival injection of recombinant MIP-1α results in the chemotaxis of mast cells into the conjunctiva in naive mice. Statistically significant elevation of total mast cell numbers in MIP-1α-injected conjunctiva compared with eotaxin-1- or sham-injected controls (4 h after injection). MIP-1α increased degranulated mast cell count proportionately. *n* = 6 per group; *P* < 0.05. Values are expressed as mean ± SEM. Eot, eotaxin-1.

mast cells indicates that these cells express CCR1 (Figure 2, A and B). The ratio of positive cells to total mast cells was approximately 1:10, corresponding to the ratio of degranulated mast cells observed in these experiments (Figure 2C). We did not observe CCR5 expression of murine conjunctival mast cells. Since the Grant laboratory has already shown that MIP-1α could profoundly stimulate mast cell activation *in vitro* (20), our mapping studies suggested that signaling from CCR1 might also play an important role in mast cell degranulation *in vivo*.

The role of MIP-1α in mast cell physiology is not clear and has been implicated in activation, differentiation, and homing (21–26). The actual role of MIP-1α in mast cell biology *in vivo* is poorly understood. To directly test whether MIP-1α might activate conjunctival mast cells *in vivo*, we first injected recombinant MIP-1α beneath the conjunctiva of naive mice. We then analyzed the morphology of resident mast cells 1 hour and 4 hours after injection by Giemsa and H&E staining. The data clearly showed that MIP-1α stimulated mast cell activation (elongation and degranulation) and was associated with an increased number of detectable mast cells at 4 hours (Figure 2C). Consistent with previous studies, MIP-1α also induced rapid recruitment of neutrophils and eosinophils as early as 1 hour after injection.

Together with the results shown in Figure 1, the data provided additional evidence that allergen-induced MIP-1α in the conjunctiva might contribute to mast cell activation. To test this more rigorously, we next analyzed allergen-induced mast cell degranulation and acute inflammation in MIP-1α-deficient mice. While the back-

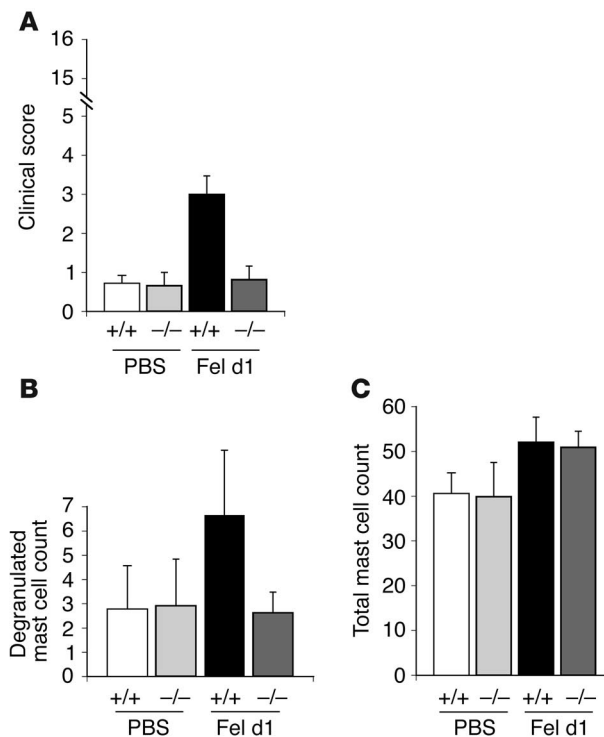
ground mouse strain developed clear parameters of acute inflammation during the sensitization period, the MIP-1α-deficient mice failed to show clinical symptoms of immediate hypersensitivity. Remarkably, MIP-1α-deficient mice were almost devoid of acute inflammation upon final challenge (Figure 3). Every symptom of clinical disease – conjunctival edema, lid edema, lid redness, tearing/discharge, and squinting – was strongly inhibited.

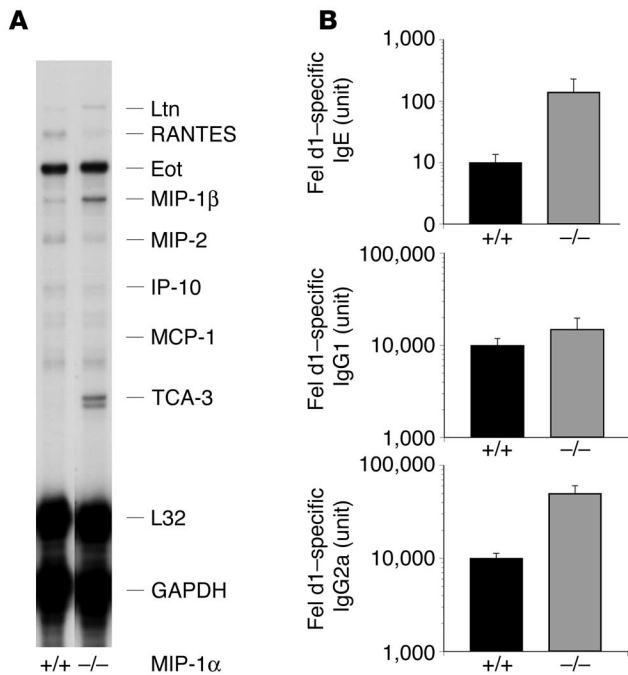
Since mast cell activation drives the acute-phase reaction, these data immediately suggested that MIP-1α deficiency was indeed impacting on mast cell activation *in vivo*. We evaluated mast cell degranulation in the challenged conjunctiva of MIP-1α<sup>+/+</sup> and MIP-1α<sup>-/-</sup> strains. Consistent with the suppression of clinical symptoms (Figure 3A), the percentage of degranulating mast cells (degranulated mast cell number / total mast cell number × 100) was significantly suppressed in MIP-1α-deficient mice (Figure 3B). Total conjunctival mast cell numbers were not significantly different (Figure 3C) between WT and MIP-1α-deficient mice (data not shown). These data directly showed that MIP-1α was somehow required for optimal mast cell activation *in vivo*.

Since the mucosal immune response is mediated by both an inductive phase and an effector phase, there were several potential

**Figure 3**

Impairment of allergen-induced immediate hypersensitivity reaction and mast cell degranulation in MIP-1α-deficient mice. (A) Immediate hypersensitivity reaction, shown here as % maximal clinical score (defined as sum of each clinical symptom scores), was abolished in MIP-1α-deficient (–/–) mice. *n* = 13 per group; *P* < 0.05. (B) Degranulation of mast cells was significantly impaired in MIP-1α-deficient mice. (C) Total mast cell counts in these mice were not affected by MIP-1α deficiency. Values are expressed as mean ± SEM.



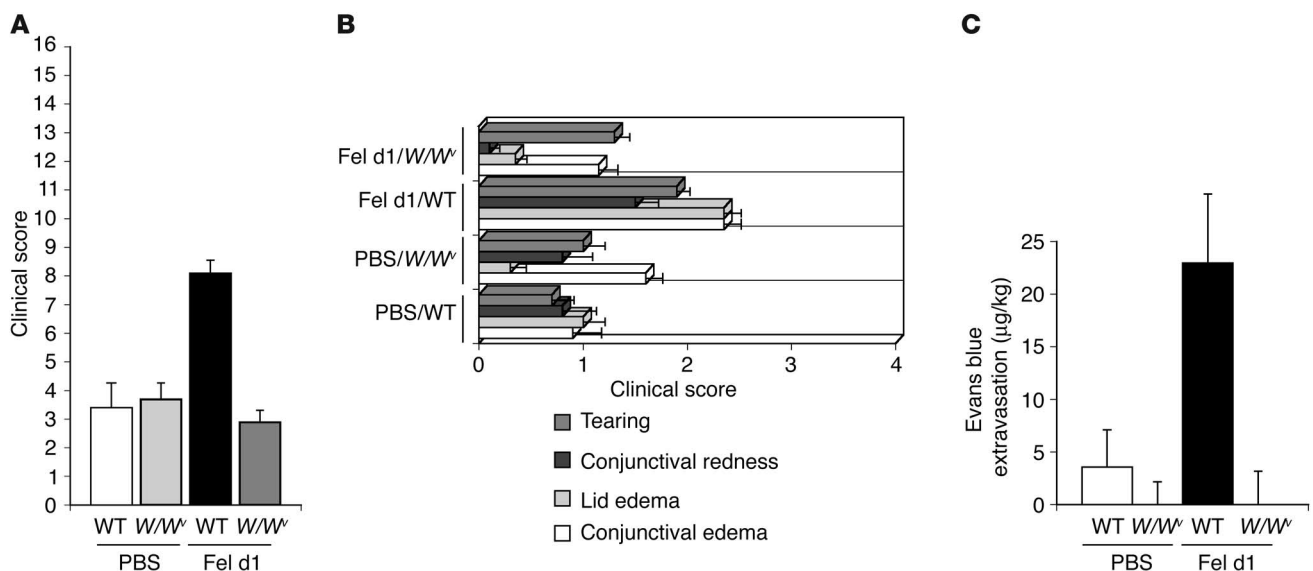


**Figure 4**

Local chemokine production and allergen-specific Ig synthesis in wild-type and MIP-1α-deficient mice. **(A)** Profiles of chemokine induction in MIP-1α-deficient eye homogenates by RNase protection assay. MIP-1α deficiency did not affect induction of eotaxin-1, MIP-2, MCP-1, or IFN-γ-inducible protein 10 (IP-10) 24 hours after allergen challenge. Each lane represents RNAs isolated from 2 representative eyes in each group. Ltn, lymphotactin. **(B)** Allergen-specific serum Ig levels in immunized mice. MIP-1α deficiency did not impair the synthesis of serum Igs (IgE, IgG1, IgG2a). *n* = 9 per group. Levels of allergen-specific Igs (IgE, IgG1, IgG2a) in the mock-immunized mice were below detection limits (data not shown). Values are expressed as mean ± SEM.

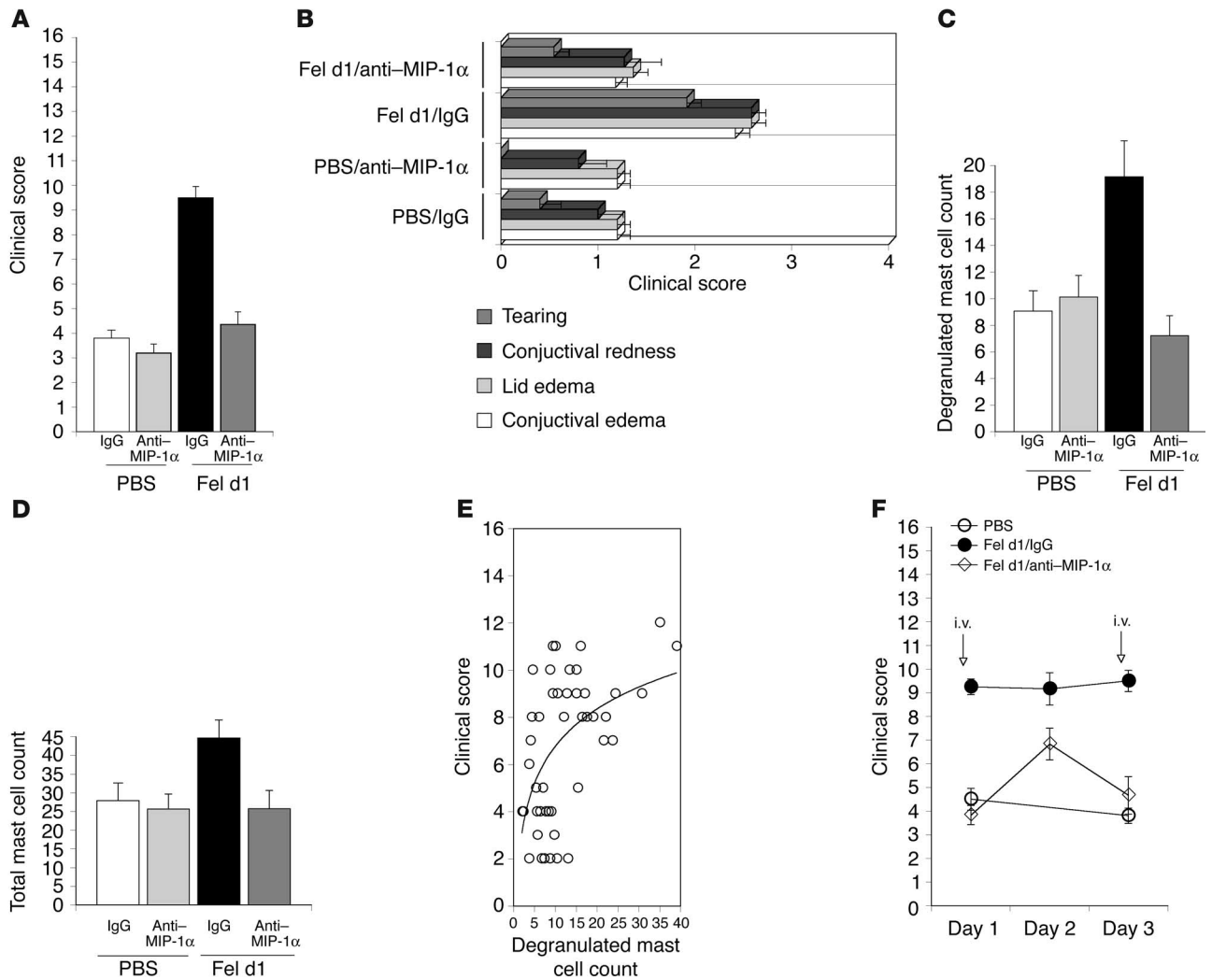
biological pathways (e.g., mast cell development or Ig synthesis) by which MIP-1α deficiency might result in depressed mast cell activation. Our subsequent experiments were focused on determining which arm of this response is impaired by MIP-1α deficiency. In the inductive phase, a complex series of cellular and molecular events results in the generation of elevated levels of allergen-specific IgE. This requires proper antigen processing and presentation, B cell maturation, and class switching driven by Th2

cells. One clear possibility was that MIP-1α-deficient mice had a defect in one of these steps, which would result in decreased levels of allergen-specific IgE. This would in turn manifest in depressed mast cell degranulation upon challenge, as the level of mast cell receptor for antigen would be limiting. Our direct analysis of all possible antibodies – IgE, IgG1, or IgG2a (Figure 4B) – revealed that there is no deficit in antibodies in MIP-1α-deficient mice. To the contrary, production of the allergen-specific IgE was markedly enhanced in these mice. Since the observed magnitude of increases in specific IgE would be predicted to augment mast cell activation, the data indicate that the impaired mast cell activation was not related to levels of Ig. MIP-1α deficiency could decrease mast cell activation and clinical symptoms in a direct or indirect manner. RNase protection analysis of RNAs isolated from the conjunctivas of allergen-challenged MIP-1α-deficient mice revealed that the induction of RANTES and MIP-2 is impaired, whereas MIP-1β, lymphotactin, and TCA-3 are induced (Figure 4A). Impaired mast cell degranulation in MIP-1α-deficient mice may be explained then either by MIP-1α deficiency or by reduction of RANTES or other CCR1 ligands.



**Figure 5**

Clinical scores are reduced and immediate sensitivity reactions impaired in mast cell-deficient *W/W<sup>c</sup>* mice. **(A)** Clinical scores were significantly suppressed in *W/W<sup>c</sup>* mice as compared with mast cell-competent WT mice (*n* = 20 per group; *P* < 0.05). **(B)** Each clinical symptom, including tearing, conjunctival redness, lid edema, and conjunctival edema was also suppressed in *W/W<sup>c</sup>* mice. **(C)** Evans blue dye extravasation 90 minutes after allergen challenge was assessed in *W/W<sup>c</sup>* and WT mice. *W/W<sup>c</sup>* mice show minimal dye extravasation, while WT mice showed a significant increase in extravasation in response to allergen challenge (*n* = 8 per group; *P* < 0.05). Values are expressed as mean ± SEM.

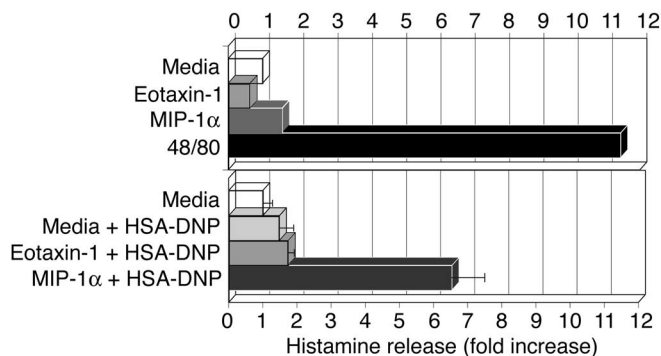


**Figure 6**

**In vivo neutralization of MIP-1α inhibits mast cell activation and clinical symptoms in the allergen-challenged conjunctiva. (A)** Suppression of allergen-induced immediate hypersensitivity by anti-MIP-1α antibody treatment. Mice primed for immediate hypersensitivity reaction were administered anti-MIP-1α monoclonal antibody (30 μg/injection) intravenously 1 hour before allergen challenge. Clinical scores assessed on day 3 were significantly reduced ( $n = 12$  per group;  $P < 0.05$ ). **(B)** Each clinical symptom, including conjunctival edema, lid edema, conjunctival redness, and tearing, was reduced by antibody treatment. **(C)** The frequency of degranulated mast cells following allergen challenge was also significantly reduced ( $n = 12$  per group;  $P < 0.05$ ). **(D)** Late-phase recruitment of mast cells was also assessed in WT mice and following MIP-1α blockade at 24 hours after challenge. Mast cell recruitment was significantly suppressed by antibody treatment ( $n = 12$  per group;  $P < 0.05$ ). **(E)** An analysis of mast cell degranulation and clinical scores showed a positive correlation between these 2 indices. **(F)** Kinetics of inhibitory effect of MIP-1α antibody treatment on clinical scores (100 μg/injection). Clinical scores were significantly suppressed on days 1, 2, and 3 ( $P < 0.05$ ). Values are expressed as mean ± SEM.

Since early-phase type I (Gel and Coombs classification) hypersensitivity reactions can be either mast cell dependent or independent (depending on sensitization schemes), we confirmed that the early-phase reaction is indeed mast cell dependent in this model. Mast cell-deficient  $WBB6F_1\text{-Kit}^{W/Kit^{W-v}}$  ( $W/W^v$ ) mice (27) or WT  $WBB6F_1^{+/+}$  mice were immunized following our standard protocol and assessed for immediate hypersensitivity reaction to allergen challenge according to 3 criteria: clinical symptoms, mast cell degranulation, and plasma exudation (Figure 5). The  $W/W^v$  mice (despite mounting a normal IgE response) clearly exhibited greatly decreased early-phase responses, regardless of the criterion monitored (Figure 5A). Induction of each symptom was similarly

impaired in  $W/W^v$  mice, which supports the view that each of these ophthalmic criteria are good indicators of mast cell function (Figure 5B). Histologic analysis clearly showed mast cells undergoing “anaphylactic” degranulation in  $WBB6F_1^{+/+}$  mice, whereas there were no mast cells detected in the conjunctiva of  $W/W^v$  mice (data not shown). Despite the lack of mast cells in the conjunctiva of allergen-challenged  $W/W^v$  mice, both late-phase eosinophilic and neutrophilic responses remained intact in these animals (data not shown). Finally, Evans blue dye extravasation was used to evaluate plasma exudation in response to allergen (Figure 5C). Allergen challenge of  $WBB6F_1^{+/+}$  mice show a 6-fold increase of Evans blue extravasation relative to mock-immunized mice, while virtually

**Figure 7**

Augmentation of allergen-induced histamine release by MIP-1 $\alpha$ . The experimental design involved a passive sensitization assay using isolated conjunctival tissue from naive mice. The upper panel shows histamine release from the in vitro-sensitized tissue challenged with eotaxin-1 (100 ng/ml), MIP-1 $\alpha$  (100 ng/ml), or compound 48/80 (1 mg/ml) without HSA-DNP (allergen) addition. The lower panel shows histamine release from the in vitro-sensitized tissue challenged with HSA-DNP in media, eotaxin-1 (100 ng/ml), or MIP-1 $\alpha$  (100 ng/ml). MIP-1 $\alpha$  supplementation significantly augmented allergen-induced (HSA-DNP-induced) histamine release of in vitro-sensitized conjunctival mast cells, while eotaxin-1 or vehicle had no effect. Compound 48/80 solution served as a positive control of mast cell degranulation. Values are expressed as mean  $\pm$  SEM.

no extravasation was observed upon allergen challenge of  $W/W^u$  mice. Taken together, these data support the view that the early-phase response in this model is heavily mast cell dependent. This also suggests that the impaired early-phase response in MIP-1 $\alpha$ -deficient mice is likely to manifest due to effects on the conjunctival mast cell. This conclusion is supported by the virtual lack of basophils in the allergen-challenged conjunctiva, as determined by electron microscopic analysis (data not shown).

To further probe MIP-1 $\alpha$  contribution to the effector phase, sensitized A/J mice were treated systemically with neutralizing antibody specific for MIP-1 $\alpha$ . Groups of mice were randomly chosen for treatment with neutralizing MIP-1 $\alpha$  antibody or control IgG via intravenous injection 1 hour prior to allergen challenges. Consistent with the data obtained using MIP-1 $\alpha$ -deficient mice, the immediate hypersensitivity reaction was significantly suppressed by neutralizing MIP-1 $\alpha$  antibody treatment. The data in Figure 6A show that the composite clinical score is reduced in mice receiving the neutralizing antibody. In addition, each standard ophthalmic subscore — for conjunctival edema, lid edema, conjunctival edema, and tearing — was decreased in antibody-treated mice (Figure 6B). Degranulated mast cell counts in these mice were also significantly suppressed (Figure 6C). Consistent with the data shown in Figure 5, the series of experiments also demonstrate that the response in this model is mast cell dependent and that our criteria for scoring of clinical symptoms correlates well with mast cell degranulation (Spearman correlation analysis,  $P < 0.001$ ) (Figure 6E). Since MIP-1 $\alpha$  blockade may also affect mast cell homing (Figure 2), the percentage of mast cell degranulation in these groups (calculated as degranulated mast cell count / total mast cell count  $\times$  100) was also assessed. The percentage of mast cell degranulation was  $35\% \pm 3\%$  for the Fel d1/IgG group and  $17\% \pm 2\%$  for the PBS/IgG group. MIP-1 $\alpha$  antibody treatment significantly suppressed the mast cell degranulation index ( $17\% \pm 3\%$  for the Fel d1/anti-MIP-1 $\alpha$  group).

We extended our in vivo analyses to include a kinetic study of clinical scores relative to pulses of MIP-1 $\alpha$ -neutralizing antibody treatment during the course of disease (Figure 6F). Injections of neutralizing antibody on days 1 and 3 led to decreased clinical symptoms. This suppressive effect was diminished in between injections on day 2. The synchronicity of disease attenuation with the injections is again supportive of a direct role of MIP-1 $\alpha$  on clinical symptoms. It is noteworthy that our analysis of serum IgE levels showed no differences between the MIP-1 $\alpha$  antibody group and control group (Fel d1/IgG group:  $1,682 \pm 243$  ng/ml; Fel d1/antibody group:  $1,758 \pm 151$  ng/ml;  $n = 8$ ), which indicates that sensitization to allergen was not affected by MIP-1 $\alpha$  neutralization. Taken together, these data strongly suggest that the suppression of clinical symptoms and mast cell degranulation by MIP-1 $\alpha$  blockade or deficiency is due to direct effects of MIP-1 $\alpha$  on the mast cell.

At the effector phase, it was also possible that MIP-1 $\alpha$  was important for terminal mast cell development and/or homing in the conjunctiva. Despite extensive morphological analyses (including analyses of differential stains), we found no evidence that MIP-1 $\alpha$  deficiency affected naive mast cell homeostasis in terms of homing to or terminal differentiation in the conjunctiva. Taken together, the data suggest that MIP-1 $\alpha$  contributes directly to the effector phase of the immediate hypersensitivity reaction, i.e., in mast cell activation upon exposure to allergen. Consistent with our findings, there are no reports indicating that MIP-1 $\alpha$ -deficient mice have an impaired humoral response or defects in mast cell homing or development (28, 29).

The next series of experiments tested whether MIP-1 $\alpha$  could in fact provide a direct costimulatory signal to mast cells. To address this issue, we employed an in vitro histamine release assay using isolated conjunctival mast cells. In this system, passively sensitized conjunctival mast cells properly degranulate and release histamine rapidly within 30 minutes after in vitro allergen challenge. We tested whether addition of exogenous MIP-1 $\alpha$  could stimulate conjunctival mast cell activation by measuring histamine release. In the assay, naive mouse conjunctiva was used for in vitro sensitization to exclude the possibility of any inductive phase effect in the mucosa. MIP-1 $\alpha$  significantly augmented IgE-dependent histamine release dose dependently, and the fold increase reached a plateau at 67% of the maximum release achieved with compound 48/80 (at 100 ng/ml of MIP-1 $\alpha$ , compound 48/80 used as positive control) (Figure 7). As a control, eotaxin-1 had no effect on IgE-dependent histamine release. Addition of MIP-1 $\alpha$  in the absence of IgE cross-linking only resulted in low levels of histamine release. Taken together, these data support the view that MIP-1 $\alpha$  provides an important costimulatory signal to conjunctival mast cells.

To receive costimulatory signals from MIP-1 $\alpha$  directly, conjunctival mast cells would need to express CCR1 or CCR5 receptors. While previous in vitro experiments showing direct activation of mast cells by MIP-1 $\alpha$  strongly suggest that these cells are positive for CCR1 or CCR5, this needed to be confirmed for conjunctival mast cells (20). As indicated previously, our experiments confirmed published reports that conjunctival mast cells express CCR1 but do not appear to express CCR5 (Figure 2A).

Since the previous passive sensitization experiments were performed using ex vivo conjunctival mast cell preparations, it is possible that addition of MIP-1 $\alpha$  might have stimulated mast cell activation indirectly by inducing the expression of another factor from



a bystander cell. However, our recent demonstration that MIP-1 $\alpha$  provides a strong costimulatory signal to CCR1<sup>+</sup> RBL-2H3 argues for a direct effect on the mast cell (30).

## Discussion

In summary our results indicate that (a) MIP-1 $\alpha$  is not required for the inductive phase of mucosal immunity; (b) MIP-1 $\alpha$  signaling does not affect mast cell homing and maturation in the conjunctiva; and (c) MIP-1 $\alpha$  is required for physiologically relevant levels of mast cell activation *in vivo*. The profound decrease in mast cell degranulation and near loss of clinical symptoms in the acute-phase reaction indicates that this is a biologically relevant mast cell activation signal in the conjunctiva. Data from several experiments strongly suggest that the MIP-1 $\alpha$  signal is a direct costimulatory signal to the mast cell operating via CCR1. Our results may help explain a correlation between MIP-1 $\alpha$  responsiveness and exacerbated disease in allergic subjects (20).

Elegant experiments by Wymann and colleagues have shown that PI3K $\gamma$  pathway is essential for amplification of mast cell function (31). Importantly, the authors found that heterotrimeric G<sub>i</sub> proteins, coupled to PI3K $\gamma$ , amplify Ca<sup>2+</sup> influx stimulated by allergen. Stimulation by MIP-1 $\alpha$  or RANTES was also shown to activate this augmentation pathway of bone marrow-derived mast cells. These findings are clearly in agreement with our own data, which showed that CCR-mediated signals are an important stimulus for mast cell activation *in vivo*.

Our data are also relevant with respect to mast cell homing and maturation. Mast cell precursor cells (MCPs) are derived from pluripotent precursor cells in bone marrow. MCPs home into the mucosal or connective tissue and differentiate into mature form *in situ*. Previous reports have indicated that CCR1, CCR3, and CCR5 are expressed on mast cells as well as the progenitor cell (12, 32, 33). The data have also indicated a role for these receptors in mast cell homing and differentiation. Our finding that MIP-1 $\alpha$ , a ligand of CCR1, is critical for mast cell activation but not differentiation may indicate that chemokine redundancy with respect to CCR1 binding leaves mast cell homing and differentiation intact in MIP-1 $\alpha$ -deficient mice. Clearly this redundancy is not operative during mast cell activation in the acute phase reaction in this tissue.

The data also support the emerging view that antagonizing the chemokine/chemokine receptor interaction or signaling from chemokine receptors hold promise for the treatment of both acute- and late-phase reactions. In a similar vein, our demonstration that IL-1 receptor antagonist or CpG oligonucleotides can prevent allergic conjunctivitis in this mouse model indicates that such antagonism of key molecular pathways in the pathogenesis of allergic disease have real potential (17, 34).

## Methods

**Animals.** A/J, BALB/c, and C57BL/6 mice were obtained from The Jackson Laboratory. MIP-1 $\alpha$ -deficient mice, on a C57BL/6 background, were obtained from The Jackson Laboratory. Control WT mice were age- and sex-matched and maintained under identical conditions. Genetically mast cell-deficient WBB6F<sub>1</sub>-*Kir<sup>W</sup>/Kir<sup>W-v</sup>* (W/W<sup>v</sup>) mice and the congenic normal WBB6F<sub>1</sub><sup>+/+</sup> mice were purchased from Shimizu Laboratory Supplies Co. The present study conformed to all regulations for laboratory animal research outlined by the Animal Welfare Act, NIH guidelines, the Association for Research in Vision and Ophthalmology statement regarding the experimental use of animals, and was approved by the Home Office (London, United Kingdom).

**Induction of allergic inflammation in the conjunctiva.** A/J, W/W<sup>v</sup>, or WBB6F<sub>1</sub> mice were sensitized with Fel d1 using the following protocol based on modification of our previously reported protocols (14, 16, 17): mice were injected intraperitoneally with 1 mg of aluminum hydroxide conjugated with Fel d1 extract (2,000 AU/mouse; ALK Laboratories) on days 1, 14, and 24. Concomitantly, aluminum hydroxide-conjugated (25 mg/eye) Fel d1 extract (200 AU/ml) was topically administered into the eye on days 1, 2, 3, 7, and 14. Thereafter, mice were topically challenged with Fel d1 extract (200 AU/ml) without alum once per week. Eight weeks after the initial sensitization, affinity-purified Fel d1 (0.5 mg/ml) was instilled into the mouse eyes (5  $\mu$ l/eye) for 3 consecutive days for the final challenge. For C57BL/6 mice, final challenges were made at week 10. Control mice were sensitized in a similar manner but challenged using PBS instead of antigen solution. The specificity of the responses was confirmed via challenge of sensitized mice with irrelevant antigen. After the final challenge, the clinical responses were recorded within the first 45 minutes and graded using the criteria described in our previous reports, with modifications detailed here (13, 16, 17, 35).

The evaluation criteria we have used in the determination of clinical scores is based on well-established protocols employed by us and others for clinical phase II trials of ophthalmic drugs for ocular allergy, including antihistamines, cyclosporin, and FK506. All symptoms evaluated (conjunctival edema, lid edema/redness, tearing, squinting/face washing), reflect distinctive categories of the inflammatory response, being provoked by distinct contributions from inflammatory mediators. The symptoms were evaluated in a double-blind fashion by teams of 3–4 individuals and graded 0 to 4 by an ophthalmologist unaware of the identity of each mouse. Specifically, mice were placed unperturbed after allergen challenge in a laminar flow hood under ambient light conditions for evaluation. First, their behavioral responses were continuously recorded between 10 and 15 minutes after challenge, and squinting/face-washing score was graded based on numbers of continuous actions (grade 0: none; 1: 1–2 s; 2: 3–4 s; 3: 4–6 s; 4: 7 s or more). We videotaped the mice, which allowed cross-checking of these numerical scores. Other symptoms (conjunctival edema, lid edema/redness, tearing) were evaluated 15 minutes after challenge. Grading criteria were as follows: for conjunctival edema, 0: none, 1: focal conjunctival edema, 2: edema confined within 1 quadrant, 3: edema extending to 3 quadrants, 4: massive edema in 4 quadrants; for lid edema/redness, 0: none, 1: slightly narrowed palpebral fissure with hyperemia (three-fourths width of normal fissure), 2: narrowed palpebral fissure with edema (two-thirds width of normal fissure), 3: narrowed palpebral fissure with severe edema (one-third width of normal fissure), 4: massive edema (cornea barely visible); and for tearing, 0: minimal level of tear meniscus, 1: increased tear level with concave meniscus, 2: increased tear level with convex meniscus, 3: highly increased tear level with mucous secretion, 4: excessive tearing with copious discharge. The cumulative clinical score was calculated as the sum of the scores of each of these 4 parameters (0 to 16). Detailed assessment criteria using a similar approach is also described in our previously published report (18).

For histological evaluation of immediate hypersensitivity reactions, mice were sacrificed, and collected tissues were fixed in 4% paraformaldehyde. They were then embedded in Histo-resin (Leica Instruments GmbH), and the serial sagittal sections (3  $\mu$ m in thickness) were stained with Toluidine blue, Giemsa, or H&E. Three consecutive conjunctival tissue sections from each eye were examined, and mast cells were counted under a  $\times$ 200 field microscope by an independent scientist in a double-blind fashion.

**MIP-1 $\alpha$  blockade by antibody treatment.** Monoclonal MIP-1 $\alpha$  antibody (MAB450; R&D Systems) or isotype control rat IgG (SouthernBiotech) were intravenously administered via tail vein on days 1 and 3 of final week





of the challenges 1 hour before allergen challenge (total of 60 or 200  $\mu\text{g}$ /mouse). The inhibitory effect was evaluated by scoring of clinical symptoms and histological analysis of mast cell degranulation.

**ELISA.** For evaluation of MIP-1 $\alpha$  protein levels, eyes with attached eyelids were collected at the indicated time points after Fel d1 challenge. Eyes were removed by dissection, and isolated conjunctival tissue was minced. The samples were suspended in PBS, sonicated on ice for 1 minute using Sonifier 450 (Branson), and clarified by centrifugation at 10,000  $g$  for 15 minutes. The clarified cell lysates were assayed for MIP-1 $\alpha$  using a commercial ELISA kit (R&D Systems). The sensitivity of MIP-1 $\alpha$  ELISA was less than 1.5 pg/ml. For measurement of serum IgE, IgG1, or IgG2a levels, mice were bled, and sera were collected after final antigen challenge. Serum ELISA of IgE, IgG1, or IgG2a was performed using Opt EIA mouse IgE set or biotin-conjugated anti-mouse IgG1 or IgG2a antibodies (BD Biosciences – Pharmingen). Antigen-specific ELISA was performed using plates coated with Fel d1 instead of capture antibodies.

**Immunohistochemistry.** Serial frozen sections (10  $\mu\text{m}$  in thickness) were acetone fixed at  $-20^{\circ}\text{C}$  for 10 minutes and stained for CCR1 using polyclonal anti-CCR1 antibody (Santa Cruz Biotechnology Inc.) and VECTASTAIN ABC kit (Vector Laboratories Inc.). Positive signals were visualized by AEC substrate.

**RNase protection assay.** Ocular tissue was obtained from 4–5 animals in each group and prepared for RNA extraction. We homogenized the tissue using a tissue grinder and extracted total RNA by homogenization in RNA STAT-60 (Tel-Test Inc.) according to the manufacturer's instructions, and 20  $\mu\text{g}$  of RNA was analyzed. RNase protection assay was performed using probes of murine eotaxin-1, MIP-1 $\alpha$ , L32, GAPDH, mCK-5, and mCK-1 panels (BD Biosciences – Pharmingen). The protected, hybridized RNA was electrophoresed on a 4.5% denatured PAGE gel.

**In situ hybridization with antisense MIP-1 $\alpha$  RNA probes.** To analyze conjunctival expression of MIP-1 $\alpha$  signal, we performed in situ hybridization of MIP-1 $\alpha$  as previously described (35, 18), using frozen sections. Briefly, the full-length cDNA of murine MIP-1 $\alpha$  in pBluescript SK<sup>+</sup> were linearized, and antisense and sense RNA probes were generated using T7 and T3 RNA polymerase, respectively (Promega Corp.). The 35S-radiolabeled probes were reduced to 200–300 ribonucleotides by alkaline hydrolysis. After hybridization, slides were washed at  $65^{\circ}\text{C}$  and autoradiographed for 4 days to 5 weeks at  $4^{\circ}\text{C}$ . Then they were counterstained by H&E or Giemsa staining. We established the specificity of the hybridization using a sense probe.

**In vitro passive sensitization assay using isolated conjunctival tissue.** Conjunctival tissue was collected surgically under operating microscope from naive mice and sensitized by incubation with anti-2,4-dinitrophenol-keyhole limpet hemocyanin (anti-DNP-KLH) IgE (50 ng/ml; Sigma-Aldrich) in HBSS for 1.5 hours at  $37^{\circ}\text{C}$ . After being washed 3 times, the conjunctival tissue was challenged in vitro with HSA-DNP; (1 mg/ml) at  $37^{\circ}\text{C}$ . Supernatants were collected 30 minutes after challenge and assayed for histamine ELISA (IBL Immuno-Biological Laboratories). For MIP-1 $\alpha$  or eotaxin-1 supplementation, they were added to media 15 minutes after challenge. The conjunctival samples were processed so that mast cells could be identified by metachromatic staining. Toluidine blue staining showed the same mast cell number and degranulation in the conjunctiva

as did Giemsa staining. Mast cell degranulation after in vitro challenge was confirmed to correlate with histamine release by morphological analysis after the samples were embedded in JB-4.

**Subconjunctival injection of MIP-1 $\alpha$ .** Five microliters of recombinant murine MIP-1 $\alpha$  (10 ng/ml in 0.1% BSA/PBS) was injected into naive mice subconjunctivally using a 30G needle attached to a Hamilton syringe. The LPS levels determined by the limulus amoebocyte lysate method were less than 0.1 ng/mg. At the indicated time point after injection, they were sacrificed, and the conjunctival samples were prepared for morphological analysis.

**Passive sensitization of CCR1-transfected RBL-2H3 cells.** RBL-2H3 cells expressing human CCR1 (19) were sensitized with anti-DNP IgE (100 ng/ml) and challenged with HSA-DNP (10 ng/ml) in vitro. For MIP-1 $\alpha$  supplementation, various concentration of recombinant human MIP-1 $\alpha$  were added to media 5 minutes after challenge. Supernatants were collected 20 minutes after addition of MIP-1 $\alpha$  and assayed for  $\beta$ -hexosaminidase ELISA. The  $\beta$ -hexosaminidase in the supernatants and cell lysate was quantified by hydrolysis of p-nitrophenyl-N-acetyl- $\alpha$ -D-glucopyranoside (Sigma-Aldrich) in 0.1 M sodium citrate buffer (pH 4.5) for 60 minutes at  $37^{\circ}\text{C}$ . The percentage of  $\beta$ -hexosaminidase release was calculated as previously described (21).

**Measurement of plasma exudation.** The plasma exudation by immediate hypersensitivity was assessed by the Evans blue (EB) dye extravasation method (21). For this purpose, unanesthetized mice were submitted to tail vein injection of 1% EB in PBS (6.7  $\mu\text{l/g}$  body wt) and challenged with allergen. Mice were sacrificed 90 minutes after the allergen exposure, and eyelids and conjunctival tissues were collected. The weighed tissues were extracted for EB in 500  $\mu\text{l}$  formamide for 24 hours. The extravasated EB concentration was determined by spectrophotometry at 620 nm based on a standard curve of EB in formamide.

**Statistical analysis.** Data are summarized as mean  $\pm$  SEM. Statistical analyses were performed by unpaired Student's  $t$  test (2-tailed), Mann-Whitney  $U$  test, or ANOVA as appropriate.

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