

Critical role of IgE-dependent mast cell activation in a murine model of allergic conjunctivitis

Ken Fukuda, MD, PhD,^a Masaharu Ohbayashi, PhD,^a Kei Morohoshi, MD, PhD,^a Lane Zhang, BS,^a Fu-Tong Liu, MD, PhD,^b and Santa J. Ono, PhD^a *Atlanta, Ga, and Sacramento, Calif*

Background: Allergic conjunctivitis is characterized by allergen-specific IgE in the serum and infiltration of eosinophils into the conjunctiva. The role of IgE and mast cells in allergic conjunctivitis is largely unknown, however.

Objectives: We investigated the importance of conjunctival mast cells in a murine model of IgE-mediated allergic conjunctivitis.

Methods: IgE-mediated allergic conjunctivitis was initiated in C57BL/6-*Kit*^{+/+} wild-type mice, mast cell-deficient *Kit*^{W-sh/W-sh} mice, and *Kit*^{W-sh/W-sh} mice that had been subconjunctivally or systemically engrafted with bone marrow-derived, cultured mast cells (BMCMCs) from *Kit*^{+/+} wild-type mice, and clinical symptoms and infiltration of eosinophil of the eyes were evaluated. Total numbers of mast cells in the conjunctiva were counted, and the phenotypes of these cells were characterized by means of immunostaining and PCR analysis of murine mast cell proteases.

Results: No mast cells were detected in the conjunctiva or eyelid dermis of adult *Kit*^{W-sh/W-sh} mice. Subconjunctival injection of BMCMCs resulted in local mast cell reconstitution, with the numbers of reconstituted mast cells in the conjunctiva and eyelid dermis comparable with those observed in wild-type *Kit*^{+/+} littermates. Reconstituted and naive conjunctival mast cells expressed proteases ascribed to connective tissue-type mast cells but not mucosal mast cells. Passive transfer of ragweed-specific IgE followed by antigen challenge resulted in both early-phase clinical symptoms and late-phase eosinophilic inflammation in *Kit*^{+/+} mice. These responses, which were significantly decreased in *Kit*^{W-sh/W-sh} mice, were restored on reconstitution of the conjunctival mast cell population.

Conclusions: These results suggest a direct contribution of IgE-activated mast cells to both the early-phase reaction and late-phase inflammation during ocular allergy. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Ocular allergy, mast cells, inflammation, IgE, eosinophil, bone marrow-derived mast cells, adoptive transfer, mast cell-deficient mouse

The occurrence of ocular allergy has increased in developed countries and now affects more than 20% of the US population.¹ Several clinical forms are included in ocular allergy, including seasonal and perennial allergic conjunctivitis, vernal keratoconjunctivitis, and atopic keratoconjunctivitis. The most common form of ocular allergy is seasonal allergic conjunctivitis, which involves sensitization/exposure to airborne allergens, such as ragweed and grass pollen. The perennial form, although less intense, is more of a chronic condition, which usually involves sensitization to antigens that are present year round, such as dust mites, animal dander, mold, and air pollutants. More severe types of allergic eye disease include vernal and atopic keratoconjunctivitis characterized by a severe late-phase reaction. Despite these differences in disease classification and incidence, the allergic response in the conjunctiva is typically elicited by exposure to an environmental allergen that causes cross-linkage of membrane-bound IgE, which triggers conjunctival mast cell degranulation, releasing a cascade of allergic and inflammatory mediators. In addition to IgE-mediated mechanisms, T cell- or other structural cell-mediated responses might be involved in vernal and atopic keratoconjunctivitis.²⁻⁵

Mast cells are widely distributed throughout the vascularized connective tissues, in particular near surfaces that are exposed to the environment, including the skin, airways, gastrointestinal tract, and conjunctiva.⁶ These cells have long been regarded as an important cell type involved in allergic diseases, such as dermatitis, asthma, and ocular allergy. Ocular allergic responses have 2 phases: an early phase and a late phase. During the early-phase response, conjunctival mast cells release preformed inflammatory mediators, such as histamine, tryptases, prostaglandins, and leukotrienes, in the seconds to minutes after allergen exposure. These molecules cause many of the acute inflammatory symptoms, such as tearing, redness, conjunctival edema, and itching. In the late-phase reaction inflammatory cells, mainly eosinophils, infiltrate into conjunctiva several hours after allergen exposure. There are, however, conflicting reports with regard to the roles of IgE and mast cells in murine models of allergic conjunctivitis and asthma. Whereas some reports have demonstrated that mast cell deficiency results in attenuated eosinophilic inflammation and early-phase responses, others have shown that allergic ocular inflammation can be induced in the absence of mast cells or antigen-specific IgE.⁷⁻¹¹

To determine the precise role of conjunctival mast cells during type I ocular allergy, we reconstituted mast cells in the conjunctiva of genetically mast cell-deficient *Kit*^{W-sh/W-sh} mice by means of adoptive transfer of bone marrow-derived, cultured mast cells (BMCMCs) from congenic wild-type C57BL/6J-*Kit*^{+/+} mice.

From ^athe Department of Ophthalmology, Emory University School of Medicine, Atlanta, and ^bthe Department of Dermatology, University of California, Davis, School of Medicine, Sacramento.

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Reprint requests: Ken Fukuda, MD, PhD, Department of Ophthalmology, Emory University School of Medicine, 1365B Clifton Rd NE, Atlanta, GA. E-mail: kfukud2@emory.edu.

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Abbreviations used

BMCMC: Bone marrow–derived, cultured mast cells
 CTMC: Connective tissue mast cell
 CPA: Carboxypeptidase A
 DMEM: Dulbecco modified Eagle medium
 FITC: Fluorescein isothiocyanate
 MMC: Mucosal mouse mast cell
 mMCP: Mouse mast cell protease
 RW: Short ragweed pollen
 TNP: Trinitrophenyl

We performed passive transfer experiments with antigen-specific IgE in wild-type *Kit^{W-sh/W-sh}* mice and mast cell–reconstituted mice. This approach provides an excellent means for analyzing the role of mast cells during IgE-mediated immediate hypersensitivity reactions and late-phase inflammation in the conjunctiva. Passive sensitization with antigen-specific IgE followed by antigen challenge resulted in immediate hypersensitivity and eosinophilic inflammation in wild-type mice and mast cell–reconstituted mice but not in *Kit^{W-sh/W-sh}* mice. This suggests that IgE-dependent mast cell activation is necessary for ocular allergy.

METHODS**Mice**

Mast cell–deficient *Kit^{W-sh/W-sh}* and C57BL/6J-*Kit^{+/+}* mice were from Jackson Laboratory (Bar Harbor, Me). The present study conformed to the principles for laboratory animal research outlined by the Animal Welfare Act; the Department of Health, Education, and Welfare (National Institutes of Health) guidelines; and the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. The study protocol was approved by the Emory University Committee on Animal Welfare.

Preparation and adoptive transfer of BMCMCs into mast cell–deficient mice

BMCMCs were obtained after 4 to 6 weeks according to a previously described method¹² with slight modification. In brief, bone marrow cells from the femurs of 3 *Kit^{+/+}* mice were pooled and cultured in a 75-cm² tissue-culture flasks containing 21 mL of BMCMC culture medium (Dulbecco modified Eagle medium [DMEM] supplemented with 5 ng/mL rIL-3, 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, nonessential amino acids, 50 μ mol/L 2-mercaptoethanol, and penicillin/streptomycin) for 2 days. Non-adherent cells were transferred into a new flask, and 21 mL of BMCMC culture medium was added. Cells were transferred into a new flask at least twice per week to remove the adherent cells from culture. Suspended cells were kept at an optimal concentration of 5×10^5 cells/mL for the duration of culture. After 4 weeks, cells were assessed for cytoplasmic granule appearance by using toluidine blue staining, and expression of both Fc ϵ RI and c-Kit was assessed by means of flow cytometry. Culture was continued until greater than 95% of evaluated cells were identified as BMCMCs and the required cell numbers for experiments were reached. For mast cell reconstitution studies, BMCMCs were transferred by means of subconjunctival (5×10^5 cells in 10 μ L of DMEM per eyelid) or tail-vein intravenous (1×10^7 cells in 200 μ L of DMEM) injection into female *Kit^{W-sh/W-sh}* mice. Six to 8 weeks after subconjunctival injection or 12 weeks after intravenous injection, mice were killed, and tissues were assessed for repair of mast cell deficiency.

IgE-mediated allergic conjunctivitis in mice

IgE-mediated allergic conjunctivitis was induced in mice by means of passive immunization with short ragweed pollen (RW)–specific IgE, followed

by challenge with RW applied to the conjunctiva in eye drops.¹³ Mice were intravenously injected with RW-specific IgE¹⁴ or trinitrophenyl (TNP)–keyhole limpet hemocyanin–specific IgE (BD Biosciences, San Jose, Calif). Twenty-four hours later, the mice were challenged with RW (Greer, Inc, Lenoir, NC) in PBS (2 mg in 10 μ L) by using eye drops. RW was from *Ambrosia artemisiifolia* and contained approximately 38 pollen grains per gram. Amb a 1 was present at 1698 μ g/g RW. Fifteen minutes later, clinical symptoms were evaluated, such as lid swelling, tear/discharge, chemosis, and redness, based on previously described criteria¹⁵ in a double-blind fashion. Two hours later, the eyes were harvested for RNA extraction and histologic analysis of mast cell degranulation. Some mice were challenged with RW again 24 hours after the first antigen challenge. Twenty-four hours after the second RW challenge, the conjunctivas were harvested and snap-frozen for eosinophil counting. See the **Methods** section in this article's Online Repository at www.jacionline.org for descriptions of additional methods used in this study.

Statistical analysis

Data are presented as means $\pm$ SEMs. Results were compared between 2 groups with the unpaired Student *t* test and among 3 or more groups by using the Dunnett test or Scheffe test with GraphPad Prism (GraphPad Software, Inc, La Jolla, Calif). A *P* value of less than .05 was considered statistically significant.

RESULTS***Kit^{W-sh/W-sh}* mice are profoundly deficient for conjunctival mast cells**

We first examined the presence of mast cells in ocular tissue from adult *Kit^{W-sh/W-sh}* mice using toluidine blue staining. We readily detected mast cells in the conjunctiva and eyelid dermis in *Kit^{+/+}* mice (Fig 1, A). Naive *Kit^{W-sh/W-sh}* mice, however, were devoid of mast cells in ocular tissues, such as the conjunctiva, eyelid, and choroid (Fig 1, B).

Repair of conjunctival mast cell deficiency in *Kit^{W-sh/W-sh}* mice by adoptive transfer of *Kit^{+/+}* BMCMCs

We next examined whether ocular mast cells could be reconstituted by means of subconjunctival or intravenous injection of BMCMCs. Cultured mast cells of C57BL/6-*Kit^{+/+}* mouse origin were harvested 4 to 6 weeks after the initiation of culture and injected subconjunctivally or intravenously (into the tail vein) into *Kit^{W-sh/W-sh}* mice. Six to 8 weeks after subconjunctival transfer of BMCMCs, mice displayed significant local repair of mast cell populations in both the conjunctival substantia propria and the eyelid dermis (Fig 1, C). The numbers of reconstituted mast cells in both the conjunctiva and the eyelid dermis were not significantly different from those observed in wild-type *Kit^{+/+}* littermates (Fig 2). Transfer of 1×10^6 or 2×10^6 BMCMCs per eyelid into the conjunctiva of *Kit^{W-sh/W-sh}* mice yielded similar numbers of reconstituted mast cells in both the conjunctiva and the eyelid dermis (data not shown).

Twelve weeks after intravenous tail-vein injection of BMCMCs, mast cells were observed in the stomachs, lung parenchyma, livers, and spleens of recipient *Kit^{W-sh/W-sh}* mice, as previously reported (see Fig E1 in this article's Online Repository at www.jacionline.org).^{12,16} Mast cell populations were observed in the eyelid dermis but not in the conjunctiva after systemic intravenous transfer of BMCMCs (Fig 1, D). The reconstituted mast cells did not distribute uniformly in the eyelid but tended to localize in the rear. The number of mast cells in the eyelid dermis was

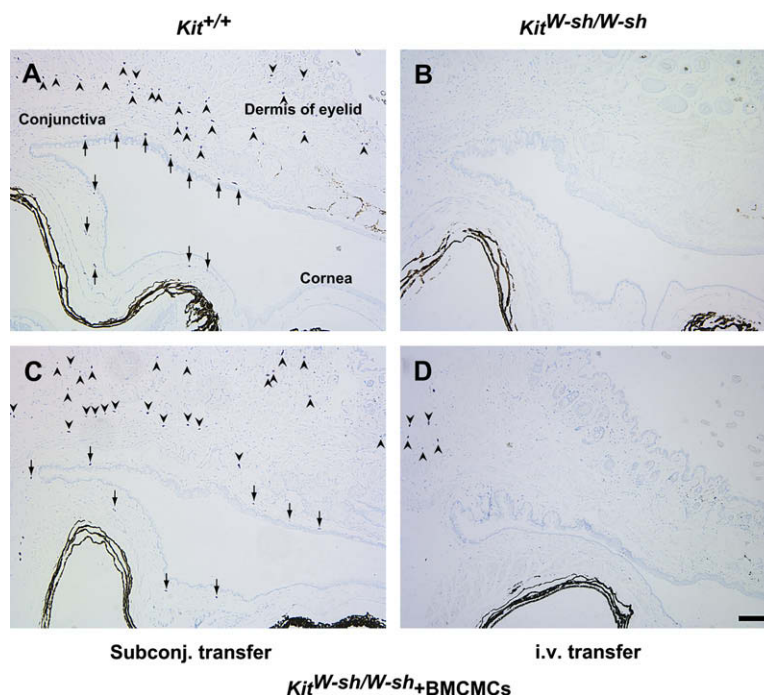


FIG 1. Representative toluidine blue-stained sections of eyes from C57BL/6-*Kit*^{+/+} (A), *Kit*^{W-sh/W-sh} (B), and mast cell-reconstituted *Kit*^{W-sh/W-sh} mice that had been subconjunctivally (C) or intravenously (D) injected with BMCMCs. Arrows, Mast cells in conjunctiva; arrowheads, mast cells in eyelid dermis. Scale bar = 100 μ m.

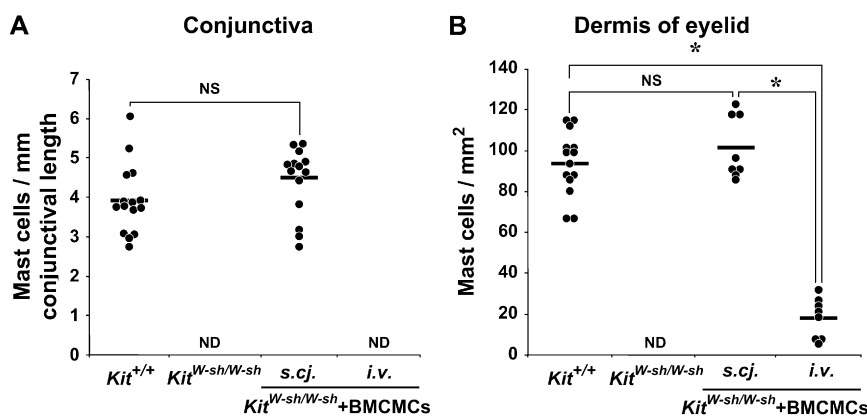


FIG 2. Mast cell numbers in conjunctiva and dermis of eyelids of C57BL/6-*Kit*^{+/+}, *Kit*^{W-sh/W-sh}, and *Kit*^{+/+} BMCMC-engrafted *Kit*^{W-sh/W-sh} mice. Dots represent the mast cell numbers from each mouse, and horizontal bars represent the overall mean values from all mice. ND, Not detected; NS, not significant. **P* < .001 (Scheffe test) versus indicated corresponding values. s.c.j., Subconjunctival; i.v., intravenous.

significantly less than observed with subconjunctival reconstitution and in wild-type *Kit*^{+/+} mice (*P* < .001, Fig 2).

Characterization of conjunctival mast cells

To determine the type of mast cell found at the ocular surfaces of these mice, we examined the phenotype of the mast cells in *Kit*^{+/+} and BMCMC-reconstituted *Kit*^{W-sh/W-sh} mice. Mast cell expression of neutral proteases typically ascribed to the mucosal mouse mast cell (MMC) subset (mouse mast cell protease [mMCP] 1 and mMCP-2) or to the connective tissue mast cell (CTMC) subset (mMCP-4, mMCP-5, mMCP-6, and

carboxypeptidase A [CPA]) was examined by means of immunohistochemistry and real-time PCR analysis. Strong reactivity to mMCP-4, mMCP-5, and mMCP-6 was observed in conjunctival and eyelid skin mast cells in wild-type *Kit*^{+/+} mice (data not shown), suggesting that these mast cells are CTMCs. No reactivity to mMCP-2 was detected in *Kit*^{+/+} mice, although mast cells in the gastric mucosa did show reactivity to mMCP-2 (data not shown). In BMCMC-reconstituted *Kit*^{W-sh/W-sh} mice, the reconstituted mast cells in the conjunctiva and eyelid dermis stained strongly for mMCP-4, mMCP-5, and mMCP-6, but not mMCP-2 (Fig 3), suggesting that these cells are phenotypically similar to those in *Kit*^{+/+} mice.

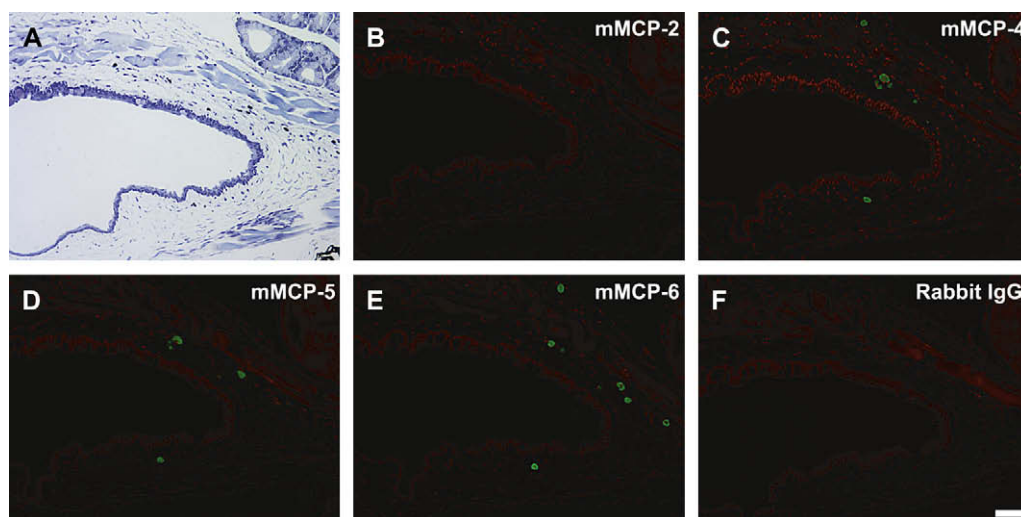


FIG 3. Characterization of conjunctival mast cells in *Kit*^{+/+} BMCMC-engrafted *Kit*^{W-sh/W-sh} mice. Reconstituted mast cells in the conjunctiva and dermis of the eyelid were positively stained with the antibodies for mMCP-4 (C), mMCP-5 (D), and mMCP-6 (E) but not with mMCP-2 (B). A, Toluidine blue staining. F, conjunctiva stained with normal rabbit IgG for negative control. Scale bar = 50 μ m.

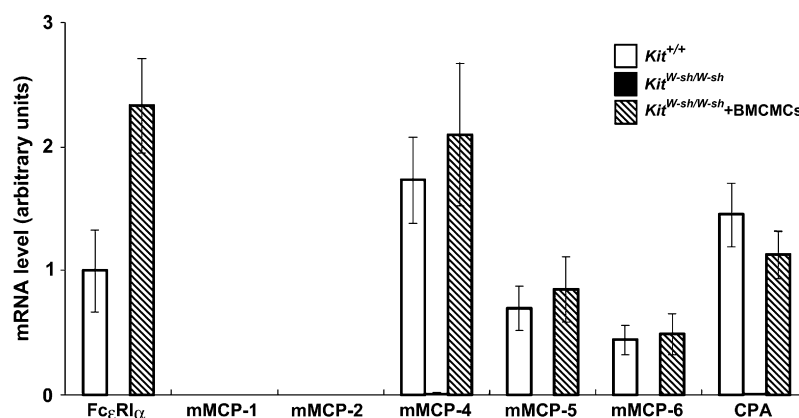


FIG 4. Expression of mRNAs for various mMCPs in conjunctiva of C57BL/6-*Kit*^{+/+}, *Kit*^{W-sh/W-sh}, and *Kit*^{W-sh/W-sh} BMCMC-engrafted *Kit*^{W-sh/W-sh} mice. Amounts of mRNA in the conjunctivas (n = 5 per group) were determined by means of reverse transcription and real-time PCR analysis. Levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase mRNA and are expressed as mean arbitrary units $\pm$ SEMs.

By using PCR analysis, we detected mRNA for FcεRIα and the CTMC proteases mMCP-4, mMCP-5, mMCP-6, and CPA but not the MMC proteases mMCP-1 and mMCP-2 in the eyelids of wild-type *Kit*^{+/+} mice (Fig 4). Transcripts for FcεRI and the mast cell proteases were undetectable in the conjunctiva of *Kit*^{W-sh/W-sh} mice. Transcripts for the CTMC proteases were detected in the conjunctiva of mast cell-reconstituted mice at similar levels to those of wild-type *Kit*^{+/+} mice, whereas mRNAs for MMC proteases were not detected after mast cell reconstitution (Fig 4).

Role of mast cells in IgE-mediated allergic conjunctivitis

Next, we determined whether the reconstituted mast cells were functionally capable of mediating ocular allergic reactions *in vivo*. IgE-mediated allergic conjunctivitis was induced in mice by means of passive immunization with RW-specific IgE, followed by RW or PBS challenge onto the conjunctiva by means

of eye drops. The severity of clinical symptoms, including conjunctival edema, conjunctival redness, tearing, and lid swelling, in the early-phase reaction was scored 15 minutes after the administration of RW or PBS eye drops. PBS challenge did not induce any clinical symptoms in these mice, regardless of the sensitization with TNP-IgE or RW-IgE (data not shown). As shown in Fig 5, wild-type mice sensitized with RW-specific IgE followed by RW displayed significantly more severe symptoms than mice sensitized with TNP-specific IgE, suggesting that these symptoms were due to an antigen-specific allergic reaction. The clinical scores for RW-IgE-sensitized *Kit*^{W-sh/W-sh} mice were significantly lower after allergen challenge than those for RW-IgE-sensitized wild-type mice. However, when RW-IgE-sensitized, mast cell-reconstituted *Kit*^{W-sh/W-sh} mice were challenged with RW, clinical signs of allergic inflammation were fully restored (Fig 5).

Because the degree of mast cell degranulation is linearly correlated with the clinical scores in wild-type, mast cell–

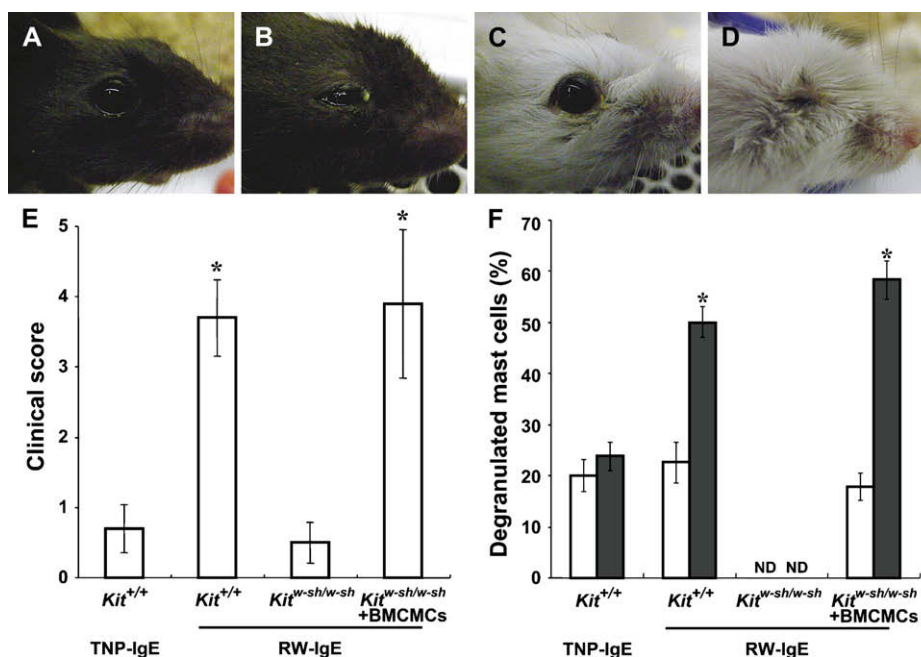


FIG 5. IgE-mediated early-phase clinical symptoms and mast cell degranulation in C57BL/6-*Kit*^{+/+} (A and B), *Kit*^{W-sh/W-sh} (C), and *Kit*^{+/+} BMCMC-reconstituted *Kit*^{W-sh/W-sh} mice (D). Clinical appearances (Fig 5, A-D) and scores (E, average $\pm$ SE) of early-phase clinical responses are shown. F, The ratio of degranulated mast cells in conjunctiva with passive sensitization by TNP- or RW-specific IgE followed by PBS (open bars) or RW (solid bars) challenge. ND, Not detected. * $P < .05$ versus corresponding values for C57BL/6-*Kit*^{+/+} mice sensitized with TNP-IgE.

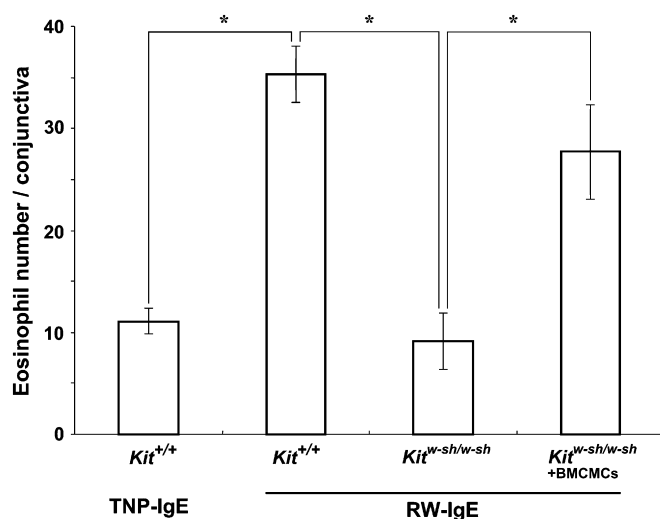


FIG 6. IgE-mediated late-phase inflammation in C57BL/6-*Kit*^{+/+}, *Kit*^{W-sh/W-sh}, and *Kit*^{+/+} BMCMC-reconstituted *Kit*^{W-sh/W-sh} mice. Mice were passively sensitized by means of transfer of TNP- or RW-specific IgE followed by RW challenge through eye drops. Twenty-four hours after antigen challenge, conjunctivas were harvested for histologic analyses. Infiltrating eosinophils were counted in the lamina propriae. The averages $\pm$ SEMs of 5 mice per group are shown. * $P < .05$ versus indicated values.

sufficient mice,¹⁷ we examined the ratio of degranulated mast cells in both *Kit*^{+/+} mice and BMCMC-reconstituted *Kit*^{W-sh/W-sh} mice. Mast cell degranulation in the conjunctiva, which was undetectable in *Kit*^{W-sh/W-sh} mice, was observed in both wild-type mice and BMCMC-reconstituted *Kit*^{W-sh/W-sh} mice. The RW-IgE-sensitized *Kit*^{+/+} mice had significantly more allergen-

specific mast cell degranulation than the TNP-IgE-sensitized mice (24% vs 50%, $P < .001$; Fig 5). The ratio of degranulated mast cells for BMCMC-reconstituted *Kit*^{W-sh/W-sh} mice was equivalent to that of RW-IgE-sensitized *Kit*^{+/+} mice. These results suggest that conjunctival mast cells are essential for early-phase clinical responses after allergen challenge and that reconstituted mast cells in the conjunctiva can induce early-phase reactions comparable with those observed in wild-type mice.

Finally, we examined the role of mast cells in late-phase conjunctival inflammation 24 hours after the last antigen challenge. PBS challenge did not increase the number of eosinophils in these mice, regardless of the sensitization with TNP-IgE or RW-IgE (data not shown). As shown in Fig 6, the number of infiltrated eosinophils after RW challenge was increased in RW-IgE-sensitized *Kit*^{+/+} mice compared with the number seen in those sensitized with TNP-IgE. No increase in conjunctival infiltration of eosinophils was observed for RW-IgE-sensitized and RW-challenged *Kit*^{W-sh/W-sh} mice. In RW-IgE-sensitized, mast cell-reconstituted *Kit*^{W-sh/W-sh} mice, the eosinophil infiltration response was restored. These results suggest that mast cells are essential not only for the early-phase reaction but also for late-phase inflammation in IgE-mediated allergic conjunctivitis.

DISCUSSION

Our data demonstrate that *Kit*^{W-sh/W-sh} mice exhibit a profound mast cell deficiency in ocular tissue and that they can be selectively repaired of this deficiency by means of subconjunctival injection of genetically compatible BMCMCs. The locally injected BMCMCs display the tissue distribution, numbers, and phenotypic characteristics of conjunctival mast cell populations in wild-type mice. Using

a passive sensitization model with transfer of antigen-specific IgE into wild-type, $Kit^{W-sh/W-sh}$, and mast cell–reconstituted $Kit^{W-sh/W-sh}$ mice, we have shown that conjunctival mast cells play critical roles not only in the early-phase reaction but also in late-phase inflammation during IgE-mediated allergic conjunctivitis *in vivo*.

The importance of mast cells in the development of allergen-induced hypersensitivity and inflammation associated with allergic conjunctivitis in mice has been controversial. For example, eosinophil infiltration can be elicited in murine models in the absence of IgE antibodies^{7,18} or mast cells.¹¹ However, it is also true that ocular allergic symptoms and late-phase reactions can be induced in mice by means of IgE-dependent^{8,13,19} and mast cell–dependent^{9,10} mechanisms. Similarly, there are some conflicting articles regarding the role of mast cells in murine models of allergic asthma.^{20–24} Notably, the extent to which mast cells can contribute to the hyperreactivity and inflammation associated with models of allergic conjunctivitis and asthma in mice is highly dependent on the specific experimental model investigated. In studies in which mice were sensitized with antigen mixed with an artificial adjuvant, such as aluminum hydroxide, mast cell–deficient mice did not display a significant reduction in allergic responses compared with wild-type mice.^{11,22,23} The sensitization protocol using aluminum hydroxide can promote a strong “nonspecific” antibody response or have other effects that can influence features of the asthma or conjunctivitis model, thereby masking the contribution of mast cells to allergic responses. A sensitization and challenge protocol using low doses of antigen without adjuvant has been used for a murine model of IgE- and mast cell–mediated asthma,^{21,24} but no such technique has previously been established for a murine model of ocular allergy. Our procedure of transferring antigen-specific IgE into genetically mast cell–deficient, congenic normal, and mast cell–reconstituted mice presents a powerful tool for examining mast cell function in allergic conjunctivitis. Using this model, we found that mast cells play a critical role in various features of allergic conjunctivitis. We hope that a similar model for chronic model ocular allergy that exhibits features of human vernal keratoconjunctivitis will be established in the near future.

Although mice that exclusively lack mast cells have not been reported, Kit mutant mice, which are deficient in mast cells but have several other phenotypic abnormalities, can be used to analyze the *in vivo* functions of mast cells. The most commonly used animals are the WBB6F1- $Kit^{W/W-v}$ mice and the more recently characterized $Kit^{W-sh/W-sh}$ mice. Both of these types of mice are profoundly deficient for mast cells and melanocytes.¹² We have previously reported that $Kit^{W/W-v}$ mice lack conjunctival mast cells and that conjunctival mast cells can be reconstituted by locally injecting mature, connective tissue–type mast cells isolated from conjunctiva of wild-type mice.⁹ $Kit^{W/W-v}$ mice have several other phenotypic abnormalities, such as anemia, a decrease in the number of neutrophils, development of stomach papillomas and ulcers, development of idiopathic dermatitis, a deficiency in intraepithelial lymphocytes in the small intestine, and sterility. In contrast, $Kit^{W-sh/W-sh}$ mice do not have these abnormalities.¹² Because the Kit -related phenotypic abnormalities that affect lineages other than mast cells are generally milder in $Kit^{W-sh/W-sh}$ mice than in $Kit^{W/W-v}$ mice and because $Kit^{W-sh/W-sh}$ mice are fertile and on the well-studied BL6 background, we chose this strain to elucidate the roles of mast cells *in vivo*. Like $Kit^{W/W-v}$ mice,²⁵ $Kit^{W-sh/W-sh}$ mice can be reconstituted with mast cells with normal c-kit function through adoptive transfer of such cells

into the peritoneal cavity or through the intradermal or intravenous routes.^{12,16,26} We have also confirmed that ocular mast cells could be reconstituted by retro-orbital intravenous injection of BMCMCs in both $Kit^{W/W-v}$ and $Kit^{W-sh/W-sh}$ mice (Aye et al, unpublished data). In this article we have first shown that conjunctival mast cells in $Kit^{W-sh/W-sh}$ mice can be selectively restored by local adoptive transfer of *in vitro* derived immature mast cells. Consistent with previous reports,^{12,16} intravenous transfer of BMCMCs through the tail vein in $Kit^{W-sh/W-sh}$ mice resulted in mast cell repopulation of the lung, stomach, spleen, and liver, but no mast cells were found in conjunctiva by using this delivery technique. Furthermore, systemic intravenous transfer of BMCMCs through the tail vein resulted in significantly lower numbers of mast cells in the dermis of the eyelid than those observed with local subconjunctival reconstitution. These results are similar to those of a previous study in which intravenous transfer of BMCMCs resulted in significantly lower numbers of mast cells in the dermis of back skin than those observed with local intradermal reconstitution.¹² It is therefore better to use local subconjunctival transfer to reconstitute mast cells for the analysis of ocular allergy.

Mast cells are a heterogeneous population of tissue-resident bone marrow–derived cells; distinct mast cell subpopulations are situated at specific microanatomic locations.²⁷ In human subjects 2 types of mast cells have been identified on the basis of protease expression. One phenotype is positive only for tryptase, designated MCT, and the other is positive for both tryptase and chymase, designated MCTC. Most of the human mast cells found in the conjunctiva and eyelid skin are reported to be MCTC.⁶ Murine mast cells are divided into 2 subgroups: MMC and CTMC. Murine MMCs preferentially express the chymases mMCP-1 and mMCP-2, whereas murine CTMCs preferentially express a different combination of the proteases, including the chymases mMCP-4 and mMCP-5 and the tryptases mMCP-6 and CPA. This is the first detailed description of the phenotype of murine conjunctival mast cells. We show that conjunctival mast cells, both wild-type and reconstituted, display a CTMC phenotype, expressing mMCP-4, mMCP-5, mMCP-6, and CPA but not mMCP-1 or mMCP-2.

In summary, our data show that $Kit^{W-sh/W-sh}$ mice are profoundly deficient in ocular mast cells and that mast cell populations in ocular tissue can be restored by means of local injection of wild-type BMCMCs. These reconstituted conjunctival mast cells have phenotypic and functional characteristics identical to those of naive conjunctival mast cells. This model might therefore represent a useful tool for analysis of mast cell function in ocular allergy. Because the reconstituted mast cells can be derived from mice deficient for genes of interest, this technique will allow *in vivo* analysis of the roles of specific mediators in mast cell function during ocular allergy.

Key message

- We define the direct contribution of IgE-activated mast cells to both the early-phase reaction and late-phase inflammation during ocular allergy using mast cell reconstitution in $Kit^{W-sh/W-sh}$ mice.

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METHODS

Reagents

DMEM, FBS, PBS, L-glutamine, sodium pyruvate, nonessential amino acids, and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, Calif), and cell-culture dishes were from Becton Dickinson (Franklin Lakes, NJ). Mouse recombinant IL-3 was from Peprotech (Rocky Hill, NJ). The reverse transcription kit and QuantiTect SYBR Green PCR kit were from Qiagen (Valencia, Calif). RW was from Greer, Inc.

Staining and quantification of mast cells

Mice were killed, and eyes (including eyelids), ear pinnae, lungs, spleens, stomachs, and livers were fixed in 4% paraformaldehyde and embedded in paraffin, ensuring a cross-sectional orientation of all tissues. Sections were cut to a thickness of 4 μ m. Mast cells were stained metachromatically with 0.1% toluidine blue. Mast cells were quantified according to area (in square millimeters for eyelid dermis) or expressed in numbers per millimeter length (conjunctiva) by using computer-generated image analysis (Image Pro Plus; Media Cybernetics, Silver Spring, Md).

For immunostaining of mMCPs, paraffin sections (4 μ m) were deparaffinized. Slides were then incubated with epitope retrieval solution (Dako, Glostrup, Denmark) at 95°C for 40 minutes. After incubation with 10% normal rabbit serum for 30 minutes at room temperature, sections were treated overnight at 4°C with rabbit polyclonal antibodies to mMCP-2, mMCP-4, mMCP-5, and mMCP-6 (generous gifts from Dr Michael F. Gurish, Brigham and Women's Hospital and Harvard Medical School). Control tissues were treated with the same concentrations of normal rabbit IgG. Immunoreactivity was detected with Alexa488-conjugated goat anti-rabbit IgG (Invitrogen) incubated for 1 hour at room temperature. Sections were mounted with VECTASHIELD mounting medium with propidium iodide (Vector Laboratories, Burlingame, Calif). Images were captured with a fluorescence microscope (BX51; Olympus, Center Valley, Pa).

Flow cytometric analysis

Cells were blocked with unconjugated anti-Fc γ RII/III (2.4G2; BD PharMingen, San Diego, Calif) and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-Fc ϵ R1 α , FITC-conjugated anti-c-kit, or FITC-conjugated-isotype-matched control immunoglobulin (eBioscience, San Diego, Calif). Cells were analyzed by using FACSsort (BD Biosciences), and data were analyzed with Flow Jo Software (TreeStar, San Carlos, Calif).

Quantitative RT-PCR

RNA was extracted from conjunctival tissues by using TRIzol reagent (Invitrogen), and cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen). Relative expression levels of RNAs were determined by using real-time PCR with the QuantiTect SYBR Green RT-PCR kit (Qiagen) and an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif), according to the manufacturer's instructions. Primers corresponding to mMCPs and glyceraldehyde-3-phosphate dehydrogenase were described previously.^{E1} Other primers were from Qiagen. Relative RNA expression was determined by using the comparative method of relative quantification (2^{-DDCt}) and normalized to glyceraldehyde-3-phosphate dehydrogenase levels.

Immunoperoxidase staining for eosinophils

The extent of eosinophil infiltration in the conjunctiva was determined as described previously.^{E2} Briefly, the eyes were immediately frozen in Optimal Cutting Temperature compound (Sakura Finetek USA, Torrance, Calif). Tangential frozen sections 8 μ m thick were cut and fixed by using 30% methanol in acetone for 10 minutes at 4°C. After blocking with 10% normal goat serum in PBS for 30 minutes at room temperature, sections were incubated with rat anti-mouse major basic protein (a generous gift from Dr Lee; Mayo Clinic, Rochester, Minn) overnight at 4°C in a humidity chamber. After washing with PBS, endogenous peroxidase activity was blocked with 1% H₂O₂ in 100% methanol for 20 minutes at room temperature. Sections were washed again with PBS and then incubated with horseradish peroxidase-labeled secondary antibody by using the Histofine Simple Stain Mouse MAX PO (Rat) kit (Nichirei Biosciences, Tokyo, Japan). Horseradish peroxidase localization was visualized by means of incubation with a diaminobenzidine in TRIS-buffered saline and counterstained with methyl green. Infiltrating eosinophils in the lamina propria mucosae of tarsal and bulbar conjunctivas were counted by a blinded observer using the bright field setting of the microscope (BX51, Olympus).

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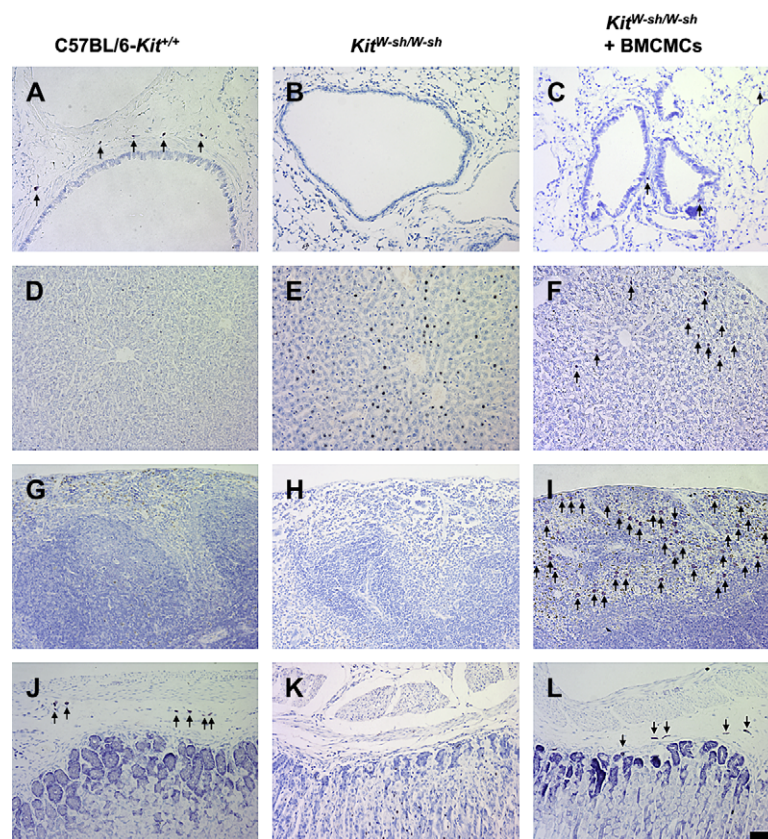


FIG E1. Representative toluidine blue–stained sections of lungs (A–C), livers (D–F), spleens (G–I), and stomachs (J–L) from C57BL/6-*Kit*^{+/+} (Fig E1, A, D, G, and J), *Kit*^{W-sh/W-sh} (Fig E1, B, E, H, and K), and mast cell–reconstituted *Kit*^{W-sh/W-sh} (Fig E1, C, F, I, and L) mice that had been intravenously injected with BMCMCs. Arrows, Mast cells. Scale bar = 100 μ m.