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IL-10 confers protection from mast cell degranulation in a mouse model of allergic conjunctivitis

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Abstract

IL-10 is a regulatory cytokine known to inhibit allergic and inflammatory events. Mast cells (MC) are effector cells which when stimulated release histamine, chemokines and cytokines that initiate the allergic inflammatory response. Recent studies have shown that IL-10 regulates MC function by affecting cytokine production and expression of FcεR1 in in vitro assays. Using IL-10 knockout (IL10KO) mice, we examined the effect of its absence on MC susceptibility to degranulation by the basic secretagogue, Compound 48/80 (C48/80). C48/80 is a receptor mimetic that directly activates G proteins and stimulates vigorous MC degranulation. For these studies we stimulated conjunctival MC with C48/80 and found that conjunctival MC of IL10KO mice exhibit increased degranulation compared with wild type mice. Reconstitution of IL10KO mice by adding rIL-10 twenty-four hours prior to challenge with C48/80 conferred increased resistance of MC to the degranulatory effects of C48/80. The protective effect therefore appears to be due to the presence of IL-10. This is the first in vivo rodent study which reports a novel role of IL-10 in stabilizing mast cells from degranulation by a secretagogue, by as of yet an unknown mechanism.

Keywords: allergic conjunctivitis; mast cell; mouse model; compound 48/80

Ocular allergy is a spectrum of clinical diseases characterized by an immunopathological reaction of ocular tissues to external stimuli. The incidence of allergic diseases, including ocular allergies has been increasing in the past 30 years. A recent study revealed that 35% of families surveyed have experienced allergic reactions, with more than 50% of this group reporting associated eye symptoms (Stahl et al., 2004).

Mast cell activation and degranulation is central to the immediate response in allergic eye disease. The common pathway of mast cell activation and degranulation is through the crosslinking of specific IgE bound to FcεRI receptors. A second pathway, the response to basic secretagogues such as Compound 48/80 (C48/80) and substance P has also been described (Lagunoff et al., 1983). These secretagogues bypass the IgE triggering pathway and act as receptor mimetic agents, effecting mast cell degranulation by directly activating the Gi proteins that control exocytosis (Aridor et al., 1990; Mousli et al., 1990). While the inflammatory changes and the clinical manifestations are similar for both pathways, the signaling pathway used by C48/80 for evoking degranulation and release of class I mediators (e.g. histamine) appears different compared to IgE crosslinking. Histamine release is faster with C48/80 induction and does not require the presence of external Ca^{2+} or Syk kinase (Sagi-Eisenberg, 2002). However, the two pathways appear to converge when stimulating production and release from mast cells of class II mediators (e.g. leukotrienes) and possibly class III mediators (e.g. cytokines) (Sagi-Eisenberg, 2002). For reasons that are still unclear, these secretagogues have been shown to activate connective-tissue type, but not mucosal mast cells. Mast cells show both physical and functional heterogeneity within a single species (Metcalf, et al. 1997).

In humans, mast cells are divided into subsets that contain tryptase (MC_T) and those that contain tryptase and chymase (MC_{TC}) (Irani et al., 1989). In rodents, mucosal mast cells contain the protease chymase, whereas connective tissue mast cells have chymase, tryptase and other proteases (Befus et al., 1995). Human MC_{TC}, like the rodent connective tissue mast cells, are susceptible to the degranulating effect of C48/80.

Compound 48/80 is a mixed polymer of phenethylamine cross-linked by formaldehyde (Lagunoff et al. 1983). Baltzly et al. (1949) synthesized C48/80 as a hypotensive agent. The effect was later discovered to be due to the release of histamine by the degranulating mast cells (Paton, 1951). C48/80 was previously reported to mimic allergic conjunctivitis in the mouse model (Li et al., 1996). The resulting inflammatory changes have been associated with histamine release and increased nitric oxide production (Allansmith et al., 1989; Meijer et al., 1996; Ko et al., 2000; Tiligada et al., 2000). A high concentration of this compound can induce almost a 90% release of histamine from mast cells, compared to antigen-antibody-complement induced histamine release which rarely reaches 50% (Johnson et al., 1969). Mast cells in the conjunctiva are located beneath the epithelium and adjacent to small venules and are particularly susceptible to C48/80 degranulation since they are predominantly of the connective tissue type (Cook et al., 2001; Li et al., 1996; Irani et al., 1990). The use of C48/80 to induce and mimic allergic disease in the mouse allows the investigator to directly evaluate the contribution of mast cells to the allergic eye disease process without any confounding factors associated with IgE crosslinking.

Interleukin-10 (IL-10) has been demonstrated to suppress allergic and inflammatory events, and has been reported to regulate mast cell function, development, proliferation and protease gene expression (Ghildyal et al., 1993; Thompson-Snipes et al., 1991). In addition, IL-10 has been shown to inhibit Kit expression and function, and FcεRI expression in murine mast cells (Gillespie et al., 2004; Mirmonsef et al., 1999). The nature and number of signal transduction pathways and kinases acted upon by IL-10 are not yet clear, and the mitogen-activated protein kinase (MAPK) components are currently being investigated as potential targets (Haddad et al., 2003).

The purpose of the study presented here is to examine the effect of IL-10 on regulating the early stages of allergic inflammation, specifically on the effector mast cell population. To test this: C57BL mice genetically deficient for IL-10 (IL10KO) and C57BL wild type (WT) control mice (Taconic, Germantown, NY) were used. The studies reported here conform to the principles for laboratory animal research outlined by the Animal Welfare Act (NIH/DHHS) guidelines for the experimental use of animals and were approved by the National Institutes of Health Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experiments were performed a minimum of three separate times using 4-8 mice per group in each experiment.

To assess IL-10 production in these strains, splenocytes were cultured as reported by Kitagaki et al. (2006), stimulated with either media or Con A (5ug/ml; SigmaChemical, St. Louis, MO) and supernatants harvested after 48 hours. Murine IL-10 was measured by ELISA (OptEIA, BD Biosciences, San Diego, CA) according to

manufacturer's instructions. IL-10 was detected in the WT strain with stimulation by media, and was elevated with the non-specific T-cell mitogen Con A. As expected, the IL10KO strain did not produce IL-10 under any conditions tested (data not shown).

In preparation for ocular challenge, the mice were anesthetized with 90 mg/kg ketamine (Ketaset, 100 mg/ml, Fort Dodge, IA) and 10 mg/kg xylazine (Xyla-Ject, Phoenix Pharmaceutical, St. Joseph, MO) given intraperitoneally. Five minutes later, either phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA) or C48/80 (Sigma Chemical, St. Louis, MO) was applied to the surface of each eye. For C48/80, two mg in a 5 μ l volume were delivered in each eye, as has been previously reported (Li et al., 1996). After one hour, the animals were sacrificed, and their eyes fixed *in situ* with 10% formalin (EMD Chemicals, Gibbstown, NJ) prior to enucleation to prevent further mast cell degranulation and goblet cell secretion due to mechanical manipulation of the eye during removal. Eyes were immediately fixed in 10% formalin to preserve goblet cells and to prevent additional non-specific mechanical mast cell degranulation prior to removal. The eyes with lids intact including the bulbar, fornical and tarsal conjunctivae were removed and placed in 10% formalin for a minimum of 24 hours prior to further processing. Five sagittal sections of conjunctival tissue with approximate 4-um thickness from each individual mouse were stained with Giemsa (American Histolabs, Gaithersburg, MD) and examined by light microscopy at 40-1000x.

The eyes of the WT and IL10KO mouse strains before and after instillation with C48/80 can be seen in Figure 1, top row. Twenty minutes after ocular challenge with C48/80, ocular symptoms such as chemosis, blepharitis and mucous discharge were more

pronounced in the susceptible IL10KO compared to the relatively resistant WT mice. The pathologic changes in IL10KO mice were maintained up to 48 hours after challenge. Control mice which received vehicle alone (PBS) in all strains did not develop any clinical manifestations (Figure 1). Histological examination (middle and bottom rows) revealed marked mast cell degranulation and conjunctival edema in susceptible mice after challenge with C48/80 (Figure 1).

Since IL-10 has been shown to regulate mast cell function, development and proliferation in vitro (Ghildyal et al., 1993; Thompson-Snipes et al., 1991) the baseline number of intact and degranulating mast cells was evaluated in naïve (data not shown) and PBS challenged mice (Figure 2) for each mouse strain. Briefly, mast cells within a 400x magnification area close to the conjunctival fornix were counted for both the upper and lower eyelids of both eyes by an observer masked to the group's treatments. There was no significant difference between naïve mice and animals post-PBS challenge in any of the mouse strains (data not shown). However, results show that IL10KO mice are more susceptible to C48/80-induced degranulation compared with control WT animals, as IL10KO ($p < 0.001$) mice had significantly fewer intact mast cells compared with their respective PBS-challenged controls. Conversely, WT mice treated with C48/80 did not have any significant changes in intact mast cell numbers ($p > 0.05$) (Figure 2), suggesting that the C57BL background may be resistant to C48/80 degranulation.

To further evaluate differences in mast cell degranulation between mouse strains in vitro, we attempted to culture mast cells from these strains. Bone marrow cells were obtained from their femurs and were cultured for 21 days at 37°C in bone marrow-

derived mast cell (BMMC) media. This media is RPMI-1640 (Invitrogen) supplemented with rIL-3 (Peprotech, Rocky Hill, NJ) at 30 ng/ml and culture conditions and identification of cells were done as previously described (Razin et al., 1983). Bone marrow cells from both WT and IL10KO strains developed into BMDCs and were assayed for β -hexosaminidase release after C48/80 challenge. The release of β -hexosaminidase was assayed by hydrolysis of p -nitrophenyl- β -D-glucosamide (Sigma, St. Louis, Missouri) as previously described (Schwartz et al., 1979). Plates were read using the Spectramax colorimetric plate reader at 405 nm (Molecular Devices, Sunnyvale, CA). Mast cell degranulation in the IL10KO strain did not show a significant difference from WT using this assay (data not shown), suggesting that there is something intrinsic to the IL-10 deficiency in vivo that is either lacking (or compensated for) in our in vitro BMMC assays. The combination of rIL-4 and SCF has been shown by Karimi et al., (1999) to favor growth of connective tissue-like MC, which are the MC type that degranulate with exposure to C48/80. Culturing bone marrow cells in RPMI-1640 supplemented with rIL-4 (Peprotech) at 600 ng/ml and stem cell factor (Peprotech) at 50 ng/ml, gave similar results as studies completed with media supplemented with IL-3. This inability of in vitro culture to mimic in vivo conditions may be due to the absence of unknown factor(s), and warrants further study. Arock et al., (1996) saw similar discordant results employing in vitro assays using rIL-10 and IgE crosslinking in mast cells from Balb/c mice. An additional shortcoming of in vitro culture that we noted is that the BMMC media supplemented with rIL-3 does not support growth and development of mast cells from bone marrow cells of the A/J mouse strain, suggesting

that the in vitro culture conditions we use do not essentially generate a mature mast cell that will act the same way as it does in vivo.

Since IL-10 appears to confer a protective effect from mast cell degranulation by C48/80, we performed IL-10 reconstitution experiments on the IL10KO strain. Either PBS or rIL-10 (Sigma, St. Louis, Missouri) at 10 to 100 ng/mouse were given to these groups of mice intraperitoneally at a volume of 100 μ l and ocular challenge with C48/80 given the next day. No gross morphological or cellular changes were noted in the ocular tissues when 10 - 100 ng of rIL-10 were administered (data not shown). Twenty-four hours after administration of rIL-10, the baseline number of conjunctival mast cells was not significantly affected in the IL10KO strain (data not shown). Mice that were given PBS exhibited the same pattern of response to C48/80 ocular challenge as in previously mentioned experiments (Figure 3). However, mice that received 100 ng of rIL-10 one day prior to challenge showed a significant decrease ($p < 0.05$) in susceptibility to C48/80-induced degranulation compared with those who did not receive rIL-10, as seen by the increased average number of intact mast cells (Figure 3).

Li et al. (1996) have shown that ocular application of C48/80 in mice can induce a pathology mimicking human allergic conjunctivitis. The amount of inflammation was also shown to vary among the different strains of mice. The cytokine IL-10 has pleiotropic effects mainly directed at regulating both the inflammatory and allergic response. As might be expected, IL-10 has been shown to regulate mast cell function. Recent studies indicate that IL-10 may be a major component of Th2-mediated mast cell homeostasis; largely through its ability to block c-Kit and Fc ϵ RI expression, and

induction of mast cell apoptosis along with IL-4 (Gillespie et al., 2004; Bouton et al., 2004). Royer et al. (2001) demonstrated that IL-10 can inhibit IgE-dependent activation of human mast cells, although this has not been observed in rodent mast cells. We now report, in an in vivo rodent model, that IL-10 can also inhibit activation of mast cells by a basic secretagogue C48/80.

Our studies indicate that mast cells from the Th1 polarized C57BL WT strain, are innately more resistant to C48/80-induced MC activation than previously published Th2 polarized mice (Li et al., 1996; Toda et al., 1989; Jacobs et al., 1996; Shibuya et al., 1998; Heinzl et al., 1989). Depletion or absence of IL-10 does not affect mast cell numbers in the conjunctiva at baseline, yet appears to cause a substantial increase in mast cell response to C48/80-induced degranulation. In vivo administration of rIL-10 in the IL-10 knockout mouse can significantly decrease MC susceptibility to degranulation. This function of IL-10 may be one among the cumulative effects that this cytokine has in regulating the allergic inflammatory response.

The molecular mechanism by which IL-10 modifies mast cell degranulation is still not well understood. This is the first in vivo study which reports a novel role of the cytokine IL-10 in stabilizing mast cells from degranulation by a secretagogue. These findings suggest IL-10 may prove to be a potent therapeutic for ocular allergic disease and other diseases such as mastocytosis that are dependent upon mast cell degranulation. Further studies to understand this mechanism will illuminate a different down-modulating effect of this cytokine on the immune response, this time early on in a key player of the allergic inflammation process.

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Figure legends

Fig. 1 Top row : Mouse eyes after challenge with either PBS or C48/80. When challenged with C48/80, the mouse develops a transient corneal opacity that resolves within 24 hours.

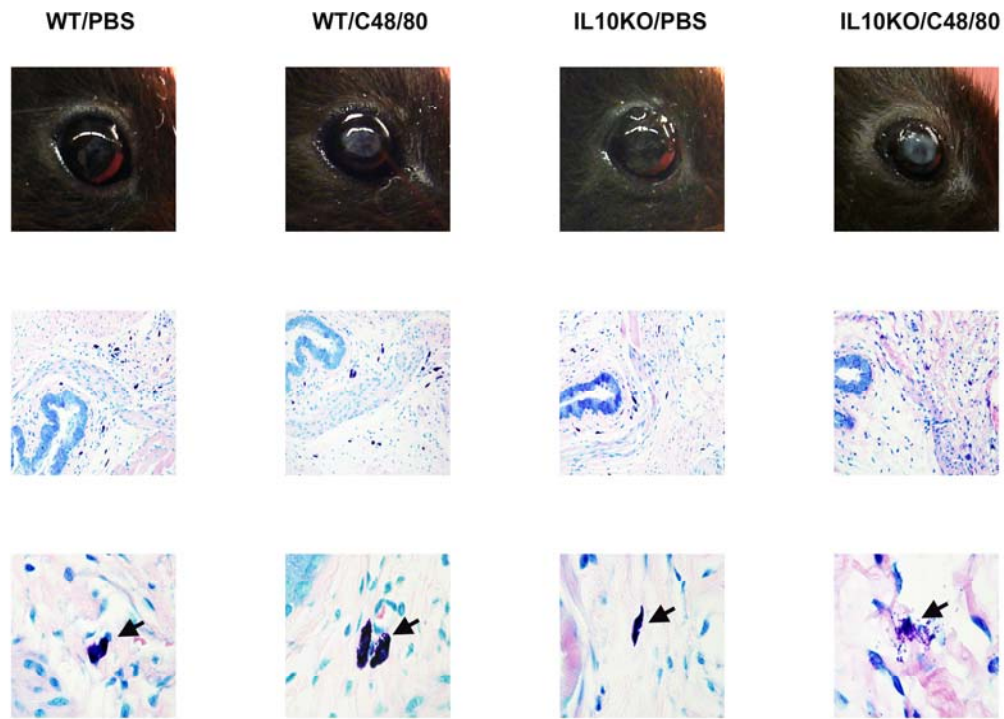
Middle row: Photomicrographs of the conjunctiva 1 hr after installation with C48/80. Giemsa. 100x.

Bottom row: Photomicrographs of the conjunctiva, 1000x. The arrows point to the mast cell in the conjunctival tissue. Note the exocytosis of secretory granule contents from the mast cell from the IL10KO mouse challenged with C48/80.

Fig. 2. Results of ocular challenge with either PBS or C48/80 on different mouse strains. Results are graphed with Graph Pad Prism (Graph Pad, San Diego, CA).

* $p < 0.001$, ANOVA analysis (Tukey post test) comparing WT with KO after C48/80 challenge. Error bars denote $\pm$ std. error.

Fig 3. Results of ocular challenge in IL-10KO mice with either PBS or C48/80. Mice were given either PBS or varying doses of rIL-10. * $p < 0.05$, ANOVA analysis (Tukey post test). Error bars denote $\pm$ std. error.



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