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Epithelium-derived IL-33 activates mast cells to initiate neutrophil recruitment following corneal injury.

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Abstract

Purpose: Neutrophils play a critical role in defending against threats such as microbial infection, yet their activation during innate immune response incites collateral damage to healthy tissues. We have previously shown that corneal injury induces mast cells to express the neutrophil chemoattractant CXCL2. Here we delineate the mechanism of injury-induced, non-IgE-mediated mast cell activation at the ocular surface.

Methods: Corneal injury was induced by mechanical removal of the epithelium and anterior stroma in mast cell deficient ($cKit^{W\text{-}sh}$) and C57BL/6 mice using Algerbrush II. Corneas were analyzed for frequencies of total $CD45^+$ inflammatory cells, $CD11b^+Ly6G^+$ neutrophils, and $cKit^+Fc\varepsilon R1^+$ mast cells using flow cytometry. Mast cells were stimulated with different inflammatory factors known to increase during corneal injury (IL-33, IL-1β, IL-36γ, IL-6, SDF1α and Substance P) and assessed for the secretion of β-hexosaminidase, tryptase and CXCL2 using ELISA. IL-33 neutralizing antibody (1 mg/ml) was administered locally for mast cell inhibition *in vivo*.

Results: Mast cell deficient mice failed to recruit early neutrophils to the injured corneas. IL-33 stimulation upregulated CXCL2 secretion by mast cells. Corneal injury resulted in amplified expression of IL-33 at the cornea and epithelium was identified as its primary source. Topical neutralization of IL-33 at the ocular surface inhibited mast cell activation, limited neutrophil infiltration and reduced corneal inflammatory haze, normalizing tissue architecture following ocular injury.

Conclusions: These data implicate IL-33 in mast cell activation and early neutrophil recruitment in non-allergic inflammation, suggesting IL-33 as a potential therapeutic target in inflammatory disorders of the ocular surface.

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Author contributions:

E.E. assisted in designing the study, performed *in vivo* and *in vitro* experiments, analyzed the data and wrote the manuscript. S.K.M. assisted in designing experiments, analyzed the data, and wrote the manuscript. W.F. and W.C. assisted in manuscript writing. S.K.C. contributed the underlying hypothesis, designed the study, analyzed data and wrote the manuscript.

Introduction

Mast cells are long-lived tissue-resident immune cells that are distributed throughout vascularized tissues in the body, and are particularly abundant at mucosal surfaces that interface with the outside environment (e.g. skin, airways and gastrointestinal tract).[1,2] Mast cell activation, for example in the setting of allergy or host defense to parasitic infection, results in the rapid release of preformed and *de novo* synthesized inflammatory mediators into the extracellular environment.[3,4] These mediators include various cytokines, growth factors, amines, and enzymes such as tryptase and β-hexosaminidase.[3,5] Yet beyond their well-established role as effector cells in pathogenic IgE- and IgG-mediated responses, mast cells are now recognized for their critical function in modulating both innate and adaptive immunity, particularly during the early phases of the inflammatory response.[6-9] Work from our own laboratory has shown that following corneal trauma, tissue-resident mast cells initiate neutrophil recruitment to the site of injury via secretion of the CXC chemokine receptor 2-binding chemoattractant CXCL2.[6] However, while the pathway of mast cell activation in allergic diseases is well defined, the underlying mechanism of mast cell activation following tissue injury have not been fully deciphered.

Neutrophils are the first cells recruited to sites of acute inflammation.[10,11] These motile phagocytes, long understood as the ‘foot soldiers’ of innate immunity, serve a critical function in eliminating microbial pathogens.[12,13] More recently, the uncontrolled effector function of neutrophils has been implicated in chronic inflammatory diseases.[14-16] During the inflammatory response, excessive release of reactive mediators by neutrophils results in collateral damage to adjacent healthy tissues.[14,17,18] Accordingly, there is significant interest in the mechanisms that govern neutrophil infiltration, particularly the contribution of tissue-resident mast cells to this process, which act as sentinels, triggering neutrophil recruitment.

We have previously reported that mast cell activation initiates neutrophil infiltration of the cornea following injury.[6] In the current study, we observed that mast cell deficient mice [19] failed to recruit neutrophils to the injured corneas, and performed a series of *in vitro* and *in vivo* experiments to delineate the mechanism of mast cell activation that induces the early recruitment of neutrophils. Our *in vivo* experiments employed a mouse model of corneal injury, which due to the relative avascularity and scarcity of resident immune cells, provides a highly informative system to evaluate mast cell activation and neutrophil recruitment.[6]

In this study, we report the novel observation that corneal epithelium-derived IL-33 activates mast cell to promote early neutrophil recruitment to the ocular surface following injury. Specifically, we identify epithelium as the primary source of IL-33 following ocular surface injury, and demonstrate that IL-33 promotes mast cell activation. Moreover, we show that *in vivo* neutralization of IL-33 inhibits mast cell activation and abrogates early neutrophil infiltration reducing corneal inflammatory haze, restoring the tissue architecture following ocular injury.

Materials and Methods

Animals

Six- to eight-week-old male and female C57BL/6 wild-type and mast cell deficient cKit$^{w\text{-}sh}$ mice (Stock No: 012861; Jackson Laboratory, Bar Harbor, ME) were purchased for use in the described experiments. The mice were housed in the Schepens Eye Research Institute animal vivarium. All experiments were reviewed and approved by the Schepens Eye Research Institute Animal Care and Use Committee. The mice were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal Injury Model

Corneal injury was performed on deeply anesthetized mice as previously described.[6,20] Briefly, the central cornea of the right eye was demarcated using a 2 mm trephine. The tip of a handheld motor brush (Algerbrush II; Alger Company, Inc., Lago Vista, TX, USA) was used to remove the corneal epithelium and anterior stroma. Following completion of this procedure, a triple antibiotic ointment was applied topically. Mice were administered a subcutaneous injection of buprenorphine in order to mitigate injury-induced pain. In order to evaluate the *in vivo* effects of mast cells, 2.5 μL of either IL33 blocking antibody (1 mg/ml in PBS; Biolegend) or 2% cromolyn sodium in PBS (Sigma-Aldrich Corp., St. Louis, MO, USA) were administered topically at four time points – one hour prior to injury, at the time of injury, one hour post-injury and 3 hours post-injury. The PBS treated injured mice and uninjured normal mice served as controls. Ocular surface tear wash was collected at 6 hours post-injury. Subsequently, mice were euthanized and their corneas (including the corneal limbus) were harvested for further analyses.

Pharmaceutical intervention

Topical administration of 2% cromolyn sodium (Sigma-Aldrich, St. Louis, MO) eye drops or αIL-33 (1 mg/ml; R&D systems, Minneapolis, MN) were applied to the injured cornea prior to injury, at the time of injury as well as at 1 and 3 hours after injury. A group of control mice were administered phosphate-buffered saline (PBS) eye drops.

Corneal Tissue Isolation and Digestion

Single cell suspensions were prepared from corneas as previously described.[7,21,22] Briefly, corneas were digested in RPMI media (Lonza, Walkersville, MD, USA) containing 4 mg/mL collagenase type IV (Sigma-Aldrich, St. Louis, MO) and 2 mg/mL DNase I (Roche, Basel, Switzerland) for 45 minutes at 37°C. Following this, cells were filtered through a 70-mμ cell strainer.

Cell Culture Assays

Mast cells were generated by culturing bone marrow cells in the presence of stem cell factor (SCF; 50 ng/mL) and IL-3 (10 ng/mL) for 3 to 4 weeks.[6] Media was changed every 3 to 4 days. This method of cell culture generates a mast cell population of >95% purity. For the mast cell stimulation assays, mast cells were cultured either in medium alone, or were stimulated with 1 μM concentration of inflammatory factors IL-1β, IL-6, IL-33, IL-36γ,

SDF1α or Substance P for 6 hours at 37°C. The cells and respective supernatants were harvested, and levels of CXCL2, β-hexosaminidase and tryptase were evaluated using the methods described below.

Flow Cytometry

Single cells suspensions were prepared by enzymatic digestion of 3-4 corneas as described above. The cells were stained with fluorochrome-conjugated anti-CD45, anti-CD11b, anti-Ly6G, anti-c-Kit and anti-FcεR1 antibodies for flow cytometry analysis. Bone marrow mast cells were stained with anti-ST2 antibodies. Appropriate isotypes were utilized as antibody controls. Antibodies and isotype controls were acquired from Biolegend (San Diego, CA, USA). LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and Summit software (Dako Colorado, Inc., Fort Collins, CO, USA) were used to analyze stained cells.

Real-Time PCR

RNeasy Micro Kits (Qiagen, Valencia, CA, USA) were used to isolate total RNA, which was reverse transcribed into cDNA using Superscript III (Invitrogen, Carlsbad, CA, USA) [23]. Quantitative real-time PCR was conducted using Taqman Universal PCR Mastermix and preformulated primers for murine IL-33 (Mm00505403_m1) and glyceraldehype-3-phosphate dehydrogenase (GAPDH, Mm99999915_gl) in a Mastercycler Realplex 2 (Eppendorf, Hamburg, Germany). The comparative threshold cycle method was used to analyze the results, which were normalized to GAPDH as an internal control.

β-hexosaminidase Assays

β-n-acetylglucosaminidase assay kits (Sigma-Aldrich) were used to quantify levels of β-hexosaminidase enzyme. These kits are based on the hydrolysis of 4-Nitrophenyl N-acetyl-β-d-glucosaminide (NP-GlcNAc).[25] In brief, ocular surface tears and culture supernatants were incubated with 0.1 mg/mL NP-GlcNAc (substrate) for 1 hour at 37°C. Subsequently, the enzyme-substrate reaction was stopped with 5 mg/mL sodium carbonate. A SpectraMax Plus 384 Microplate Reader (Molecular Devices, San Jose, CA, USA) was used to measure absorbance at 405 nm. β-hexosaminidase levels were estimated using the formula: U/mL = (A405sample - A405blank) × 0.05 × 0.3 × DF/A405standard × time × volume of sample in milliliters.

Tryptase Assays

Mast Cell Degranulation Assay Kits (Sigma-Aldrich) were used to quantify levels of tryptase enzyme. These kits are based on the spectophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate tosyl-gly-pro-lys-pNA.[26] In brief, ocular surface tear and culture supernatants were incubated with 0.1 mg/mL tosyl-gly-pro-lys-pNA (substrate) for 2 hours at 37°C. A SpectraMax Plus 384 Microplate Reader (Molecular Devices, San Jose, CA, USA) was used to quantify free pNA at 405 nm.

ELISA

Commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to quantify levels of CXCL2 or IL-33 in the ocular surface tear wash and supernatants harvested from mast cell cultures, as per the manufacturer's instructions. Exposure to ethylenediamine-tetraacetic acid (EDTA) at 37°C for 30 minutes was used to separate epithelial and stromal layers from corneas harvested from naïve C57BL/6 mice. Subsequently, isolated epithelium and stromal tissues were lysed to evaluate IL-33 levels by ELISA.

Immunohistochemistry and Histology

For immunohistochemistry analysis, corneas with limbus and bulbar conjunctivae were harvested and fixed with 4% paraformaldehyde to stain with Texas red avidin (Company). Stained corneas were mounted on slides and visualized using a confocal microscope (Leica TCS-SP5; Buffalo Grove, IL, USA) at 5X magnification for the detection of avidin-labeled mast cells. For H&E staining, cross sections were prepared from formalin-fixed whole eye ball harvested at 48 hours post-injury as previously described.[27] Corneal tissue structure was analyzed under bright field microscope (Nikon Eclipse E800; Nikon Instruments, Melville, NY, USA) at 20X magnification.

Slit-lamp microscopy

Slit lamp biomicroscopy (with photographs) was used to evaluate inflammation-induced corneal haze following injury, as previously described.[22,28] PBS-treated injured eyes were used as controls. Corneas were visualized in bright field to examine development of haze at 6 hours following injury (Day 0) as well as on Days 1 and 2 post-injury.

Statistical Analysis

Unpaired two-tailed Student t-tests were used to compare means between two groups. The significance level was set at $p < 0.05$. Data are presented as the mean ± standard deviation. Results shown are representative of at least three independent experiments. Samples sizes were estimated on the basis of previous studies of corneal injury and inflammation.[21,22,29,30]

Results

Mast cell activation is associated with early neutrophil infiltration following corneal injury

To evaluate the distribution of mast cells, corneas were harvested from C57BL/6 mice and stained with a fluorescent conjugate of avidin.[31] Mast cells were observed predominantly in the peripheral cornea and limbus (Fig. 1A). Next, we investigated the effect of corneal injury on mast cell activation at the ocular surface. Corneal injury was performed using an Algerbrush, with 2 mm diameter trephination of the central corneal epithelium and anterior stroma (Fig. 1B). Tear wash was collected at 3-hours following injury. Naïve mice were used as controls. Mast cell activation was assessed by evaluating two markers of mast cell degranulation; β-hexosaminidase and tryptase.[5] Our data demonstrate that both β-hexosaminidase and tryptase levels in the ocular surface tear wash were significantly

elevated in the injured mice relative to naïve (2 fold increase [p = 0.003] and 4.5 fold increase [p = 0.018], respectively) (Fig. 1C & 1D). These data indicate that ocular surface injury triggers mast cell activation.

Next, we investigated neutrophil infiltration into the cornea following ocular injury. Mice were sacrificed at 3- and 6-hours following injury. Single cell suspensions of harvested corneas were prepared and immunostained with CD11b and Ly6G antibodies. Consistent with the results from our previous study,[6] flow cytometry data demonstrated a progressive increase in $CD11b^{+}Ly6G^{+}$ neutrophil frequencies following corneal injury (3.4 fold increase at 3 hours [p = 0.005] and 5 fold increase at 6 hours [p < 0.001]) (Fig. 1E). The CXC chemokine receptor 2-binding chemokine CXCL2 is recognized as a potent chemoattractant that induces neutrophil recruitment.[17] Accordingly, we evaluated levels of CXCL2 in tear wash at 6 hours following injury relative to naïve controls. Our data show a significant increase in CXCL2 levels following injury (5 fold increase [p = 0.001]). Taken together, our data indicate that corneal injury results in early mast cell activation and neutrophil infiltration to the cornea.

Mast cell deficiency abrogates early neutrophil infiltration

Having demonstrated that corneal injury triggers the activation of mast cells and infiltration of neutrophils, we sought to determine whether activation of mast cells is the cause of the increased neutrophil frequencies observed in the cornea following injury. To investigate this, we employed mast cell-deficient mice ($cKit^{w\text{-}sh}$). The absence of mast cells was confirmed by evaluating frequencies of $cKit^{+}FC\varepsilon R1^{+}$ cells in the ocular surface and peritoneum of $cKit^{w\text{-}sh}$ mice, with wild type C57BL/6 mice as controls (Fig. 2A). Following the confirmation of mast cell deficiency, corneal injury was performed on $cKit^{w\text{-}sh}$ mice. Mice were sacrificed at 6 hours-post injury, single cell suspensions were prepared from harvested corneas, and frequencies of total $CD45^{+}$ inflammatory cells as well as $CD11b^{+}Ly6G^{+}$ neutrophils were analyzed by flow cytometry. The frequencies of $CD45^{+}$ cells observed in the injured corneas harvested from $cKit^{w\text{-}sh}$ mice were significantly lower than those detected in injured corneas from C57BL/6 mice (p < 0.03; Fig. 2B). Similarly, the frequencies of $CD11b^{+}Ly6G^{+}$ neutrophils in corneas derived from $cKit^{w\text{-}sh}$ mice were significantly lower (3-fold) than those from wild type controls (p < 0.05; Fig. 2C). However, no significant differences were observed in numbers of total leukocytes ($CD45^{+}$ cells) and neutrophils ($CD11b^{+}Ly6G^{+}$) in in the bone marrows of C57BL/6 and $cKit^{w\text{-}sh}$ mice (Fig. S1). These data suggest that mast cells play a critical role in promoting early neutrophil infiltration of the injured cornea.

IL-33 activates mast cells to secrete high levels of the neutrophil chemoattractant CXCL2

Having determined that mast cells are critical in the promotion of early neutrophil infiltration following corneal injury, we next investigated the mechanism of mast cell activation at the ocular surface. Due to the cornea harboring very low frequencies of mast cells, we employed bone marrow-derived mast cells that were generated by culturing bone marrow cells with mast cell lineage cytokines, stem cell factor and IL-3 (purity > 95%).[6] We performed *in vitro* mast cell activation assays in which bone marrow-derived mast cells were stimulated for 6 hours with a variety of pro-inflammatory factors previously reported to

be upregulated following ocular surface inflammation (IL-1 β, IL-6, IL-33, IL-36γ, SDF-1α and Substance P).[22,32-37] ELISA analyses demonstrated highest levels of CXCL2 in supernatants of IL-33-stimulated cultures, with negligible expression of CXCL2 observed in unstimulated controls (Fig. 3A). Stimulation of mast cells with other pro-inflammatory factors including IL-1β, IL-6, IL-36γ, SDF-1α and Substance P failed to upregulate CXCL2 expression (Fig. S2). Next, we evaluated whether IL-33 activates mast cells using β-hexosaminidase and tryptase assays. IL-33 stimulation of mast cells resulted in a significant increase in both β-hexosaminidase ($p = 0.036$) and tryptase levels ($p = 0.028$), compared to unstimulated controls (Fig. 3B and 3C, respectively). Furthermore, expression of the IL-33 receptor ST2 on mast cells was analyzed using flow cytometry. Our data demonstrate that mast cells constitutively express IL-33R (>99%; Fig. 3D). Collectively these results indicate that IL-33 stimulates mast cells to secrete the neutrophil chemoattractant CXCL2, as well as the markers of mast cell activation β-hexosaminidase and tryptase.

Corneal epithelium releases prestored IL-33 during injury

Our next investigation sought to determine whether corneal injury promotes expression of IL-33 at the ocular surface. Whole corneas were harvested at 6 hours post-injury to isolate total mRNA, and expression of IL-33 was evaluated by real-time PCR. Naïve, non-injured corneas were used as controls. Our data demonstrate a substantial increase in IL-33 expression following corneal injury relative to naïve controls ($p = 0.033$) (Fig. 4A). The upregulated expression of IL-33 following corneal injury was confirmed by analyzing tear washes using ELISA, with a 3-fold increase in IL-33 levels relative to the naïve ($p = 0.022$) (Fig. 4B). Finally, in order to identify the cellular source of IL-33, corneal epithelial and stromal layers were separated and lysed to quantify IL-33 levels using ELISA. Corneal epithelium was observed to express 4-fold higher levels of IL-33 compared to the stroma ($p < 0.001$, Fig. 4C), suggesting epithelium to be the primary source of prestored IL-33 released following corneal injury (Fig. 4C).

In vivo blockade of IL-33 suppresses mast cell activation and neutrophil infiltration to the injured cornea

Finally, we investigated whether blockade of IL-33-mediated mast cell activation prevents the early infiltration of neutrophils following corneal injury. Corneal injury was created as shown in Fig. 1B. Anti-IL-33 antibody (1mg/ml) was applied to the cornea at four time points: one hour prior to injury, at the time of injury, as well as at one- and three-hours following injury. Mice treated at the same time points with cromoglycate (a known mast cell inhibitor[38]) or PBS were used as controls. Both topical blockade of IL-33 and administration of cromoglycate inhibited mast cell activation, as demonstrated by a significant reduction in levels of tryptase in the tear wash collected 6 hours following injury ($p = 0.002$ and $p = 0.003$ respectively, Fig. 5A). To evaluate early neutrophil infiltration, single cell suspensions were prepared from corneas harvested at 6 hours post-injury, and flow cytometry was performed. Similar to the observed inhibitory effect on mast cell activation, topical application of both anti-IL-33 antibody and cromoglycate limited the infiltration of $CD11b^{+}Ly6G^{+}$ neutrophils to the injured cornea ($p = 0.027$ and $p = 0.012$ respectively, Fig. 5B). To further confirm the inhibitory effect of IL-33 neutralization on corneal inflammation, bright-field images were captured on day 0 (day of injury) as well as

on day 1 and 2 post-injury. Topical blockade of IL-33 reduced injury-induced inflammatory haze compared to PBS treated controls (Fig. 5C). Moreover, analysis from corneal cross-sections stained with H&E show that treatment with anti-IL-33 antibody normalize corneal architecture by reducing stromal thickness, edema and disorganization of stromal lamellae, which are associated with injury (Fig. 5D).[39] Taken together, our data indicate that blockade of IL-33 prevents injury-induced mast cell activation, inhibits neutrophil infiltration, and protects against both inflammatory corneal haze and disorganization of tissue architecture following injury.

Discussion

Neutrophils play vital roles in host defence against noxious pathogens, yet paradoxically, neutrophils are also involved in a range of inflammatory diseases.[40] Indeed, the release of cytotoxic molecules into the extracellular milieu by neutrophils can result in substantial host tissue damage.[41] The functional toll of this collateral tissue damage is clearly evidenced in inflammatory diseases of the ocular surface, where corneal opacification results in loss of vision.[42,43] Previous work by our laboratory[6,24] and others[44] has demonstrated that mast cells are implicated in early neutrophil recruitment, yet the mechanisms that govern mast cell activation and its subsequent neutrophil recruitment following tissue injury have not been defined. Here, using a murine model of corneal injury, we show that: (i) injury-induced mast cell activation is associated with both heightened levels of the neutrophil chemoattractant chemokine CXCL2 and increased neutrophil frequencies; (ii) epithelium-derived IL-33 stimulates mast cells to secrete higher levels of CXCL2 following injury; and (iii) *in vivo* blockade of IL-33 abrogates mast cell activation and neutrophil infiltration at early time points following injury.

Mast cells are notorious for their role in initiating the allergic immune response by releasing cytokines and chemokines that recruit a variety of leukocytes including eosinophils, basophils and T cells to the site of allergen exposure.[45,46] Recently, using a non-allergic ocular surface inflammation model, we reported that topical application of cromoglycate (a mast cell inhibitor formulated as eye drops for the treatment of allergic conjunctivitis[47]) following corneal injury significantly reduces local infiltration of inflammatory cells.[6] In the current study we developed this work further by employing mast cell-deficient cKit$^{W\text{-}sh}$ mice[48] and investigated the mechanisms by which sterile injury of the ocular surface leads to mast cell-mediated neutrophil recruitment. Our data indicate that mast cells secrete CXCL2 at early timepoints following injury, which recruits neutrophils to the injured tissue. Corroborating the importance of mast cell activation in promoting early neutrophil recruitment, our data demonstrate that mast cell deficiency substantially reduces the frequencies of infiltrating neutrophils following corneal injury, relative to wild type controls. We considered the possibility that cKit$^{W\text{-}sh}$ mice may have reduced total numbers of neutrophils, and that this might be exerting a confounding effect on our data, but we observed similar frequencies of both total CD45^{+} immune cells and CD11b^{+}Ly6G^{+} neutrophils in c-Kit knockout and wild type C57BL/6 mice (Fig. S1).

IL-33, a member of the IL-1 family, is a pleiotropic cytokine that has been shown to modulate type 1, type 2 and regulatory immune responses [49]. In our study, we screened a

range of inflammatory mediators known to be released following tissue injury (including IL-1β, IL-6, IL-33, IL-36γ, SDF-1α and Substance P)[22,32-37] for their capacity to stimulate the secretion of CXCL2 by mast cells. We detected substantially higher secretion of CXCL2 following stimulation with IL-33 compared to other candidate factors. IL-33 is known to be released into the extracellular environment during necrosis or tissue injury, where it binds to the receptor complex of ST2 and IL-1 receptor accessory protein.[50] Accordingly, we evaluated the expression of IL-33 at the cornea, and found it to be substantially upregulated following injury relative to naïve controls. This is consistent with reports showing heightened expression of IL-33 following acute kidney injury [51] and acute hepatitis [52]. We traced the source of prestored IL-33 in the cornea, and found it to be primarily stored in epithelial cells. In order to further assess whether the IL-33/ST2 axis drives the mast cell activation following injury, we evaluated the surface expression of ST2 by mast cells, and found near-uniform expression of ST2. Finally to confirm *in vivo* whether ligation of IL33 with ST2 leads to mast cell activation and subsequent early neutrophil recruitment to the injured site, we topically administered neutralizing anti-IL33 antibody to the injured ocular surface. We observed that IL-33 neutralization substantially reduced both mast cell activation and neutrophil infiltration following injury. We found the scale of these effects to be comparable to topical application of the mast cell inhibitor cromoglycate. Moreover, we observed that blockade of IL-33 resulted in reduced inflammatory corneal haze following injury, and protected against disruption of the corneal tissue architecture.

In summary, these findings provide novel insights on the role of mast cells in ocular mucosal inflammation. Injury to the ocular surface leads to upregulation of epithelium-derived IL-33 that activates mast cells to secrete CXCL2 and initiate neutrophil infiltration. Furthermore, mast cell-deficient mice with injured ocular surface show a significant reduction in neutrophil infiltration. The demonstration of in vivo blockade of IL-33 preventing mast cell activation and neutrophil recruitment to the injured ocular surface, suggest IL-33 could be a potenital thearpeutic target for ocular surface inflammatory disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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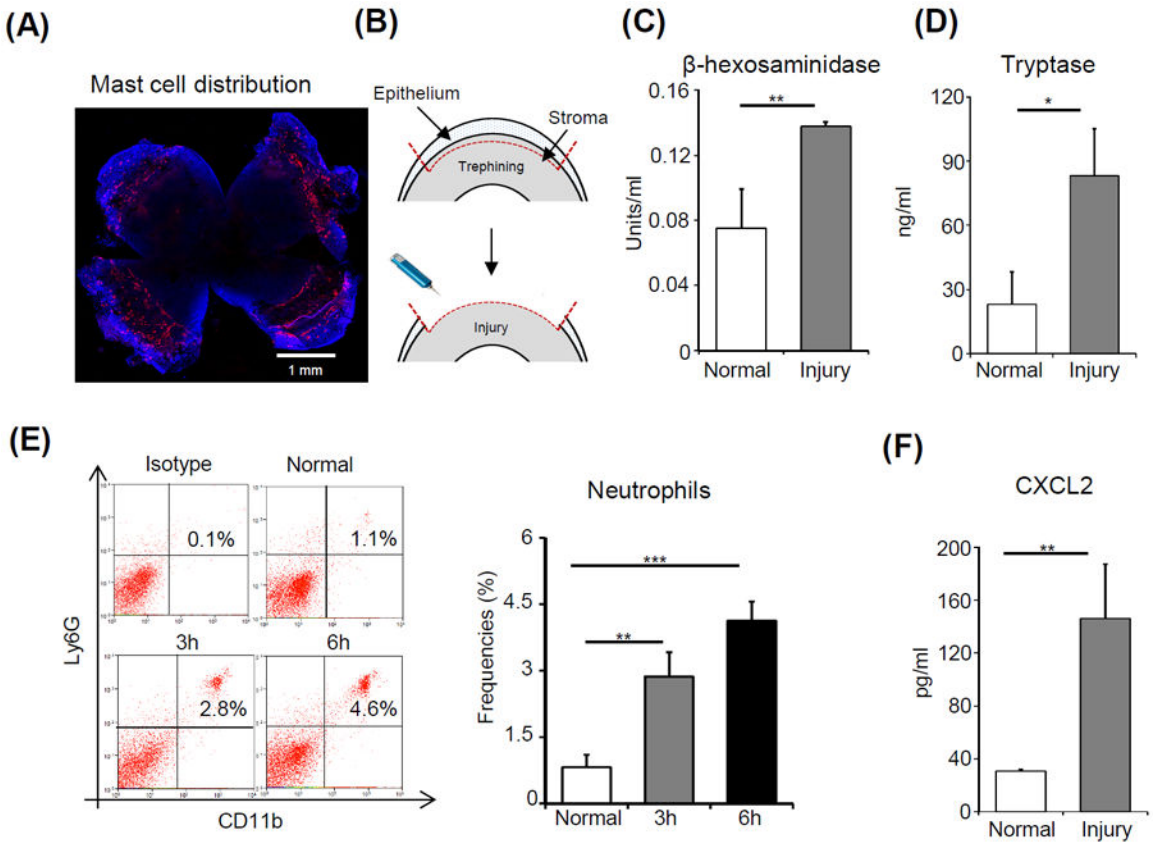

Figure 1. Mast cell activation is associated with early neutrophil infiltration of the injured cornea.

(A) Representative image showing a C57BL/6 murine cornea stained with a fluorescent conjugate of avidin (Texas Red; scale bar: 1 mm). (B) Schematic diagram depicting mouse model of corneal injury. Ocular surface tear wash was collected at 6 hours after injury and mast cell activation markers (C) β-hexosaminidase and (D) Tryptase were estimated relative to naïve mice. (E) Representative flow cytometric dot plots (left) and cumulative bar chart (right) showing the frequencies of $CD11b^{+}Ly6G^{+}$ neutrophils in the cornea at 3 and 6 hours after injury, compared to normal control animals. (F) Bar chart depicting the secretion levels of CXCL2 in the ocular surface tear wash at 6 hours following injury as estimated by ELISA, relative to normal mice. Representative data from three independent experiments are shown, and each experiment consisted of four to six animals. Data presented are mean ± SD (error bar). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

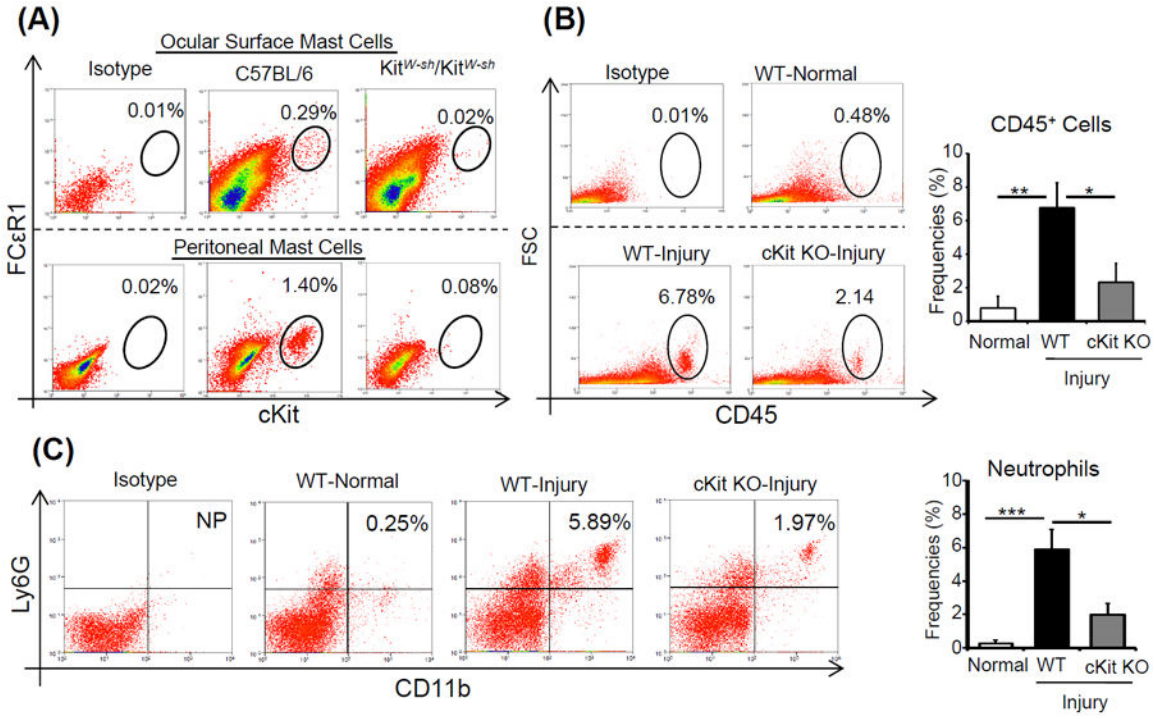

Figure 2. Mast cell deficiency abrogates early neutrophil recruitment

(A) Representative flow cytometry dot plots showing the frequencies of ckit^{+}FcεR1^{+} mast cells in the ocular surface tissues (upper panels) and peritoneum (lower panels) of cKit$^{W\text{-}sh}$ mice, relative to wild type mice. (B) Representative flow cytometry dot plots (left) and cumulative bar chart (right) showing the frequencies of CD45^{+} inflammatory cells in the corneas of cKit$^{W\text{-}sh}$ mice and wild type mice at 6 hours after injury, relative to normal control mice. (C) Representative flow cytometry dot plots (left) and cumulative bar chart (right) showing the frequencies of CD11b^{+}Ly6G^{+} neutrophils in the corneas of cKit$^{W\text{-}sh}$ mice and wild type mice at 6 hours after injury, relative to normal mice. Representative data from two independent experiments are shown, and each experiment consisted of three to four animals. Data presented are mean ± SD (error bar). *p < 0.05, **p < 0.01, ***p < 0.001.

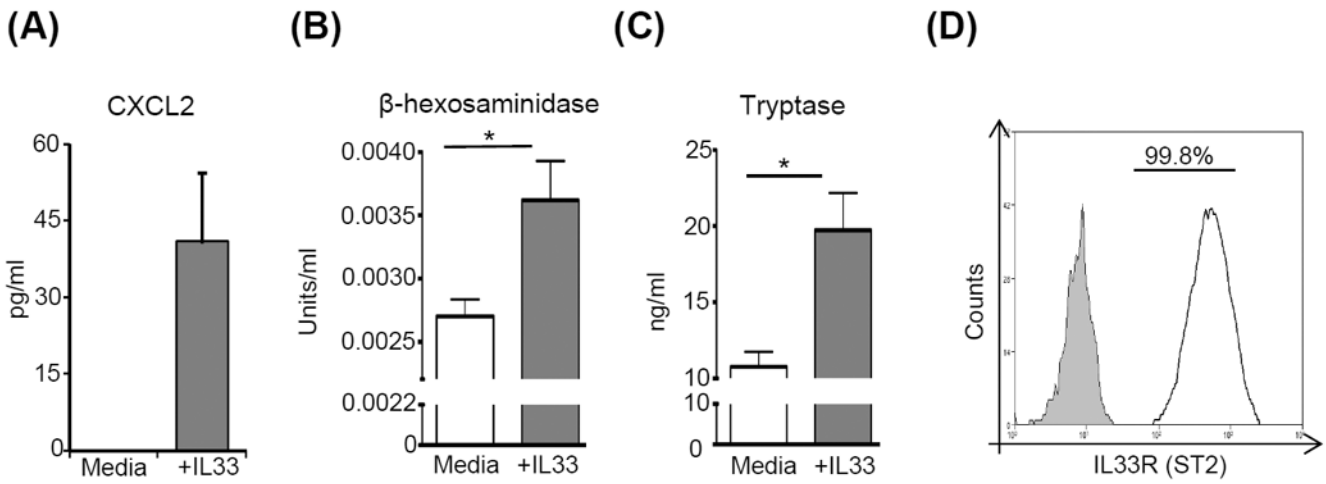

Figure 3. IL-33 activates mast cells and promotes mast cell expression of CXCL2

(A) ELISA analysis of CXCL2 levels in the supernatants of mast cells stimulated with IL-33 compared to unstimulated cells. Supernatants from cultures of mast cells stimulated with IL-33 were analyzed for mast cell activation using (B) β-hexosaminidase (C) tryptase. Supernatant from unstimulated mast cells were used as controls. (D) Representative flow cytometry histogram showing the surface expression of IL33R (ST2; White) on mast cells as compared to isotype control (Grey). Representative data from three independent experiments are shown, and each experiment was repeated three times. Data presented are mean ± SD (error bar). *$p < 0.05$.

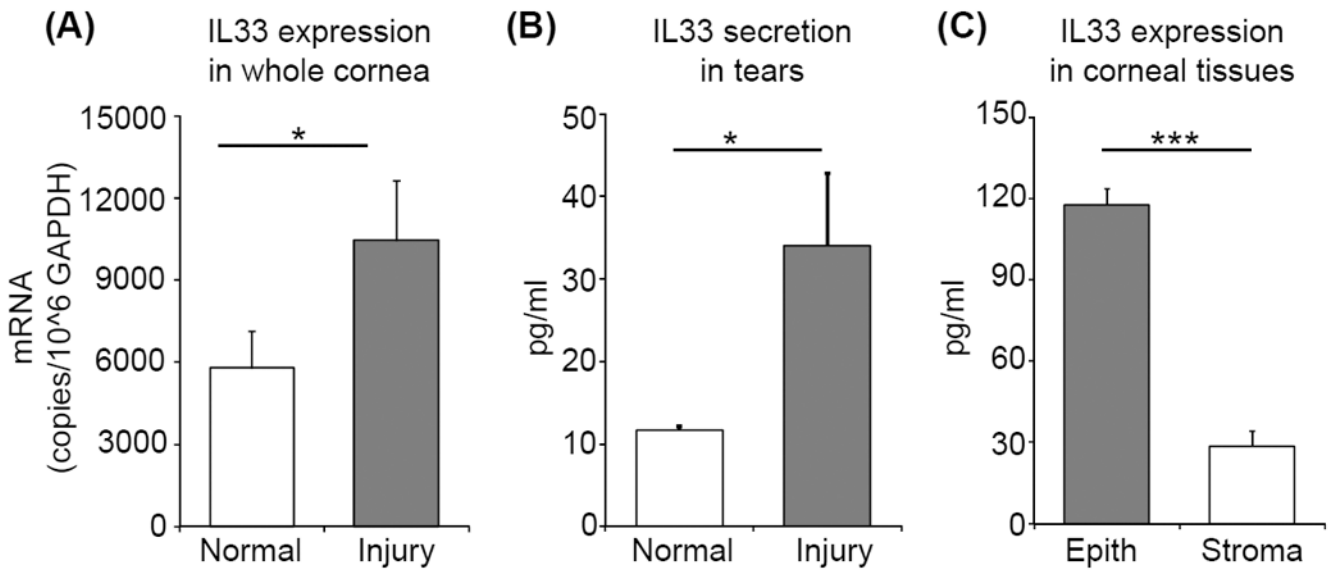

Figure 4. IL-33 released from the cornea following injury is prestored in epithelium

(A) Bar chart depicting mRNA expression of IL-33 in lysate of whole corneas harvested at 6 hours post-injury (normalized to GAPDH) relative to naïve cornea lysate, as quantified by real-time PCR. (B) Bar chart depicting ELISA analysis of IL-33 levels in the ocular surface tear wash at 6 hours post-injury relative to naïve mice. (C) Bar chart depicting ELISA analysis of IL-33 levels in the lysates derived from separated corneal epithelium and stromal tissue lysates. Representative data from three independent experiments are shown, and each experiment consisted of four to six animals. Data presented are mean ± SD (error bar). *$p < 0.05$, ***$p < 0.001$.

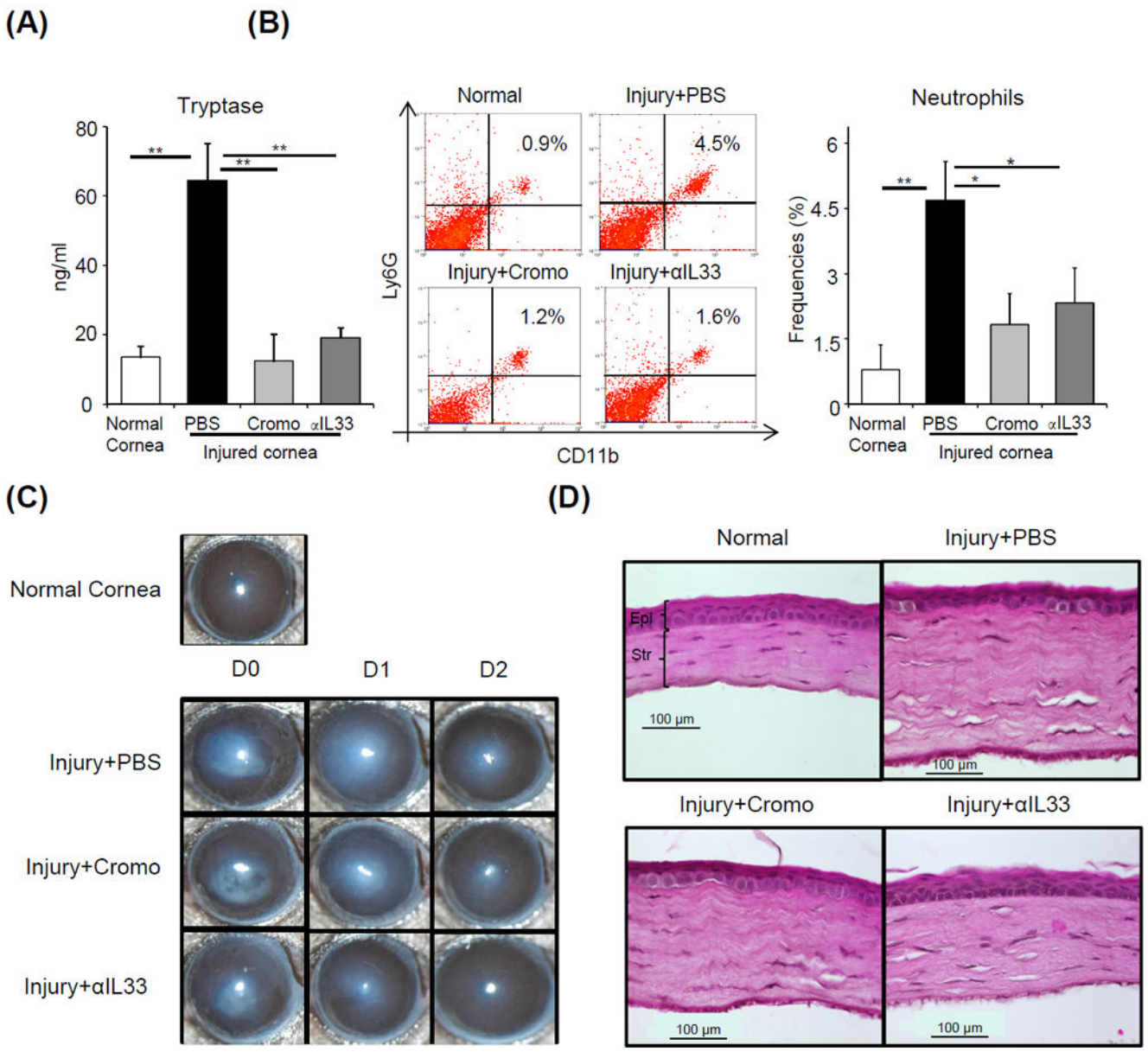

Figure 5. *In vivo* blockade of IL-33 suppresses mast cell activation and post-injury corneal inflammation.

C57BL/6 mice were treated topically with either cromoglycate (2%) or αIL-33 (1 mg/ml) at 1 hour prior to injury, at the time of injury as well as 1 and 3 hours after injury. (A) Bar chart depicting tryptase levels in ocular surface tear wash at 6 hours post-injury in the indicated groups relative to normal mice. (B) Representative flow cytometry dot plots (left) and cumulative bar chart (right) showing the frequencies of $CD11b^{+}Ly6G^{+}$ neutrophils in the cornea at 6 hours after injury in the indicated groups, relative to normal mice. (C) Representative bright-field microscopic images of corneas of indicated groups at Day 0, 1 and 2 post-injury. (D) Cross-sections stained with hematoxylin and eosin to visualize corneal thickness, edema and stromal lamellae (Epi: epithelium; Str: Stroma; scale bar: 100 μm). Representative data from three independent experiments are shown, and each experiment

consisted of four to six animals. Data presented are mean ± SD (error bar). *p < 0.05, **p < 0.01.