RESEARCH ARTICLE

Eosinophils promote corneal wound healing via the 12/15-lipoxygenase pathway

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Abstract
Lipid mediators play important roles in regulating inflammatory responses and tissue homeostasis. Since 12/15-lipoxygenase (12/15-LOX)-derived lipid mediators such as lipoxin A4 (LXA4) and protectin D1 (PD1) protect against corneal epithelial cell damage, the major cell types that express 12/15-LOX and contribute to the corneal wound healing process are of particular interest. Here, we found that eosinophils were the major cell type expressing 12/15-LOX during the corneal wound healing process. Eosinophils were recruited into the conjunctiva after corneal epithelium wounding, and eosinophil-deficient and/or eosinophil-specific 12/15-LOX knockout mice showed delayed corneal wound healing compared with wild-type mice. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based mediator lipidomics revealed that a series of 12/15-LOX-derived mediators were significantly decreased in eosinophil-deficient mice and topical application of 17-hydroxydocosahexaenoic acid (17-HDoHE), a major 12/15-LOX-derived product, restored the phenotype. These results indicate that 12/15-LOX-expressing eosinophils, by locally producing pro-resolving mediators, significantly contribute to the corneal wound healing process in the eye.

KEYWORDS
alox15, corneal wound healing, eosinophils, lipid mediator, lipoxygenase, metabolomics

Abbreviations: 12/15-LOX, 12/15-lipoxygenase; AA, arachidonic acid; CCR3, C-C chemokine receptor type 3; CD, cluster of differentiation; DAPI, 4’,6-diamidino-2-phenylindole; DHA, docosahexaenoic acid; dHDoHE, dihydroxy docosahexaenoic acid; dHETE, dihydroxyeicosatetraenoic acid; DSS, Dextran sulfate sodium; EET, epoxyeicosatrienoic acid; EGF, epidermal growth factor; EpDPE, epoxydocosapentaenoic acid; EpETE, epoxyeicosatetraenoic acid; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBSS, Hank’s Balanced Salt Solution; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HHT, hydroxyheptadecatrienoic acid; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LSCD, limbal stem cell deficiency; LT, leukotriene; LXA4, lipoxin A4; MRN, multiple reaction monitoring; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocytes; PD1, protectin D1; PG, prostaglandin; SEM, standard error of the mean; SPF, specific-pathogen-free; SPMs, specialized pro-resolving mediators; WT, Wild-type.

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Lipid mediators play essential roles in host defense and restoring homeostasis during inflammatory responses. 1 12/15-Lipoxygenase (12/15-LOX) is the enzyme that locally produces polyunsaturated fatty acid-derived lipid mediators such as lipoxin A4 (LXA4) and protectin D1 (PD1), which are classified as specialized pro-resolving mediators (SPMs). 2 SPMs exhibit potent anti-inflammatory and pro-resolving roles in many disease models. 3, 4 The importance of 12/15-LOX is supported by the previous work indicating that 12/15-LOX-deficient mice show delayed corneal wound healing. 5 The corneal epithelium is a stratified squamous epithelium that constitutes a major part of the ocular surface in the eye and is continually exposed to various stresses such as dryness, infection, and chemical and mechanical stress that generate small wounds in the epithelium. 6 Therefore, corneal wound healing is important for eye homeostasis. Indeed, some clinical symptoms are associated with impaired corneal wound healing, such as limbal stem cell deficiency (LSCD). LSCD is characterized by a loss or deficiency of the stem cells in the limbus caused by traumatic or inflammatory insults such as ocular burns, Stevens-Johnson syndrome, and chemical traumas. Severe LSCD is difficult to cure and persistent epithelial defects develop due to the limited supply of epithelium for repair. 7 Therefore, a better understanding of corneal wound healing mechanisms and the development of efficient ways to promote the repair process are clinically important.

Eosinophils are a distinct granulocyte lineage that is involved in the innate immune response to helmint infection and in allergic responses. 8 We previously reported that eosinophils contribute to the resolution process of acute peritonitis through the local production of 12/15-LOX-derived pro-resolving mediators. 9 In addition, eosinophils protect against dextran sulfate sodium (DSS)-induced colitis. 10 Thus, eosinophils may modulate the epithelial wound healing process, in addition to their host defense functions. In this study, we demonstrated that eosinophils contribute to corneal wound healing through the local production of 12/15-LOX-derived lipid mediators in the eye.

Materials and Methods

Animals

Wild-type (WT) C57BL/6J mice (CLEA, Tokyo, JAPAN) were bred in our facility under specific pathogen-free (SPF) conditions and were placed on a standard rodent diet. 12/15-LOX-deficient mice were obtained from the Jackson Laboratory (002778, Bar Harbor, ME, USA). ΔdblGATA mice obtained from The Jackson Laboratory (005653) were backcrossed with C57BL/6J mice and bred in our animal facility. Knock-in mice with an ORF encoding a Cre recombinase into the eosinophil peroxidase locus (eoCRE) were originally established by Dr James J. Lee (Mayo Clinic Arizona, USA). 11 12/15-LOX-floxed mice were generated through homologous recombination in ES cells 12 by the targeting vector (12/15-LOXtm1a(KOMP)Wtsi) (Figure S1) obtained from the trans-NIH Knock-Out Mouse Project (KOMP). EoCRE × 12/15-LOX-floxed mice were generated and bred in the RIKEN Institute. For in vivo animal studies, male mice aged 8-14 weeks were used. All mouse studies were performed in full compliance with RIKEN-approved protocols (29-009-4) and institutional guidelines. All animal studies were approved by the RIKEN Committee and were performed according to the guidelines of the Association for Research in Vision and Ophthalmology.

2.2 | Corneal epithelium removal experiments

Mice were anesthetized via intraperitoneal injection of ketamine (50 mg/kg) and xylazine (20 mg/kg) prior to corneal scratch induction. 5 The corneal epithelium up to the limbus was removed using a spatula under slit-lamp biomicroscopy.

2.3 | Wound healing assessment

Mice were anesthetized and corneas were subjectively assessed 24, 48, 72, 96, and 120 hours after injury for corneal epithelium defects by slit-lamp biomicroscopy. Corneas were stained with fluorescein as a direct marker of the epithelial defect and photographed with a Leica Application Suite ver. 4.8 camera. The digital images of the fluorescein-stained wound area were then quantitated using image analysis software (FIJI, ImageJ).

2.4 | Topical application

After the removal of the epithelium, we topically applied treatments to the eye (1 eye drop; 10 µL) comprising 1 µg of 17-HDoHE (Cayman Chemical, Ann Arbor, MI, USA) in 10 µL of 100% sterile Hank’s Balanced Salt Solution (HBSS) (pH 7.4). The topical application was repeated three times a day for 0-120 hours. Right eyes were selected to receive treatment, while left eyes were assigned to receive the placebo (100% sterile HBSS at pH 7.4).

2.5 | Flow cytometric analyses

Corneal and conjunctival single-cell suspensions were acquired as previously described. 13 Single-cell suspensions were blocked with anti-mouse cluster of differentiation (CD) 16/32 blocking
antibody (93; BioLegend, San Diego, CA, USA) and stained with CD45 BV (30-F11; BioLegend), Gr-1 APC-cy7 (RB6-8C5; BioLegend), F4/80 APC (BM8; BioLegend), Zombie Aqua Pacific Orange (BioLegend), C-C chemokine receptor type 3 (CCR3) FITC (83101; RD Systems, Minneapolis, MN, USA), Siglec-F PE (E50-2440; BD PharMingen, San Jose, CA, USA), and CD11b PerCP-Cy5.5 (M1/70; BD PharMingen) antibodies for 15 minutes at 4°C. For 12/15-LOX and Ki67 staining, the cells were fixed and permeabilized according to the instructions of the BD Foxp3/Transcription Factor Staining Buffer kit (00-5523-00; Thermo Fisher Scientific, Waltham, MA, USA) and stained with rabbit anti-mouse 12/15-LOX antibody for 60 minutes and then Alexa Fluor 488-conjugated (Thermo Fisher Scientific) donkey anti-rabbit IgG, and FITC anti-mouse Ki67 antibody (16-A8; BioLegend) for 30 minutes. All flow samples were acquired on a FACSCanto II Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and were analyzed using FlowJo v. 9.9.6. Cell counts were calculated as the numbers of cells gated on live cells.

2.6 | Immunohistochemistry

Embedded frozen sections were fixed with 4% paraformaldehyde for 20 minutes, then permeabilized three times for 5 minutes each with 0.3% Triton X-100/phosphate-buffered saline (PBS) and blocked in PBS solution containing 5% bovine serum albumin and 0.3% Triton X-100, followed by overnight incubation at 4°C with rabbit anti-12/15-LOX polyclonal antibody, rat anti-Siglec-F monoclonal antibody (E50-2440; BD PharMingen), rabbit anti-Ki67 polyclonal antibody (ab15580; Abcam, Cambridge, UK), and chicken anti-Keratin 14 polyclonal antibody (19053; BioLegend). After three washes in PBS, the sections were incubated for 1 hour with Alexa Fluor 488-conjugated anti-rabbit IgG, Alexa Fluor 594-conjugated anti-rat IgG, Alexa Fluor 594-conjugated anti-rabbit IgG (Thermo Fisher Scientific), Alexa Fluor 488-conjugated anti-chicken IgY (Abcam), and DAPI (4′,6-diamidino-2-phenylindole). All antibodies were used at a 1:100 dilution. Images were obtained using a BZ-X700 microscope (Keyence, Osaka, Osaka, Japan).

2.7 | Mediator lipidomics

Lipidomics was performed as previously described. Lipid metabolites were extracted by solid-phase extraction using MonoSpin C18-AX cartridges (GL Science, Shinjuku, Tokyo, Japan) in the presence of deuterated internal standard: 1 ng of arachidonic acid (AA)-d8, 15-hydroxyeicosatetraenoic acid (HETE)-d8, leukotriene B4 (LTB4)-d4, LTD4-d5, prostaglandin E2 (PGE2)-d4, PGB2-d4, and 9-iso-PGF2α-d4. For LC-MS/MS analysis, a triple-quadrupole linear ion-trap mass spectrometer (5500QTRAP; Sciex, Framingham, MA, USA) equipped with an ACQUITY UPLC BEH C18 column (1.0 × 150 mm, 1.7-μm particle size; Waters, Milford, MA, USA) was used. Samples were eluted with a mobile phase composed of water/acetate (100:0.1, v/v) and acetonitrile/methanol (4:1, v/v), 73:27, for 5 minutes, ramped to 30:70 after 15 minutes, to 20:80 after 25 minutes and held for 10 minutes, to 5:95 after 35 minutes and held for 9 minutes, and to 0:100 after 39 minutes and held for 1 minutes, with flow rates of 50 µL/minutes (0-30 minutes), 80 µL/minutes (30-33 minutes), and 100 µL/minutes (33-40 minutes). MS/MS analyses were conducted in a negative ion mode, and lipid metabolites were identified and quantified by multiple reaction monitoring (MRM). Calibration curves between 1 and 1000 pg and the LC retention times for each compound were established with synthetic standards.

2.8 | Quantitative real-time PCR

Corneal and conjunctival tissues were harvested and homogenized at 6,500 rpm for 15 seconds twice in TRIzol using a tissue homogenizer. RNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany). cDNA synthesis was performed using RT Master Mix (Takara, Kusatsu, Shiga, Japan). Real-time PCR was performed by 40-cycle amplification using gene-specific 12/15-LOX primers (forward: TACACGTTCCCCTGTTACCG; reverse: GATTGTGCCATCCCTCCAGT) and TB Green Premix Ex Taq kit (Takara) on a StepOnePlus (Applied Biosystems). Measurements were normalized to endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. Relative fold changes in expression were calculated using the ∆∆CT method.

2.9 | Statistical analysis

Data were analyzed using GraphPad Prism software 6.0e. Results are expressed as means and standard error of the mean (SEM). Statistical significance was determined using a two-tailed Student’s t-test.

3 | RESULTS

3.1 | 12/15-LOX-expressing eosinophils are recruited in response to corneal epithelial wounds

The local inflammatory response is characterized by the recruitment of different types of leukocytes that become activated at the inflamed site. To elucidate the cellular events during corneal wound healing, we used flow cytometry to investigate the temporal changes in leukocytes in a mouse...
corneal epithelium scratch model. After the removal of the corneal epithelium, the total numbers of leukocytes in the cornea and conjunctiva were increased for 6 hours. Leukocytes at 6 hours predominantly comprised PMNs, as determined by Gr-1hi CD11bhi. As the PMN numbers decreased, F4/80hi CD11bhi cells, which are often classified as macrophages, were gradually increased at 48-72 hours. In addition, the number of eosinophils characterized by Siglec-Fhi CD11bhi was increased, representing 14.4% ± 7.4% and 9.8% ± 1.0% of the total cells at 48 and 72 hours, respectively (Figure ). Flow cytometric analysis revealed that eosinophils highly expressed 12/15-LOX, whereas other cell types such as macrophages and PMNs lacked 12/15-LOX expression (Figure 2A). Immunostaining of tissue sections identified Siglec-F- and 12/15-LOX-positive eosinophils present around the limbus after injury (Figure 2B). These results indicated that eosinophils were the major cell type expressing 12/15-LOX during corneal wound healing.

3.2 | Delayed corneal wound healing in eosinophil-deficient mice

To determine the role of eosinophils in corneal wound healing, we analyzed eosinophil-deficient ΔdblGATA mice.8 In the steady state, there were no differences in the morphology, structure, or transparency of the cornea between WT and ΔdblGATA mice (data not shown). Flow cytometric analyses revealed that Siglec-Fhi CCR3hi eosinophils were present in the eye 48 hours after epithelial injury in WT but not ΔdblGATA mice (Figure 3A). Under this condition, the 12/15-LOX mRNA level was significantly decreased in ΔdblGATA mice compared with WT mice (Figure 3B). Eosinophil-deficient ΔdblGATA mice showed delayed corneal wound healing compared with WT mice (Figure 3C). LC-MS/MS-based mediator lipidomics revealed that a series of 12/15-LOX-derived mediators were significantly reduced in ΔdblGATA mice (Figure 3D). These results indicated that the local production of 12/15-LOX-derived mediators by eosinophils might facilitate the local mediator environment to support corneal wound healing.

3.3 | Eosinophils promote corneal wound healing via the 12/15-LOX pathway

Next, we determined the corneal wound healing process in eosinophil-specific 12/15-LOX-deficient mice. These mice were generated by crossing eoCre mice11 with 12/15-LOX-floxed mice (see the details in the Materials and Methods). Immunostaining confirmed the absence of

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**FIGURE 1** Eosinophils are recruited in response to corneal epithelial wounding. A. Time course of the ocular surface leukocyte composition during wound healing. After removal of the corneal epithelium, ocular surface tissues, including the cornea and conjunctiva, were collected at the indicated time points. Cells were stained with CD45 for leukocytes, Gr-1hi CD11bhi for neutrophils, F4/80hi CD11bhi for macrophages, and Siglec-Fhi CD11bhi for eosinophils. Numbers of cells were calculated as the numbers of cells gated on live CD45-positive cells. Results are shown as means ± SEM (n = 3)
12/15-LOX protein expression in SiglecF-positive eosinophils in EoCRE × 12/15-LOX-floxed mice (Figure 4A). These eosinophil-specific 12/15-LOX-deficient mice showed delayed corneal wound healing and attenuated proliferation of corneal epithelial cells in response to injury (Figure 4B,C,D). LC-MS/MS-based lipidomics revealed a significant reduction in 12/15-LOX-derived mediators such as 17-HDoHE, which is generated from docosahexaenoic acid (DHA), in the eyes at 48 hours after injury (Figure 4E). Topical application of 17-HDoHE significantly restored the delayed corneal wound healing in eosinophil-deficient ΔdblGATA mice (Figure 5). Collectively, these results strongly support the theory that eosinophils promote corneal wound healing via the 12/15-LOX-mediated biosynthetic route and that a major 12/15-LOX-derived product, namely 17-HDoHE, displays a potent pro-resolving action to promote the wound healing process in vivo.
In this study, we demonstrated for the first time that eosinophil depletion and/or eosinophil-specific 12/15-LOX deficiency causes delayed corneal wound healing in mice. Eosinophils were actively recruited into the cornea in response to the wound, thereby producing 12/15-LOX-derived lipid mediators that facilitate the local mediator environment to support the wound healing process.

Eosinophils have multiple functions in regulating immunological responses and inflammatory processes. The numbers of eosinophils in tissues and blood are increased during...
FIGURE 4  Delayed corneal wound healing in eosinophil-specific 12/15-LOX-deficient mice. A, Immunostaining of 12/15-LOX and Siglec-F in WT and EoCRE × 12/15-LOX-floxed mice 48h after corneal epithelium wounding. B, Delayed corneal wound healing in eosinophil-specific 12/15-LOX-deficient mice. Results are mean ± SEM (n = 4-5). **P < .01. Representative images of fluorescein-stained cornea 72 hours after corneal epithelium wounding are provided. C, Immunostaining of cornea and conjunctiva by anti-Ki67 (red), anti-Keratin 14 (green), and DAPI (blue) 48 hours after corneal epithelium wounding. D, Numbers of Ki67-positive corneal epithelial cells as measured by flow cytometry. Results are shown as means ± SEM (n = 3-4). **P < .01. E, LC-MS/MS-based mediator lipidomics at 48 hours after corneal epithelium wounding. Results are mean ± SEM (n = 3-4). *P < .05
specific immune responses such as helminth infections and allergic conditions. Indeed, the conjunctiva and cornea from allergic conjunctivitis patients and model mice display a massive infiltration of eosinophils. Activated eosinophils release a series of granule proteins, such as major basic protein 1, eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase, to confer effector cell functions. In addition, recent studies have revealed that eosinophils facilitate tissue regeneration in response to muscle and liver injury and promote inflammation resolution in acute peritonitis and colitis. Accordingly, it was of particular interest whether eosinophils recruited around the limbus in the ocular surface play important roles in the corneal wound healing process.

Corneal epithelial cells recognize the insult at the edge of wound, which leads to cell migration, proliferation, differentiation, stratification, and desquamation. Epidermal growth factor (EGF) initiates the migration and proliferation of corneal epithelial cells through activation of its receptor. In addition, lipid mediators play regulatory roles in controlling wound healing processes. For example, 12/15-LOX-deficient mice show delayed corneal wound healing. EGF stimulates LXA₄ synthesis during corneal repair through extracellular signal-regulated kinase (ERK) and p38 activation. In addition, a cyclooxygenase-derived product 12-hydroxyheptadecatenoic acid (HHT) promotes corneal wound healing via LTB₄ type 2 (BLT2) receptor activation. In this study, our comprehensive mediator lipidomics revealed that 12/15-LOX-derived mediators were selectively reduced in eosinophil-deficient mice. Indeed, eosinophils were the major cell type expressing 12/15-LOX during corneal wound healing. Moreover, eosinophil-specific 12/15-LOX-deficient mice displayed delayed wound healing, supporting the theory that 12/15-LOX-expressing eosinophils play important roles in the corneal wound healing process by locally producing pro-resolving mediators. Interestingly, a recent study demonstrated that 12/15-LOX played a protective role in bacterial keratitis by promoting phagocyte functions. 12/15-LOX-expressing eosinophils may also play such an immunoregulatory role in the host defense mechanisms in the eye.

DHA-derived 17-HDoHE was found to be one of the most abundant mediators produced by eosinophils during the corneal healing process. This was consistent with previous work showing that 17-HDoHE was the major 12/15-LOX-derived metabolite in the eye and that its topical application promoted corneal wound healing. In addition, in this study, topical application of 17-HDoHE restored the delayed corneal wound healing in eosinophil-deficient mice. These results indicate that locally recruited eosinophils actively convert DHA into pro-resolving mediator 17-HDoHE during the corneal healing process. As a local mediator, 17-HDoHE is reported to promote the antibody-mediated immune response and macrophage phagocytosis, reduce obesity-associated inflammation, prevent hyperalgesia in adjuvant-induced arthritis. In these studies, 17-HDoHE reduced inflammatory cytokines and shifted immune cell functions such as B cell differentiation toward the antibody-secreting phenotype and macrophages to M2 polarization. Our results might prove that 12/15-LOX of eosinophils accelerate the proliferation of CD3-CD69+ cells.

![Figure 5](image.png)

**Figure 5** Topical application of 17-HDoHE restores delayed corneal wound healing in eosinophil-deficient mice. 17-HDoHE (1 µg) (black bar) or vehicle (gray bar) was administered to the wounded cornea in eosinophil-deficient ΔdblGATA mice three times a day. Results are mean ± SEM (n = 5-6). *P < .05, **P < .01. Representative images of fluorescein-stained cornea during the process of corneal wound healing are provided.
of corneal epithelium from corneal stem cells. The detailed molecular mechanism underlying how 17-HDoHE promotes corneal wound healing remains to be elucidated. Also, the present results do not exclude the possibility of other 12/15-LOX-derived metabolites such as 12-HETE, 15-HETE, 15-HEPE, 14-HDoHE, and SPMs that may play roles in corneal wound healing as well.

Taken together, our results suggest a novel effector function of eosinophils that facilitates corneal wound healing via the local production of lipid mediators in the eye. These findings lead to a better understanding of the cellular and molecular mechanisms underlying the wound healing process in the corneal epithelium. Moreover, these results open up a new therapeutic strategy for corneal disorders involving the control of eosinophil functions and/or modulation of lipid mediator balance in the ocular surface.

ACKNOWLEDGMENTS

This study was supported by the Japan Society for the Promotion of Science (KAKENHI JP15H05897, 15H05898, and 20H00495 to MA), Program for the Advancement of Research in Core Projects in the Longevity Initiative at Keio University Global Research Institute from Keio University (MA), RIKEN Junior Research Associate Program, and Keio University Doctorate Student Grant-In-Aid Program (MO). The authors wish to thank Dr James J. Lee for sharing the EoCRE mice maintained in his colony. The authors wish to thank Dr Satoshi Iwamoto (Juntendo University) for assistance with corneal wound healing model. The authors also wish to thank Dr Tetsuya Kawakita (Keio University School of Medicine) for constructive comments on this research.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

M. Arita designed research, analyzed data, and wrote the paper; M. Ogawa performed experiments, analyzed data, and wrote the paper; T. Ishihara and Y. Isobe performed experiments and analyzed the data; T. Kato, K. Kuba, and Y. Imai contributed to generate 12/15-LOX-floxed mice; K. Tsubota and Y. Uchino were involved in data analysis and discussion.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Ogawa M, Ishihara T, Isobe Y, et al. Eosinophils promote corneal wound healing via the 12/15-lipoxygenase pathway. The FASEB Journal. 2020;34:12492–12501. https://doi.org/10.1096/fj.20200483R