

Endogenous LXA₄ Circuits Are Determinants of Pathological Angiogenesis in Response to Chronic Injury

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Inflammation and angiogenesis are intimately linked, and their dysregulation leads to pathological angiogenesis in human diseases. 15-lipoxygenase (15-LOX) and lipoxin A₄ receptors (ALX) constitute a LXA₄ circuit that is a key feature of inflammatory resolution. LXA₄ analogs have been shown to regulate vascular endothelial growth factor (VEGF)-A-induced angiogenic response *in vitro*. 15-LOX and ALX are highly expressed in the avascular and immune-privileged cornea. However, the role of this endogenous LXA₄ circuit in pathological neovascularization has not been determined. We report that suture-induced chronic injury in the cornea triggered polymorphonuclear leukocytes (PMN) infiltration, pathological neovascularization, and up-regulation of mediators of inflammatory angiogenesis, namely VEGF-A and the VEGF-3 receptor (FLT4). Up-regulation of the VEGF circuit and neovascularization correlated with selective changes in both 15-LOX (Alox15) and ALX (Fpr2) expression and a temporally defined increase in basal 15-LOX activity. More importantly, genetic deletion of 15-LOX or 5-LOX, key and obligatory enzymes in the formation of LXA₄, respectively, led to exacerbated inflammatory neovascularization coincident with increased VEGF-A and FLT4 expression. Direct topical treatment with LXA₄, but not its metabolic precursor 15-hydroxyeicosatetraenoic acid, reduced expression of VEGF-A and FLT4 and inflammatory angiogenesis and rescued 15-LOX knockout mice from exacerbated angiogenesis. In summary, our findings and the prominent expression of 15-LOX and ALX in epithelial cells and macrophages place the LXA₄ circuit as an endogenous regulator of pathological angiogenesis. (*Am J Pathol* 2010, 176:74–84; DOI: 10.2353/ajpath.2010.090678)

Formation of a new functional microvasculature, neovascularization, is a fundamental response to ischemia and a salient feature of wound healing. The primary function of newly formed blood vessels is to increase tissue oxygen tension and delivery of essential nutrients and effector cells to restore normal function. However, aberrant neovascularization is a hallmark feature of chronic inflammation and is associated with numerous pathological conditions that include diabetic retinopathy, Crohn's Disease, atherosclerosis, and cancer.^{1–3}

The growth of microvessels from existing vessels, angiogenesis, is tightly controlled by a range of angiogenic factors and inhibitors; a circuit that is highly evolved in avascular tissues such as the cornea. The vascular endothelial growth factor (VEGF) family of angiogenic factors and their receptors are key mediators of this process, which has led to the recent development and clinical use of anti-VEGF strategies for the treatment of pathological neovascularization in the retina, colon cancer, and lung cancer.^{2–5} Many insights into the endogenous role of the VEGF network have been gained by using the cornea,^{5,6} which maintains an immune-privileged and avascular state despite expression of VEGF-A and its immediate proximity to the vasculature.⁷ Specifically, recent findings have demonstrated that avascularity of the cornea requires expression of a soluble VEGF receptor-1 (sFLT1), which traps VEGF-A.⁸ In addition, the receptor for VEGF-C/VEGF-D, namely VEGF receptor-3 (VEGFR-3, FLT4), is a critical regulator of inflammatory neovascularization.^{9–11} FLT4 is of special interest because its essential expression during development becomes restricted primarily to lymph vessels and activation of this endothelial receptor is a critical step in initiating lymphangiogenesis. However, FLT4 expression is also up-regulated in microvessels of tumors and wounds, and in macrophages and in addition is constitutively expressed in

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corneal epithelial cells.^{3,10–13} A recent report demonstrates that FLT4 is highly expressed in angiogenic sprouts and is a critical regulator of sustained heme-angiogenesis,¹² which underscores the potential key role of this receptor in pathological neovascularization.

Inflammation is intimately associated with neovascularization especially during wound healing and ischemic injury. Lipid autacoids are some of the earliest signals that are released in response to injury or insult. In this regard, the 15-lipoxygenase pathway^{14,15} is of interest as it is one of the most inducible genes in macrophages and highly expressed in mucosal and corneal epithelial cells. Macrophages and epithelial cells are important regulators of angiogenesis, especially in avascular tissue such as the cornea.^{3,10,16} Macrophages have a central and well-documented role in angiogenesis, especially in tumors and inflammatory neovascularization. In the cornea, a well-established model tissue for studying inflammatory neovascularization, VEGF-A recruits macrophages, the major cell type to generate VEGF, which drives inflammatory heme and lymphangiogenesis. Corneal epithelial cells constitutively express the receptor for VEGF-C/VEGF-D (ie, FLT4), which has been proposed as a critical pathway for regulating inflammatory neovascularization.¹⁰

Human 15-LOX (ALOX15 and ALOX15B) generate 15S-hydroxyeicosatetraenoic acid (HETE) and mouse 12/15-LOX (Alox15) generates 15S-HETE and 12S-HETE from arachidonic acid. 15-HETE and 12-HETE have been shown to induce proliferation, migration, and tube formation in endothelial cells.¹⁷ More importantly, 15-HETE is a key intermediate in the formation of the well-studied anti-inflammatory mediator lipoxin A₄ (LXA₄) that is generated via the rate-limiting enzyme 5-lipoxygenase (5-LOX). A body of work^{18–24} has established that the anti-inflammatory actions, which are associated with the up-regulation of 15-LOX and/or 15S-HETE formation are mediated by LXA₄ and its G-protein coupled receptor ALX. Recent reports have demonstrated that stable analogs of LXA₄ inhibit VEGF induced angiogenic responses in endothelial cells.^{25–27} These metabolically stable analogs are mimetics of aspirin-triggered LXA₄, an endogenous isomer whose synthesis can be triggered by aspirin-acetylated cyclooxygenase-2 rather than 15-LOX. This 15-epi isomer of LXA₄ resists metabolic inactivation and mediates its bioactions, like LXA₄, via the ALX receptor. The intimate link between inflammation and angiogenesis and the ability of LXA₄²⁸ and analogs of 15-epi LXA₄^{25–27} to inhibit VEGF-A induced angiogenic responses *in vitro* points toward a potential role of endogenous LXA₄ circuits in pathological angiogenesis. However, the endogenous role of 15-LOX in the regulation of angiogenesis remains controversial. Reports have demonstrated that the enzyme or its products promote or inhibit angiogenic responses in *in vitro* studies.^{29–32} More importantly, the *in vivo* role of the 15-LOX pathway or the LXA₄ circuit in pathological neovascularization remains to be clearly defined. To this end, we assessed the role the 15-LOX pathway and LXA₄ circuit in chronic injury-induced inflammatory neovascularization.

Here, we report that inflammatory neovascularization and up-regulation of the VEGF circuit correlate with

changes in both 15-LOX (Alox15) and LXA₄ receptor (ALX) expression and temporally defined 15-LOX activity. More importantly, genetic deletion of 15-LOX or 5-LOX, key enzymes in the formation of LXA₄, led to amplified neovascularization and expression of VEGF-A and FLT4 in the avascular cornea during chronic injury. LXA₄, but not 15S-HETE, attenuated expression of VEGF-A and FLT4 and the angiogenic response, which provides evidence that selective autacoids from the prominent 15-LOX pathway have an endogenous role in limiting pathological neovascularization.

Materials and Methods

Animals

12/15-lipoxygenase (Alox15) and 5-lipoxygenase (Alox5) deficient mice (6 to 10 weeks, female) were purchased from Jackson Laboratory (Bar Harbor, ME). These Jax Gemm strains have a targeted mutation in 12/15-LOX or in 5-LOX that is in a background C57Bl/6J inbred strain. These mice do not express a functional “leukocyte-type” 12/15-LOX (Alox15) or 5-LOX (Alox5).^{33,34} Age and gender-matched congenic C57Bl/6J stock 000664 mice (6 to 10 weeks, female) were used as controls. Mice were maintained on a 12-hour day/night cycle and fed *ad libitum* a standard diet (Rat/Mouse diet LM-485, Harlan Tekland, Madison, WI).

Corneal Neovascularization and Treatment

All animal studies have been approved by the University of California, Berkeley in accordance with the NIH Guide for the Care and Use of Laboratory Animals and in strict accord with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized with ketamine (50 mg/kg) and xylazine (20 mg/kg) intraperitoneally and a drop of tetracaine-HCL 0.5% was applied to the eye to deliver local corneal anesthesia before injury. A single sterile 8.0 silk suture was placed intrastromally extending over the corneal apex, without disrupting the iris. Selected mice were treated topically t.i.d. with LXA₄ or 15S-HETE (Cayman Chemical, Ann Arbor, MI), or sterile saline alone (PBS, pH 7.4) for two or seven days. Ethanol from the LXA₄ and 15S-HETE solutions were rapidly removed under gentle stream of nitrogen and autacoids immediately resuspended in sterile PBS and applied to the eye (5 μl drop, t.i.d.). Eyes were enucleated at the respective time points under a stereo-microscope and corneas carefully dissected on ice to remove the limbus area and all noncorneal tissue. Isolated corneas were either snap frozen for RNA or lipidomic analyses, or immediately processed for immunohistochemistry.

Assessment of Neovascularization and Inflammation

Isolated corneas were rinsed in PBS, fixed in acetone (100%) for 30 minutes, blocked in 2% bovine serum

albumin/PBS solution and incubated in PBS containing fluorescein isothiocyanate-conjugated CD31/PECAM-1 overnight (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100). Flatmounts were prepared by sectioning the cornea and fixing them to slides. The images were taken with a Zeiss Axiophot laser scanning confocal microscope and neovascularization was quantified by manually tracing the length of all vessels using Image Pro-Express software (Cyber Media) and was expressed as total pixels. Corneal flatmounts from 5-LOX KO mice and their matched congenic wild-type controls were taken with a Zeiss Axioplan 2 microscope equipped with a Ludl motorized stage and z focus. Mosaic images were taken with a AxioCam MR camera and compiled using MosaicX and Zeiss AxioVision 4.5 software.

Myeloperoxidase Activity

Myeloperoxidase (MPO) activity, an index of tissue leukocyte infiltration, was measured 48 hours and 7 days post injury.^{35,36} In brief, corneas (1 cornea/data point) were homogenized with a hand held tissue grinder in 450 μ l of 50 mmol/L potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH 6.0). This was followed by sonication, freeze-thaw three times and a second sonication. The homogenates were then centrifuged and supernatants collected. MPO activity in supernatants was measured by spectrophotometry using *o*-dianisidine dihydrochloride oxidation as a colorimetric indicator. Calibration curves for MPO activities were established with PMN collected from 12 hours zymosan A-induced peritonitis exudates in mice.

Gene and Protein Expression

RNA from mouse corneas was isolated using RNA Easy Mini Kit (Qiagen Sciences, Maryland). RNA integrity was verified using agarose gel electrophoresis and quantified by spectrophotometry. RNA was reverse transcribed using a High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA). Heme oxygenase (HO)-1 was amplified using 5'-GATAGAGCGCAACAAGCAGAA-3' and 3'-CAGTGAGGCCCATACCAGAA-5'; VEGFA 5'-TCACCAAAGCCAGCACATAGGAGA-3' and 3'-TTCGTTTAACTCAAGCTGCCTCGC-5'; Fpr1 5'-CATTTGGTTGGTTCATGTGCAA-3' and 3'-AATACAGCGGTCCAGTGCAAT-5'; Fpr-rs2 5'-GCCAGG ACTTTCGTGGAGAGAT-3' and 3'-GATGAACTGGTGCTTGAATCACT-5'; VEGF-R3 (FLT-4) 5'-CTGGCAAATGTTACTCCATGA-3' and 3'-ACAACCCGTGTGTCTTCACTG-5'; 12/15 LOX (Alox15) 5'-GCGACGCTGCCCAATCTAATC-3' and 3'-ATATGGCCACGCTGTTTTCTACC-5'; and soluble flt1 5'-AGGTGAGCACTGCGGCA-3' and 3'-ATGAGTCCTTAAATGTTTAC-5'. Nucleotide primers were selected from the Harvard Primer Bank (pga.mgh.harvard.edu/primerbank/) and verified by the NIH GenBank database or have recently been reported.³⁵⁻³⁹ Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) with a Step One Plus QPCR system (Applied Biosystems). Amplifications were run in duplicates and efficiencies for each primer pair were established.

Comparative quantification of gene expression was performed by Step One software (Applied Biosystems) using the $\Delta\Delta$ CT method. Expression of all genes is referenced to a positive mRNA control that was generated by pooling mRNA from C57Bl/6J mouse spleen and kidney.

Lipid Mediator Lipidomics

For endogenous lipid autacoid analysis, corneas were homogenized with a hand-held tissue grinder in 66% methanol (4°C). The methanol contained deuterated internal standards, prostaglandin (PG)₂-d₄, 15(S)-HETE-d₈, and leukotriene B₄ (LTB₄)-d₄ (400 pg/each), to calculate the recovery of prostanoids or mono-hydroxy- and dihydroxy-containing fatty acids. Lipid autacoids were extracted by solid phase using Accubond ODS-C18 cartridges (Agilent Technologies, Santa Clara, CA)⁴⁰ and the average extraction recovery of our class specific deuterated internal standard was 81% for PGE₂-d₄, 60% for LTB₄-d₄, and 63% for 15-HETE-d₈. Eicosanoids were identified and quantified by liquid chromatography (LC)/mass spectrometry (MS)/MS-based lipidomics.^{37,41-45} In brief, extracted samples were analyzed by a triple quadrupole linear ion trap LC/MS/MS system (MDS SCIEX 3200 QTRAP) equipped with a LUNA C18-2 minibore column using a mobile phase (methanol:water:acetate, 65:35:0.02, v:v:v) with a 0.50 ml/flow rate. MS/MS analyses were performed in negative ion mode and prominent fatty acid metabolites were quantified by multiple reaction monitoring (MRM mode) using established transitions^{37,41,42,44,45} for PGE₂ (351→271, 351→189 *m/z*), thromboxane B₂ (369→169 *m/z*), PGF₂ α (353→193 *m/z*), PGD₂ (351→271 *m/z*), 5-HETE (319→115 *m/z*), 15-HETE (319→175 *m/z*), 12-HETE (319→179 *m/z*), 5,12-DiHETE/LTB₄ (335→195 *m/z*), LXA₄ (351→115 *m/z*), PGE₂-d₄ (335→275 *m/z*), LTB₄-d₄ (339→197 *m/z*), and 15-HETE-d₈ (327→182 *m/z*). Calibration curves (1 to 1000 pg) and specific LC retention times for each compound were established with synthetic standards (Cayman Chemical, Ann Arbor, MI). Structures were confirmed for selected autacoids by MS/MS analyses using enhanced product ion mode with appropriate selection of the parent ion in quadrupole 1.

Statistics

All data are expressed as mean \pm SEM unless otherwise indicated. Student's *t*-test was used to evaluate the significance of differences between two groups and multiple comparisons were performed by regression analysis and one-way analysis of variance. *P* values of less than 0.05 were considered significant.

Results

The cornea in mice and humans is avascular and in general devoid of leukocytes; hence, it is an ideal tissue to study inflammatory neovascularization. Neovascularization is a fundamental response to severe corneal injury

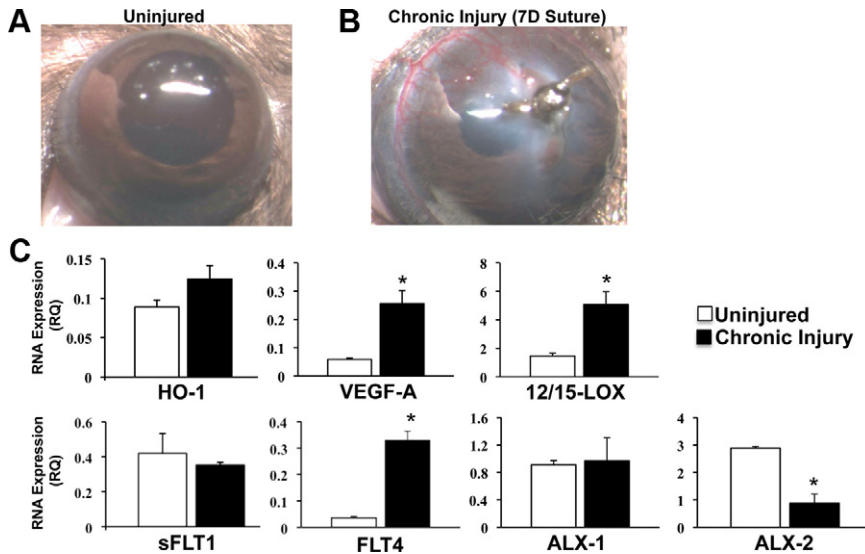


Figure 1. Inflammatory neovascularization differentially regulates expression of the LXA₄ circuit. **A:** Representative images of an uninjured cornea. **B:** An 8.0 silk suture was placed intrastromally in female C57/BL6 mice. Image of cornea with neovascularization after 7 days of chronic injury. **C:** mRNA expression of selected genes was quantified by real-time PCR analyses in corneas after 7 days of chronic injury for heme oxygenase-1 (HO-1), vascular endothelial growth factor A (VEGF-A), soluble VEGF receptor-1 (sFLT1), VEGF receptor-3 (FLT4), 12 and /15 LOX (Alox15), and after 48 hours for ALXR-1 (Fpr1) and ALXR-2 (Fpr-rs2). mRNA expression was compared with matched untreated corneas and is expressed as relative quantity (RQ) compared with a mouse positive mRNA control ($n = 4$, * $P < 0.05$) using the $\Delta\Delta CT$ method and β -actin as a reference gene.

or infection such as microbial keratitis and chemical burns and a key feature of the pathogenesis. We selected the well-established corneal suture model as it induces robust and quantifiable neovascularization as a consequence of chronic injury and irritation.^{10,11,16} Consistent with the model, the silk suture induced robust formation of new blood vessels originating from the vascular border of the cornea (limbus) that become visible by day 4 and are pronounced by day 7 (Figure 1, A–B).

Due to the small tissue size of the mouse cornea, we selected real-time PCR to quantify mRNA expression of key mediator of angiogenesis and pathways that limit the sequelae of corneal injury (Figure 1C). Chronic injury led to a significant 4.3-fold increase in mRNA levels of VEGF-A (injured = 0.256 ± 0.046 relative quantity [RQ] versus uninjured = 0.059 ± 0.005 RQ, $n = 4$) and a significant 9.2 fold increase in the receptor FLT4 (injured 0.330 ± 0.034 RQ versus uninjured = 0.036 ± 0.004 RQ, $n = 4$). In contrast, expression of the soluble FLT1 receptor (sFLT1), which traps VEGF-A in the cornea, did not change in response to injury. Consistent with the temporally defined induction of HO-1,⁴⁶ an early response and cytoprotective gene, levels of HO-1 were no longer significantly elevated after 7 days of chronic injury compared with basal expression. Chronic injury differentially altered the expression of the resident LXA₄ biosynthetic pathway and its receptor (Figure 1C), which are expressed in both recruited leukocyte and resident corneal epithelial cells.^{15,36} Specifically, both ALX receptors (Fpr1 and Fprs2) were expressed in the uninjured cornea of C57BI/6J. Expression of ALX1 (Fpr1) did not change after 2 days of chronic injury. However, expression of ALX2 (Fprs2) decreased by 70% (injured = 2.89 ± 0.61 RQ versus uninjured = 0.873 ± 0.559 RQ, $n = 4$) despite the fact that 2 days is the initial phase of PMN infiltration, a cell type that expresses both ALX1 and ALX2.⁴⁷ In sharp contrast mRNA levels for 12/15-LOX mRNA increased by 3.5 fold (injured = 5.07 ± 0.90 RQ versus uninjured = 1.46 ± 0.19 RQ, $n = 4$) as a consequence of

7-day chronic injury when directly compared with healthy corneas.

To assess if pathological angiogenesis was associated with temporally defined changes in endogenous lipid autacid formation, we used LC/MS/MS-based lipidomics analyses, which demonstrated selective formation of LOX and cyclooxygenase-derived autacoids. Consistent with the time course of neovascularization and up-regulation of 12/15-LOX, endogenous levels of 15-HETE demonstrated a temporally defined increase from 33 pg/cornea in the uninjured cornea to a peak of 94 pg/cornea by day 4 (Figure 2; uninjured = 33 ± 6 pg/cornea versus injured = 94 ± 10 pg/cornea, $n = 4$) coinciding with the first appearance of functional blood vessels. 15-HETE is a metabolic intermediate for the formation of LXA₄ in the cornea and thus a marker for the biosynthetic pathway.¹⁵ We were not able to consistently detect endogenous LXA₄ formation in isolated single corneas of C57BI/6J female mice. However, endogenous LXA₄ formation in the cornea has been reported in a different strain of mice (BALB/c, males) in a model of self-resolving abrasion injury³⁶; these differences in corneal LXA₄ levels likely reflect differences in mouse strain, gender and injury model. Moreover, our lipidomic analysis did not measure tissue levels of 15-oxo-LXA₄,⁴⁵ the primary endogenous metabolite of LXA₄, which may reflect a significant and established route of metabolic inactivation. Thus our measurements likely underestimated the corneal levels of LXA₄, especially during chronic inflammation.

12-HETE levels were higher in both the uninjured (222 ± 55 pg/cornea, $n = 4$) and injured cornea by day 7 (237 ± 61 pg/cornea, $n = 4$) compared with 15-HETE levels, which reflects the expression of several distinct 12-LOX isozymes in the mouse cornea.^{15,36} Unlike 15-HETE, levels of 12-HETE did not increase in response to chronic injury or parallel the time course of neovascularization. Consistent with previous reports, both the uninjured and injured cornea demonstrated 5-LOX activity,³⁶ a key and rate-limiting enzyme in the metabolism of 15-

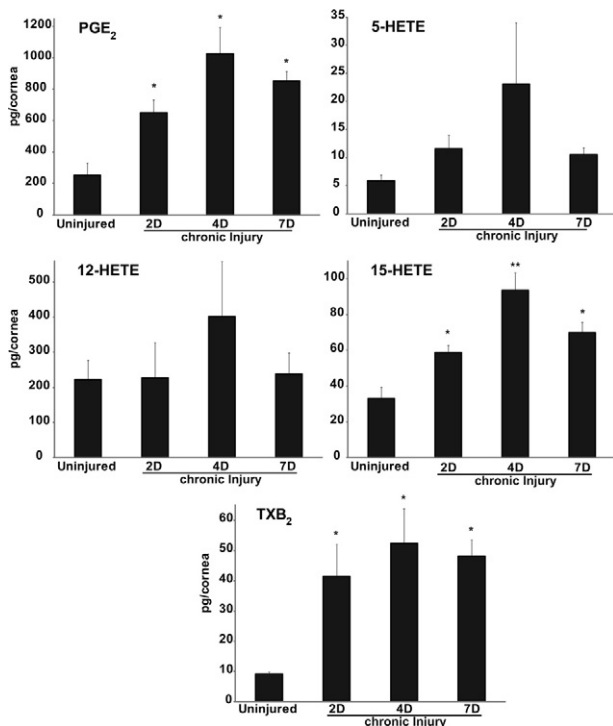


Figure 2. Inflammatory neovascularization is associated with the dynamic formation of select lipid autacoids. Endogenous lipid autacoids were quantified in corneas without injury and after 2, 4, and 7 days of chronic injury by MS based lipidomic analyses using triple quadrupole LC/MS/MS system (MDS SCIEX QTRAP 3200). Specific transition ions and multiple reaction monitoring was used to measure levels of >20 eicosanoids in a single mouse corneas. Significant levels of prostaglandin E₂ (PGE₂), 15-hydroxyeicosatetraenoic acid (15-HETE), thromboxane B₂, 5-hydroxyeicosatetraenoic acid (5-HETE), and 12S-hydroxyeicosatetraenoic acid (12-HETE) were detected in injured and uninjured corneas ($n = 4$, * $P < 0.05$ versus uninjured, ** $P < 0.05$ versus 2 D).

HETE to LXA₄.⁴⁷ 5-LOX activity (ie, 5-HETE formations) did not increase during the time course of chronic injury and consistent with a previous report³⁶ LTB₄ formation was not detected in injured or uninjured corneas. In addition to 15-HETE, formation of the cyclooxygenase metabolites PGE₂ and thromboxane B₂ demonstrated a time dependent increase in response to chronic injury and their formation paralleled that of 15-HETE and neovascularization (Figure 2). PGE₂ was the most prominent eicosanoid in the inflamed and vascularized cornea, peaking

at 1.1 ng/cornea (1030 pg ± 167, $n = 4$) by day 4, which is consistent with its role as key mediator of inflammation and angiogenesis.^{33,48}

To define the endogenous role of the 15-LOX in inflammatory neovascularization, we used the well-defined 12/15-LOX (Alox15) KO mice and matched congenic controls. *In vivo* deletion of 12/15-LOX, which is highly expressed in corneal epithelial cells and recruited leukocytes,³⁶ correlated with a 2.8-fold (12/15-LOX KO = 5125 ± 500 PMN versus wild-type = 1770 ± 1277 PMN, $n = 4$) increase in the number of PMN after 7 days of chronic injury (Figure 3A). These findings are consistent with the notion that 15-LOX is a central pathway for the formation of pro-resolving and anti-inflammatory autacoids,^{18,21,49} such as LXA₄, in the cornea.^{18,21,36} 15-LOX activity (ie, 15-HETE formation) in the injured cornea of 12/15-LOX deficient mice was decreased by 60% (Figure 3B, wild-type = 59 ± 4 pg/cornea versus 12/15-LOX KO = 23 ± 9, pg/cornea, $n = 4$). The lack of complete ablation of 15-LOX activity in the Alox15 KO mice is in agreement with the fact that mice express multiple 12-LOX isozymes in the cornea,¹⁵ which can contribute toward the overall endogenous 15-HETE formation. We have previously demonstrated that topical LXA₄, but not its metabolic intermediate 15-HETE, inhibits exacerbated inflammation in the cornea.³⁵ In the current chronic injury model topical LXA₄ (100 ng, t.i.d.) consistently inhibited PMN infiltration after 48 hours of chronic injury by 49%, when directly compared with saline treatment alone (Figure 3C, LXA₄ = 9523 ± 3226 PMN/cornea versus saline = 18,750 ± 4417 PMN/cornea).

Pathological neovascularization as a consequence of chronic injury (7 days) was markedly exacerbated in 12/15-LOX KO mice, which had a 180% increase in the total number of blood vessels, as compared with the matched congenic wild-type mice (Figure 4A, 12/15-LOX KO = 52899 ± 2477 pixels versus wild-type = 19016 ± 1490 pixels, $n = 4$). Topical LXA₄ (100 ng, 7 days, t.i.d.) consistently attenuated pathological neovascularization by 38% (11775 ± 909 pixels, $n = 3$) and was able to markedly reduce the exacerbated neovascularization by 45% in 12/15-LOX KO mice (28979 ± 1448 pixels, $n = 4$), which have an impaired LXA₄ biosynthetic pathway.³⁶ Since 15S-HETE is a metabolic intermediate in the forma-

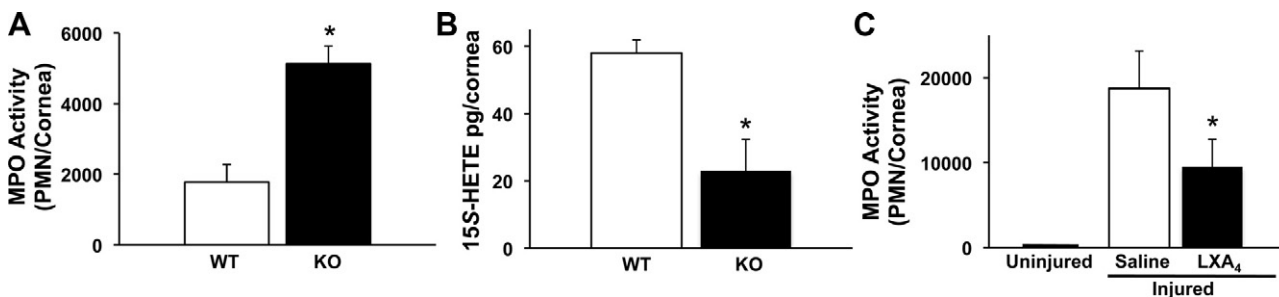


Figure 3. Deletion of a LXA₄ biosynthetic pathway or topical treatment with LXA₄ regulates PMN recruitment to the injured cornea. **A:** PMN content of corneas from 12/15-LOX KO mice and matched congenic wild-type (wt) controls was assessed by measuring tissue MPO activity after 7 days of chronic injury ($n = 4$, $P < 0.02$). A MPO calibration curve was established with inflammatory exudate peritoneal PMN and used to calculate relative tissue PMN numbers. **B:** 15-HETE formation, a metabolic precursor for LXA₄ formation, was determined by LC/MS/MS-based lipidomic analyses after 4 days of chronic injury ($n = 4$; * $P < 0.05$). **C:** Topical action of LXA₄ on PMN recruitment to the injured cornea was determined at 48 hours. Relative PMN numbers in uninjured corneas and injured corneas treated with either saline alone or LXA₄ (100 ng, t.i.d.) were assessed by measuring total tissue MPO activity ($n = 4$, * $P < 0.02$).

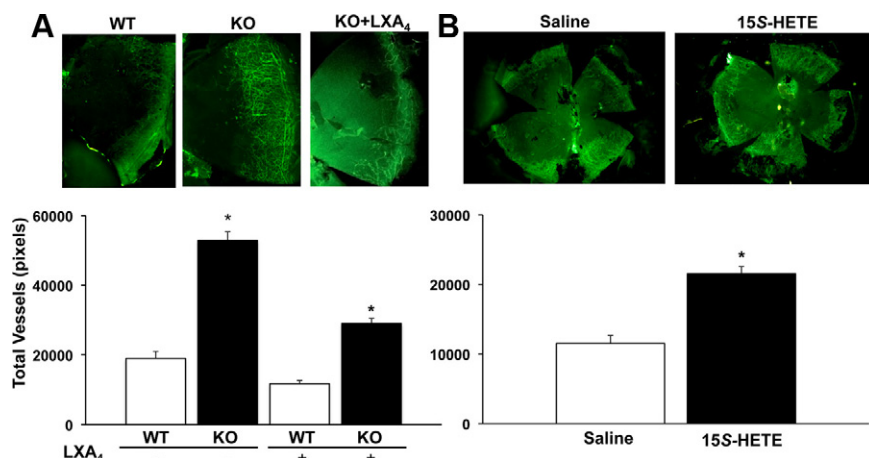


Figure 4. Topical LXA₄ attenuates inflammatory neovascularization and inhibition of its biosynthetic pathway exacerbates pathological angiogenesis. **A:** Heme-angiogenesis in corneas with 7 days of chronic injury was assessed by immunohistochemistry using CD31 as a specific endothelial antigen. Corneas from 12/15-LOX KO or matched congenic wild-type (wt) mice treated with or without topical LXA₄ (100 ng, t.i.d., 7 days) were collected and incubated in PBS containing fluorescein isothiocyanate-conjugated CD31/PECAM-1 monoclonal antibody and analyzed using a Zeiss Axiophot laser scanning confocal microscope. Images show neovascularization of representative corneal quadrants. All CD31⁺ vessels were traced manually and are expressed as total pixels (Image Pro Express 6.0) (*n* = 3 to 4; **P* < 0.05). **B:** Topical 15S-HETE exacerbates inflammatory neovascularization. Injured corneas were treated topically with either 15S-HETE (100 ng, t.i.d.) or sterile saline for 7 days. Images show representative whole corneal flat mounts (**left panel**, saline treatment alone; **right panel** 15S-HETE treatment). All CD31⁺ vessels were traced manually and are expressed as total pixels (*n* = 4; **P* < 0.05).

tion of LXA₄, we assessed if 15S-HETE shared the potent bioactions of topical LXA₄. Treatment with topical 15S-HETE (100 ng, t.i.d., 7 days), contrary to LXA₄, markedly amplified pathological neovascularization by 87% (Figure 4B, 15S-HETE = 21516 ± 1048 pixels versus saline alone = 11497 ± 1142 pixels, *n* = 3).

To obtain proof of concept for a role of endogenous LXA₄ in pathological neovascularization, we used 5-LOX KO mice, which are LXA₄ knockout mice^{49–51} since the mouse genome only contains one 5-LOX enzyme, the obligatory and rate-limiting enzyme for LXA₄ formation.

5-LOX is a well-studied and prominent enzyme in most leukocytes, especially PMN and macrophages.³⁴ Recent reports have established expression and activity of this enzyme in uninjured and inflamed mouse corneas³⁶ and RNA expression in bovine corneas and human corneal epithelium.⁵² Deletion of the LXA₄ biosynthetic pathway in 5-LOX KO mice was associated with a 148% increase in pathological neovascularization (Figure 5) when directly compared with matched congenic wild-type mice (5-LOX KO = 164180 ± 9072 pixels versus wild-type = 66193 ± 6122 pixels, *n* = 4). Taken together, *in vivo* deletion of two key biosynthetic pathways for formation of endogenous LXA₄ in the cornea (ie, 15-LOX and 5-LOX) provides strong evidence for a key role of endogenous LXA₄ circuit as a key regulator of inflammatory neovascularization.

Real-time PCR analysis was used to assess the impact of impaired 12/15-LOX activity on endogenous pathways that are markers of inflammatory neovascularization and stress responses during the initial stage of the angiogenic response (48 hours). mRNA expression of key mediators of inflammatory angiogenesis was markedly amplified in 12/15-LOX KO mice when directly compared with matched congenic wild-type mice (Figure 6). In accordance with exacerbated neovascularization VEGF-A (KO = 8.80 ± 1.32-fold versus wild-type = 4.60 ± 0.64-fold, *n* = 4), sFLT1 (KO = 7.79 ± 1.64-fold versus wild-type = 1.51 ± 0.272 fold, *n* = 4), and FLT4 (KO = 31.7 ± 9.27 versus wild-type = 10.4 ± 1.17-fold, *n* = 4) mRNA levels increased 91%, 413% and 210% in 12/15-LOX KO

mice, respectively. Up-regulation of HO-1 and sFLT1 suggest a compensatory mechanism in this avascular and immune-privileged tissue to counter exacerbated neovascularization in the 12/15-LOX KO mice.

Recent reports have demonstrated that stable analogs of LXA₄ inhibit VEGF-A-induced angiogenic responses in isolated endothelial cells^{25,26,28} and the murine airpouch model²⁷ by inhibiting downstream signals of the VEGF-2 receptor (FLK1). In view of intimate and dynamic interactions of inflammatory and angiogenic pathways in physiological responses to injury, we assessed the *in vivo* actions of native LXA₄ on selected pathways that regulate

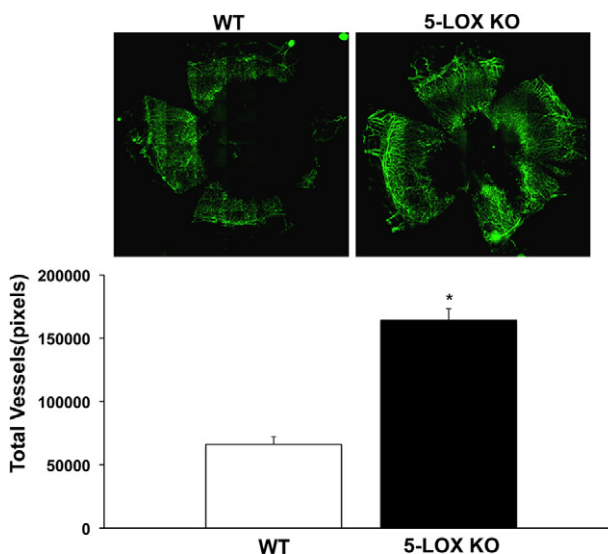


Figure 5. 5-LOX/LXA₄ KO mice have a phenotype of exacerbated neovascularization in response to chronic injury. 5-LOX KO mice, which cannot generate LXA₄, and their matched congenic wild-type (wt) controls were subjected to chronic suture injury for 7 days. Corneas were removed and pathological angiogenesis quantified by immunohistochemistry using a Zeiss Axioplan 2 microscope. Mosaic images were taken with a AxioCam MR camera and compiled using MosaiX and Zeiss AxioVision 4.5 software. **Inset** shows representative whole corneal flatmounts (**left panel**, wild-type; **right panel**, 5-LOX KO). Vessel density was documented and expressed as total pixels (Image Pro Express 6.0) (*n* = 4; **P* < 0.05).

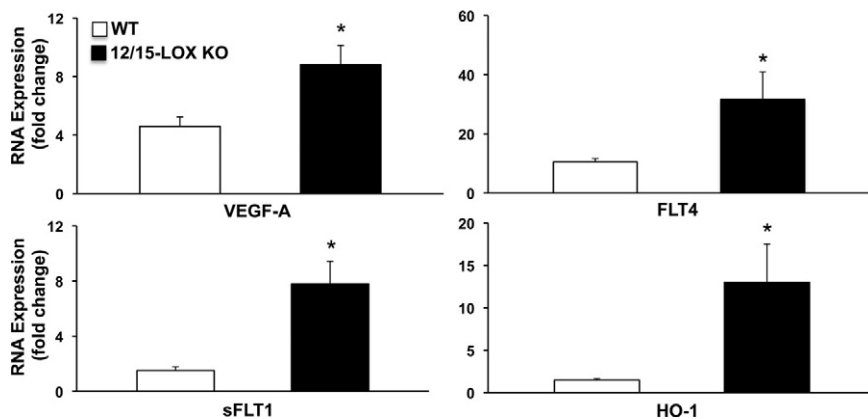


Figure 6. Deletion of 12/15 LOX up-regulates key regulators of inflammatory neovascularization. Corneas after 48 hours of chronic injury were collected from 12/15-LOX KO and matched congenic wild-type mice ($n = 4$). mRNA expression was quantified for HO-1, VEGF-A, sFLT1, and FLT4 using a Step One Plus QPCR system (Applied Biosystems). RNA expression for each gene was calculated using relative quantity (RQ) compared with a mouse positive mRNA control with the $\Delta\Delta CT$ method and β -actin as a reference gene. Data are expressed as fold change compared with the mouse positive mRNA control ($n = 4$, * $P < 0.05$).

the angiogenic and inflammatory responses to chronic injury in the cornea (Figure 7). A fundamental feature of exacerbated/aberrant inflammation, especially in the immune privileged cornea, is the up-regulation of protective pathways such as HO-1 and 12/15-LOX.^{36,38,46,53} Direct topical treatment with LXA₄ (100 ng, t.i.d., Figure 7) significantly reduced levels of HO-1 and 12/15-LOX by 4.4 fold (LXA₄ = 0.010 ± 0.002 RQ versus saline = 0.044 ± 0.042 RQ, $n = 4$) and 3.3 fold (LXA₄ = 0.015 ± 0.02 RQ versus saline = 0.050 ± 0.02 RQ, $n = 4$), respectively. In accordance with the observation that exogenous amplification of the LXA₄ circuit reduced expression of resident protective circuits in the injured cornea, topical LXA₄ also significantly reduced mRNA levels of VEGF-A and FLT4 by 66% (LXA₄ = 0.120 ± 0.024 RQ versus saline = 0.358 ± 0.001 RQ, $n = 4$) and 80% (0.015 ± 0.005 RQ versus 0.003 ± 0.001 RQ), respectively. LXA₄ mediates its established bioactions via the ALX receptor, which is expressed predominantly in the epithelium of healthy

corneas and recruited leukocytes.^{36,47} Direct corneal treatment with LXA₄ abrogated expression of both LXA₄ receptors (ALX-1, LXA₄ = 0.040 ± 0.018 RQ versus saline = 1.05 ± 0.447 RQ; ALX-2 LXA₄ = 0.274 ± 0.097 RQ versus saline = 1.710 ± 0.678 RQ, $n = 3$) in the injured cornea suggesting a ligand-dependent negative feedback loop in this tissue. This response is distinct from the innate immune responses in the peritoneal cavity,⁵⁴ where transgene expression of human ALX in a positive feed forward loop increases endogenous LXA₄ formation by the inflammatory exudate.

Discussion

A coordinated and synergistic array of pro- and anti-angiogenic signals regulates the multicellular process of angiogenesis that leads to the formation of functional blood and/or lymph vessel.³ Inflammation and angiogenesis are triggered, especially in response to injury and insult, by the same molecular events and in a reciprocal fashion these two fundamental responses sustain each other. Indeed, there is strong evidence for a close association or co-dependence between chronic inflammation and angiogenesis in several human diseases.^{2,3}

An essential feature of acute inflammation is the resolution of leukocytes and restoration of normal tissue function.⁵⁵ It is well established that dynamic lipid circuits, such as the LXA₄ circuit, have a pivotal role in the active resolution of normal and vital inflammatory responses.^{49,55-57} Hence, it stands to reason that resolution of heme- or lymphangiogenesis, like inflammation, is an active and highly coordinated process that is regulated by common or shared pathways. 12/15-lipoxygenase is a central biosynthetic pathway in mucosal tissues and the cornea for the formation of LXA₄,¹⁵ an eicosanoid that is formed in both humans and mice.^{49,55-57} In addition, 12/15-LOX is a key enzyme for the formation of ω-3 polyunsaturated fatty acid-derived mediators, namely protectins, resolvins, and maresins,^{36,58-61} which have demonstrated anti-inflammatory and anti-apoptotic and/or pro-resolving bioactions. These 15-LOX products, namely protectins and resolvins, are formed in the eye.^{36,58,60,62} Hence, in addition to LXA₄, 15-LOX can generate additional classes of potent anti-inflammatory mediators, which can contribute to the protective function of

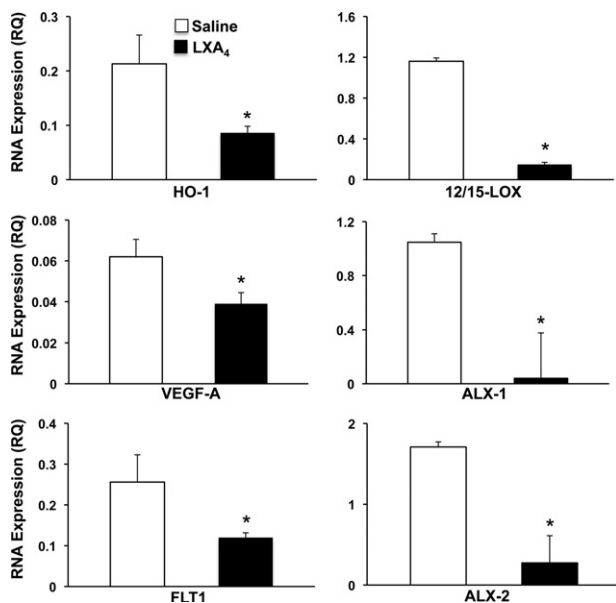


Figure 7. Direct topical treatment with LXA₄ down-regulates expression of key regulators of inflammatory angiogenesis. After suture injury corneas were treated with either saline alone or LXA₄ (100 ng, t.i.d.) for 48 hours. mRNA expression was quantified for HO-1, VEGF-A, FLT4, and both LXA₄ receptors (ALX-1, ALX-2) using a real-time PCR system. Relative expression was compared with a mouse positive mRNA control ($n = 4$, * $P < 0.05$).

this enzyme in the eye. It is of particular interest that chronic injury of the cornea and pathological angiogenesis are associated with increased expression and functional activity of 12/15-LOX (Figure 1 and 2). More importantly, *in vivo* deletion of 12/15-LOX (*Alox15*) resulted in marked amplification of inflammatory neovascularization (Figures 3 and 4), which provides direct evidence that this prominent ocular enzyme^{15,58,60,63,64} has a key role in attenuating inflammatory neovascularization. The notion that 15-LOX is a protective pathway in acute inflammatory responses is supported by studies that demonstrate that *in vivo* transfection with human 15-LOX (*ALOX15*) provides functional protection against immune-mediated renal injury.⁶⁵ Moreover, transgenic rabbits,^{54,66} which selectively express human *ALOX15* in monocytes/macrophages and PMN, demonstrate functional protection against neutrophil-mediated tissue injury, microbial inflammation, and atherosclerosis.

The endogenous role of 15-LOX in angiogenesis is not well defined and has not been explored in models of inflammatory neovascularization. *In vitro* studies that either add 15S-HETE to microvascular endothelial cell lines^{30,31} or express human 15-LOX (*ALOX15*) in rabbit skeletal muscle³² have led to conflicting conclusions, namely, that the 15-LOX pathway either induces angiogenic responses or inhibits VEGF-A induced angiogenesis, respectively. 15-lipoxygenase can initiate the potential formation of several distinct classes of lipid autacoids that have pleiotropic actions. Endogenous formation of two 15-LOX-derived products are firmly established in several animal models of inflammation and human tissue, namely 15S-HETE and LXA₄.^{14,47,49,57} A body of work^{49,55,57,67,68} has established the endogenous formation of LXA₄ and its role as a key mediator of inflammatory resolution.^{55,57} More importantly, unlike other 15-LOX metabolites, data from receptor transgenic mice and detailed structure-activity studies have established specific ALX receptors in humans (FPRL1) and mice (Fpr1 and Fpr-rs2) as a molecular mechanism for LXA₄'s protective *in vivo* actions.⁴⁷

In accordance with our previous studies in male BALB/c mice³⁶ and the original identification of ALX in human corneas,⁶⁹ we detected basal expression of ALX-1 (Fpr1) in uninjured corneas of female C57Bl/6J mice and were also able to detect expression of ALX-2 (Fpr-rs2). More importantly, our findings demonstrate that ALX-2 expression is markedly down-regulated as a consequence of chronic injury, which is coincident with pathological neovascularization. If reduced expression of ALX2 attenuates the protective actions of endogenous LXA₄ and the mechanism for the dynamic regulation of the corneal ALX2 receptor remains to be determined and are of great interest. Our findings are in line with a recent report³⁹ that confirmed expression of a LXA₄ receptor (Fpr-rs2) in the cornea of BALB/c mice. Also note, unlike this report,³⁹ we detected formation of both LXA₄ receptors in uninjured corneas and observed dynamic regulation of ALX-2, which may reflect differences in the sensitivity of analytical methods, mouse strain, and/or gender differences.

The rationale for focusing on the *in vivo* actions of LXA₄ and activation of the corneal ALX receptor^{36,70} as a likely

12/15-LOX induced circuit that attenuates inflammatory neovascularization was based on several independent lines of evidence: 1) the key observation that inflammatory neovascularization was associated with a specific decrease in one of the mouse LXA₄ receptors (Fpr-rs2, Figure 1); 2) previous findings that demonstrate that corneal formation of LXA₄ is attenuated in 12/15-LOX KO mice;³⁶ and 3) reports that have demonstrated that stable mimetics of LXA₄ inhibit VEGF-induced angiogenesis.^{25–27,39}

More importantly, *in vivo* deletion of 5-LOX, which is the obligatory and rate-limiting enzyme for LXA₄ formation^{47,49} led to pronounced amplification of neovascularization (Figure 6).

It is important to note that except for LXA₄, other 5-LOX products such as leukotrienes are potent and well-established pro-inflammatory mediators in both humans and mice.³³ Inflammation is an essential feature of injury-induced neovascularization and amplifies the angiogenic response in the cornea.^{16,71} Hence, the finding that 5-LOX deletion leads to amplified neovascularization indicates an unexpected protective role for this pathway in the immune privileged cornea, which strongly implicates LXA₄ as the metabolic product. Taken together, our data from 5-LOX KO mice, which are LXA₄ knockouts,^{50,51} and from 12/15-LOX KO mice provide strong evidence for endogenous LXA₄ as a key regulator of inflammatory neovascularization.

Topical treatment with LXA₄ initiated immediately after inducing chronic injury not only attenuated formation of blood vessels, but also critical mediators of inflammatory neovascularization, namely VEGF-A and FLT4. In addition, topical LXA₄ reduced expression of HO-1, a stress gene and a marker of inflammation, whose degree of expression directly correlates with the degree of the inflammatory response^{37,72} and is a key feature of non-resolving inflammation in the cornea.³⁸ Contrary to LXA₄, topical 15-HETE exacerbated inflammatory neovascularization (Figure 4), which supports the notion that the protective actions of 15-LOX require metabolism of 15-HETE to LXA₄ by 5-LOX, which is expressed in leukocytes⁴⁹ and the cornea.³⁶

The findings that topical LXA₄ inhibits and *in vivo* deletion of LXA₄ formation exacerbates VEGF-A and FLT4 expression provides a compelling argument for regulation of angiogenic responses by the resident LXA₄ circuit. It is important to recognize that the VEGF-A circuit, in addition to its role as a key angiogenic factor, is also involved in macrophage chemotaxis, a cell type that not only is a major source but also a target for the bioactions of VEGF-A. VEGF-A induces release of several prominent inflammatory mediators in primary endothelial cells such as interleukins 6 and 8, and tumor necrosis factor- α ²⁸ and was originally identified as a potent vascular permeability factor.⁷³ Moreover, local intravitreal injection of VEGF-A leads to local adhesion of leukocytes and monocyte chemotaxis,³ while VEGF-A traps significantly inhibit leukocyte infiltration in the suture injury model.¹⁶ These findings underscore the important role of VEGF-A as a modulator of inflammatory function. Hence, LXA₄'s ability to inhibit VEGF-A up-regulation in response to injury indicates that LXA₄ may regulate the early phase of both angiogenic and inflammatory responses. In this context it

also important to note that our model is inflammatory based neovascularization. Hence, it remains to be determined if our findings regarding the protective actions of the resident LXA₄ circuit extend to hypoxia-driven angiogenesis.

FLT4 is the endothelial receptor for VEGF-C and VEGF-D and mediates lymphangiogenesis. Recent evidence suggests that FLT4 has a key role in sprouting angiogenesis and thus may drive angiogenesis.¹² The observation that FLT4 is required for spontaneous heme-angiogenesis in the cornea of corn1 mice¹¹ support the notion for a pivotal role in pathological neovascularization. It is of particular interest that a functional FLT4 receptor is expressed in corneal epithelial cells.¹⁰ How FLT4 ligation and proven kinase activity¹⁰ regulates epithelial function remains to be defined. What is clear is that chronic injury as early as 48 hours, which is initial phase of angiogenesis, strikingly up-regulates expression of FLT4. Moreover, deletion of protective LXA₄ biosynthetic pathways correlates with amplified expression FLT4 while topical treatment with LXA₄ inhibits its expression (Figure 6, 7). Detailed studies are required to define the cellular targets for LXA₄ in the cornea (ie, FLT4 expressing endothelial cells, epithelial cells and/or macrophages) and if activation of VEGF circuits precedes inflammation or is a consequence of injury-induced inflammation.

Our findings regarding the endogenous role LXA₄ in the model of suture induced angiogenesis are in general agreement with a recent report³⁹ that assessed the therapeutic actions (subconjunctival injections) of a stable analog of LXA₄ and two ω -3 PUFA-derived anti-inflammatory autacoids. However, in regards to FLT4 expression our findings with 12/15-LOX KO mice and direct topical treatment with LXA₄ both indicate that FLT4 expression is regulated by exogenous and endogenous LXA₄ in the cornea, while the report by Lin and colleagues³⁹ shows a strong trend but not significant inhibition in male BALB/c mice. These differences are likely due to differences in the treatment protocols (ie, direct daily t.i.d. corneal treatment versus indirect treatment by subconjunctival injection every 48 hours), the use of a stable LXA₄ analog versus native LXA₄ and mouse strain differences (C57Bl/6J versus BALB/c).

ALX receptors are expressed in leukocytes, epithelial cells and vascular endothelial cells, hence, LXA₄ can regulate the function of all cell types critical to corneal neovascularization. LXA₄'s well-established anti-inflammatory actions with PMN, macrophages, and epithelial cells are consistent with our finding that therapeutic amplification attenuates while *in vivo* inhibition of endogenous LXA₄ formation exacerbates chronic inflammation. However, it is important to recognize that LXA₄ and stable analogs of LXA₄ have been shown to directly regulate signaling of the VEGF-2 (FLK1) receptor and its expression in endothelial cells.^{25,26,28} Thus, the ability of endogenous and therapeutic LXA₄ to inhibit inflammatory neovascularization is likely extends to direct regulation of angiogenesis. In this regard, it is important to recognize that angiogenic responses as a consequence of corneal injury do not require leukocyte infiltration since complete depletion of PMN and monocytes attenuates but does not

prevent angiogenesis.⁷¹ Moreover, the initiating signals or cell types for injury-induced angiogenic response have not been elucidated in the cornea. In view of the fact that the healthy cornea, especially the central cornea, contains no leukocytes the initial targets for LXA₄ are likely epithelial cells, since these cells are a predominant cell source of ALX in human and mouse corneas.^{15,36}

In summary, our current results demonstrate that resident LXA₄ circuits are key determinants for the degree of inflammatory neovascularization in response to chronic injury. Genetic deletion of LXA₄ biosynthetic pathways or amplification with exogenous LXA₄ provides strong evidence that this protective circuit controls key regulators of the angiogenic and inflammatory response in the cornea. In view of the prominent expression of 15-LOX and the ALX receptor in epithelial cells and macrophages, the endogenous role the LXA₄ circuit in pathological angiogenesis clearly warrants further studies.

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