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Novel Anti inflammatory and Pro resolving Lipid Mediators Block Inflammatory Angiogenesis

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Abstract

Purpose—Resolvins and lipoxins are lipid mediators generated from essential polyunsaturated fatty acids that are the first dual anti-inflammatory and pro-resolving signals identified in the resolution phase of inflammation. Here, we investigated the potential of aspirin-triggered lipoxin (LX)A4 analog (ATLa), resolving(Rv)D1, and RvE1, in regulating angiogenesis in a murine model.

Methods—ATLa and RvE1 receptor expression was tested in different corneal cell populations by RT-PCR. Corneal neovascularization (NV) was induced by suture or micropellet (IL-1 β , VEGF-A) placement. Mice were then treated with ATLa, RvD1, RvE1, or vehicle, subconjunctivally at 48 h intervals. Infiltration of neutrophils and macrophages were quantified after immunofluorescence staining. The mRNA expression levels of inflammatory cytokines, VEGFs and VEGFRs were analyzed by real-time PCR. Corneal NV was evaluated both intravitaly and morphometrically.

Results—The receptors for LXA4, ALX/Fpr-rs-2, and for RvE1, ChemR23, were each expressed by epithelium, stromal keratocytes and infiltrated CD11b⁺ cells in corneas. Comparing to the vehicle-treated eye, ATLa-, RvD1- and RvE1-treated eyes had reduced numbers of infiltrating neutrophils and macrophages, as well as reduced mRNA expression levels of TNF- α , IL-1 α , IL-1 β , VEGF-A, VEGF-C and VEGFR2. Animals treated with these mediators had significantly suppressed suture-induced or IL-1 β -induced hemoangiogenesis (HA), but not lymphangiogenesis. Interestingly, only ATLa application significantly suppressed VEGF-A-induced HA.

Conclusions—ATLa, RvE1 and RvD1 each reduce inflammatory corneal HA by early regulation of resolution mechanisms in innate immune responses. In addition, ATLa directly inhibits VEGF-A-mediated angiogenesis and is the most potent inhibitor of NV among this new genus of dual anti-inflammatory and pro-resolving lipid mediators.

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Conflict of interest

Brigham & Women's Hospital is the assignee of patents on lipoxins and resolvins and their stable analogs, for which Dr. Serhan is an inventor. These patents are licensed for clinical development to Bayer HealthCare and Resolvyx Pharmaceuticals and are the basis of consultancy with Dr. Serhan. Other authors have declared that no conflict of interest exists.

Introduction

The normal cornea has no blood or lymphatic vessels. This feature is essential for corneal transparency and optimal visual performance, and contributes to the immunologic privilege of the cornea. Neovascularization (NV) is a common complication secondary to various corneal diseases, including infection, degeneration, trauma and stem cell deficiency-induced insults. NV is also strongly associated with graft failure after corneal transplantation. Additionally, corneal NV as a result of viral or chlamydial (trachoma) infection is a leading cause of visual impairment worldwide.

Corneal NV is a complex response to a number of stimuli, and involves a sequence of coordinated cellular and molecular mechanisms. Dilation of the existing limbal vessels followed by adhesion and diapedesis of leukocytes, such as neutrophils and macrophages, and migration and proliferation of vascular endothelial cells (EC), in large part mediated by vascular endothelial growth factor (VEGF), are all important factors in NV pathogenesis.¹⁻³

Treatment with omega-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are known to be beneficial in a wide range of inflammatory disorders. More recently, the potential efficacy of omega-3 PUFA in inhibition of tumor angiogenesis has been demonstrated.⁴ A novel genus of potent anti-inflammatory and proresolving lipid mediators biosynthesized from PUFA, include three unique families: lipoxins/aspirin-triggered lipoxin (ATL) derived from arachidonic acid, resolvins of the D-series (RvDs) from DHA, and resolvins of the E-series (RvEs) from EPA. Recent findings indicate that these lipid mediators are produced actively during the resolution phase of inflammation to reestablish normal homeostasis. Specifically, Prostaglandin E₂ and prostaglandin D₂ stimulate the switching of arachidonic-acid-derived lipids from leukotriene B₄ production to Lipoxin A₄ production, and the switching of lipid mediator families to produce anti-inflammatory and pro-resolution lipid mediators, such as RvDs and RvEs. Lipoxin A₄, RvD1 and RvE1, these endogenous natural compounds, promote the resolution of exudates, and display potent anti-inflammatory and immunoregulatory functions.⁵ These actions include reducing neutrophil trafficking and regulation of reactive oxygen species.^{6,7} Although the contribution of these lipid mediators in resolution of inflammation and maintenance of homeostasis has been established in several disease models,⁸⁻¹³ their relative efficacy in modulation of angiogenesis has not been investigated systematically. In this study, we report that resolvinD1 (RvD1), resolvin E1 (RvE1), and a stable analog of aspirin-triggered Lipoxin A₄ (ATLa) significantly down-regulate the expression of angiogenic growth factors and their receptors, as well as the infiltration of neutrophils and macrophages concomitant with the suppression of inflammatory cytokines. These changes have a significantly greater effect in reducing hemoangiogenesis (HA) than lymphangiogenesis (LA).

Methods

Animals

Six to eight-week-old male BALB/c (Taconic Farms, Germantown, NY) mice were used in all experiments. All experimental protocols were approved by the Schepens Eye Research Institute Animal Care and Use Committee, and all animals were treated according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Suture induced Inflammatory Corneal Angiogenesis

Our standard model for induction of inflammatory corneal NV is associated with development of intrastromal vessels in close association with a mixed-cell (primarily neutrophilic) infiltrate.

^{14,15} Three interrupted sutures (11–0 nylon, Sharpoint; Vanguard, Houston, TX) were placed intrastromally with two stromal incursions extending over 120° of the corneal circumference each to induce inflammatory corneal NV, which is also associated with significant LA, as described previously.¹⁴ The corneas were followed by slit-lamp biomicroscopy for corneal NV development. NV was graded between 0 and 3, with increments of 0.5, using a grid system per each corneal quadrant based on the centripetal extent of the neovascular branches from the limbus. Scores for each quadrant then summed to derive the NV index (range 0 to 12) for each eye, as previously described.¹⁴

Corneal Micropocket Assay

The corneal micropocket assay in mice and quantification of the resultant NV has been described previously.^{16,17} In brief, 0.3 µl of hydron pellets (IFN Sciences, New Brunswick, NJ) containing 30 ng of murine IL-1β (R&D Systems, Minneapolis, MN) or 200 ng of VEGF-A (gift from BRB Preclinical Repository, National Cancer Institute) were prepared and implanted into the corneal stroma of male BALB/c mice. After 7 days the animals were sacrificed and the corneas were harvested for quantitative analysis of HA and LA.

Ocular Administration of Compounds

BALB/c mice were randomized to receive ATLa, RvD1, RvE1 or vehicle (normal saline) by subconjunctival injection in a masked fashion after suture or hydron pellet placement. The compounds were administered at a dose of 100 ng/10µl per mouse every 48 h after suture or pellet placed. For these experiments, RvD1 and RvE1 were prepared by total organic synthesis as reported previously,^{17,18} in the total organic synthesis core for NIH P-50 DE0169191. ATLa was synthesized as described previously.²⁰ The physical properties were monitored routinely via LC/MS/MS matching the reported biological and physical properties prior to analysis in present experiments.

RNA Isolation and Reverse Transcriptase (RT) PCR

Corneas were carefully dissected to ensure that the conjunctival and iris tissues were not included. To extract mRNA from whole-thickness corneas, two corneas were pooled as a sample in each group. To extract mRNA from corneal epithelial and stroma-endothelial layers separately, intact corneas were placed in 30 µl of RNA stabilization reagent (RNAlater, Qiagen, Valencia, CA) at 4°C overnight and then stored at –30°C for 2–3 days. After incubated in 250 µl 20 mM EDTA (sterile, pH 7.4) at 37°C for 30 minutes, the epithelial layers were peeled off the stroma-endothelial layers before mRNA isolation. Ten corneal epithelial layers or stroma-endothelial layers were pooled as a sample in each group. The mRNA isolated from submandibular lymph nodes were used as positive controls.

A combined-method for total RNA isolation was employed, using Trizol (Invitrogen Corp., Carlsbad, CA) and RNeasy MinElute Spin Columns (Qiagen, Valencia, CA), as described previously.²¹ Reverse transcription of total RNA was conducted using oligo(dT)₂₀ primer and Superscript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR was conducted using primer pairs for Fpr1l (sense GATGCTAGAGGGGATGTGCAC, antisense TCTTCAGGAAGTGAAGCC, 530bp), Fpr-rs2 (sense TGCTGTCAAGATCAACAGAAG, antisense TGCCAGGAGGTGAAGTAGAAC, 359bp), ChemR23 (sense ACCACACCCTCTACCTGCTG, antisense TGGTGAAGCTCCTGTGACTG, 237bp) and GAPDH (sense GAAGGGCATCTTGGGCTACAC, antisense GCAGCGAACTTTATTGATGGTATT, 373bp). The PCR conditions were 35 cycles at 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute, followed by final extension at 72°C for 10 minutes. PCR products were observed by agarose gel electrophoresis. The mean density of each band was measured by using NIH image J software. The density of each receptor

band was divided by the density of the corresponding GAPDH band to obtain the normalized band density.

Real time PCR

1 μ l of total cDNA, synthesized from 400 ng total RNA with random hexamers using SuperscriptTM III Reverse Transcriptase (Invitrogen, Carlsbad, CA), was loaded in each well and assays were performed in triplicates. Quantitative PCR was performed with Taqman Universal PCR Mastermix and FAM2MGB dye labeled predesigned primers (Applied Biosystems, Foster City, CA) for IL-1 α (Mm 99999060_ml), TNF- α (Mm99999068_ml), IL-1 β (Mm00434228_ml), VEGFR2 (Mm00440099_ml), VEGFR3 (Mm00433337_ml), VEGFA (Mm00437304_ml), VEGFC (Mm00437313_ml). PCR conditions were 2 minutes at 50°C, 10 minutes at 95°C, followed by 35 cycles of 15 second at 95°C and 60°C for 1 minute using an ABI PRISM 7900 HT (Applied Biosystems, CA). PCR amplification of the house-keeping gene encoding GAPDH (Mm99999915_gl) was performed during each run for each sample to allow normalization between samples. A nontemplate control was included in all the experiments to evaluate DNA contamination of isolated RNA and reagents. The results were analyzed by comparative threshold cycle (C_T) method. The relative expression level of each sample was expressed as fold change from normal control.

Isolation of Cornea infiltrating Cells

Forty corneas were pooled, teased with scissors, and digested with collagenase D (Roche Applied Science, 11088874103) at 37°C for 1 h in a humidified atmosphere of 5% CO₂. After incubation, corneas were disrupted by grinding with a syringe plunger.²²⁻²⁴ Total cells were then collected after passing through a steel mesh. Upon blockade by anti-FcR mAb, these cells were labeled with FITC-conjugated rat anti-mouse CD11b (granulocyte/monocyte/macrophage marker, BD Pharmingen, San Diego, CA) at 4°C for 30 minutes. CD11b⁺ cells were sorted from total cells by using MoFlo® High-Performance Cell Sorter (Cytomation, Fort Collins, CO).

MK/T-1 Cell Culture and Stimulation

MK/T-1 cells, immortalized keratocytes from the corneal stroma of C57BL/6 mouse (gift from R.L. Gendron [Memorial University of Newfoundland, St. John's, Newfoundland, Canada]), were used to identify the expression of lipid mediator receptors on the corneal keratocytes. MK/T-1 cells were grown in low-glucose Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum and 1 mM α -glutamine at 37°C in 5% CO₂. To stimulate MK/T-1 cells, 10 ng/ μ l TNF- α (R&D Systems, Minneapolis, MN) and 10 ng/ μ l IL-1 β were added in the culture medium.

Immunohistochemical Studies

Full thickness corneal tissue or 8 μ m-frozen sections were fixed in acetone for 15 minutes at room temperature. To block non-specific staining, anti-FcR mAb (CD16/CD31, Fc γ III/II receptor) or 10% goat serum was used before primary antibodies or isotype-matched control antibodies were applied at 4°C overnight. Thereafter, samples were incubated with secondary antibodies at RT. Each step was followed by three thorough washings in PBS for 5–10 minutes. Finally, the samples were covered with mounting medium (Vector Laboratories, Burlingame, CA) and analyzed by epifluorescence microscopy (Eclipse E800; Nikon, Tokyo, Japan). The following antibodies were used: FITC-conjugated rat anti-mouse CD31 (Santa Cruz Biotechnology, Santa Cruz, CA), purified rat anti-mouse neutrophil (NIMP-R14, Abcam, Cambridge, MA), purified rat anti-mouse F4/80 (Novus Biologicals, Littleton, CO) and purified rabbit anti-mouse LYVE-1 (Abcam, Cambridge, MA). The secondary antibodies were Rodamine-conjugated donkey anti-rabbit IgG and Rodamine-conjugated goat anti-rat IgG

(Santa Cruz Biotechnology, Santa Cruz, CA). Isotype controls included FITC-conjugated rat IgG2a, purified rat IgG2b, and purified rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

To quantify corneal angiogenesis, digital pictures of corneal flat-mounts were taken using an image analysis system (Spot Image Analysis, Diagnostic Instruments, Sterling Heights, MI). The areas covered by CD31^{high}/LYVE-1⁻-vessels (blood vessels) or CD31^{low}/LYVE-1^{high} vessels (lymphatics) were measured morphometrically using NIH Image J software (version 1.34s; <http://rsb.info.nih.gov/ij>). The total corneal area was outlined using the inner-most vessel of the limbal arcade as the border. The vessel density was calculated by the proportion of neovascularized area to the whole corneal area.

Statistics

All data are expressed as means $\pm$ SEM. Statistical significance between the vehicle and each lipid mediator group was analyzed by the two-tailed t-test with Prism (version 4.0; GraphPad, San Diego, CA).

Results

LipoxinA4 and Resolvin E1 Receptor Expression in the Cornea

We determined the expression of the receptors for ATLa and RvE1 in corneal tissue by RT-PCR. In the normal corneas, Fpr-rs2 (one of the murine LXA4 receptors) and ChemR23 (the RvE1 receptor), but not Fpr11 (another murine LXA4 receptor), were present in both the epithelial and stromal-endothelial layers (Figure 1). The receptor for RvD1 has not been identified to date.

To further delineate whether corneal keratocytes versus immunocytic CD11b⁺ cells (i.e., macrophages and dendritic cells) in the corneal stroma express these receptors, we cultured MK/T-1 cells (an immortalized corneal keratocyte cell line) stimulated with TNF- α and IL-1 β to mimic in situ corneal inflammation. RT-PCR results showed both Fpr-rs2 and ChemR23 were expressed by MK/T-1 cells irrespective of cytokine stimulation. In addition, the infiltrated CD11b⁺ cells, sorted from the inflamed corneas, also expressed high levels of Fpr-rs2 and ChemR23 (Figure 1).

Application with ATLa, RvD1, or RvE1 Reduces Neutrophil and Macrophage Infiltration

Next, we treated inflamed corneas with ATLa, RvD1, or RvE1 to assess the action of these lipid mediators on the infiltration of neutrophils and macrophages (Figure 2). An approximate 30% inhibition of neutrophils recruitment ($p < 0.05$) into the inflamed corneas was observed at 24 and 72 h with the administration of ATLa (neutrophils; 24 h: 53 ± 1 cells/section, 72 h: 34 ± 5 cells/section, $n=3$), RvD1 (neutrophils; 24 h: 62 ± 4 cells/section, 72 h: 40 ± 3 cells/section, $n=3$), or RvE1 (neutrophils; 24 h: 52 ± 4 cells/section, 72 h: 41 ± 4 cells/section, $n=3$), compared to the vehicle-treated group (neutrophils; 24 h: 81 ± 6 cells/section, 72 h: 57 ± 4 cells/section, $n=3$). Similarly, macrophage infiltration was also reduced, but this was only observed at 72 h after suture placement: ATLa (macrophage; 46 ± 5 cells/section, $n=3$), RvD1 (macrophage; 57 ± 4 cells/section, $n=3$), or RvE1 (macrophage; 49 ± 7 cells/section, $n=3$) resulted in a 25–40% ($p < 0.05$) reduction in macrophage infiltration compared to the vehicle control group (macrophage; 83 ± 3 cells/section, $n=3$).

Application with ATLa, RvD1, or RvE1 Reduces Inflammatory Cytokine Expression

The mRNA levels of inflammatory cytokines, IL-1 α , IL-1 β , and TNF- α , were monitored by real-time PCR at 24 h and 72 h after suture placement (Figure 3). Treatment with ATLa, RvD1 or RvE1 led to a more than 50% reduction in the increase of IL-1 β expression levels compared

to the vehicle-treated controls ($p<0.05$) after induction of corneal inflammation. Increases in TNF- α expression were also significantly suppressed by these three mediators at 24 h, but not at 72 h, except for RvE1 application which led to a significant lowering of TNF- α expression at 72 h. The expression of IL-1 α was not significantly altered by these lipid mediators (Figure 3). We also confirmed protein levels of inflammatory cytokine IL-1 β using ELISA in the different treatment groups. Similar to RNA levels, ATLa-treated group shows 70% decrease in IL-1 β protein level compared to vehicle-treated group ($P<0.001$, data not shown).

ATLa, RvD1, or RvE1 Impacts the Expression of VEGFs and VEGFRs

To determine the effect of ATLa, RvD1, and RvE1 in modulation of angiogenesis, we measured mRNA expression for the critical ligands (VEGF-A, C, D) and receptors (VEGFR-2, 3) involved in angiogenesis (Figure 4). In contrast to the vehicle-treated group, ATLa, RvD1, and RvE1 application groups uniformly had lower mRNA expressions of the angiogenic growth factors, VEGF-A, C and their receptor, VEGFR-2, at 24 h and 72 h after suture placement. However, the mRNA expression for the lymphangiogenic growth factor, VEGF-D and its receptor, VEGFR-3, were not significantly altered in the ATLa, RvD1, and RvE1 groups relative to the vehicle-treated controls.

Evaluation of Clinical Corneal NV and Histological Assessment of HA and LA

We measured the growth of corneal neovessels over a 2-week time course via slit lamp biomicroscopy. Use of corneal sutures in this model induces inflammatory NV within 2 days and peaks approximately 2 weeks post manipulation as described previously.¹⁴ We observed that application with any of the three lipid mediators led to significant suppression of the angiogenic response, relative to the vehicle control (Figure 5). We further compared the density of the blood vessels (BV) and lymphatic vessels (LV) using whole-mounted corneas harvested from the different groups, and co-stained these with anti-CD31 and anti-LYVE21 (BV are CD31^{high}/LYVE-1⁻, while the LV are CD31^{low}/LYVE-1^{high}). Consistent with our slit-lamp observations, by day 14 after suture placement the BV density was significantly suppressed with ATLa ($8.26\pm0.63\%$, $n=6$), RvD1 ($8.4\pm0.39\%$, $n=6$), or RvE1 ($10.92\pm0.53\%$, $n=6$) application, relative to vehicle application ($23.18\pm1.12\%$, $n=6$). Interestingly, we did not observe any significant changes in the density of lymphatic vessels among the lipid mediator groups relative to the vehicle controls.

ATLa, RvD1, or RvE1 Regulation of IL- β and VEGF-A induced HA

To further dissect the direct regulatory actions of these lipid mediators on VEGF-A-induced angiogenesis versus a more 'indirect' inhibitory effect on angiogenesis via suppression of innate immune responses, we measured HA and LA after intrastromal placement of micropellets loaded with IL-1 β - or VEGF-A. Quantitative analysis of corneal flat-mounts harvested from VEGF-A micropellet stimulation showed that the BV density in the group treated with ATLa ($5.9\pm0.4\%$, $n=4$), but not in the group treated with RvD1 ($10.9\pm2.3\%$, $n=4$) or RvE1 ($14.1\pm2.2\%$, $n=4$), was significantly lower relative to vehicle treatment ($14.75\pm3.8\%$, $n=4$). Vessel growth stimulated by IL-1 β -micropellets was more marked than that with VEGF-A stimulation. Nonetheless, treatment with either ATLa (BV density; $16.1\pm3.0\%$, $n=4$), RvD1 (BV density; $17.1\pm2.4\%$, $n=4$), or RvE1 (BV density; $18.6\pm2.2\%$, $n=4$) significantly impaired IL-1 β -induced BV growth, relative to vehicle treatment (BV density; $26.8\pm2.0\%$, $n=4$). Interestingly, and corroborating with our previous observations in suture-induced corneal NV, no significant reduction of LA stimulated by either IL-1 β - or VEGF-A was observed with any of these mediator treatments (Figure 6).

Discussion

Here, we report that the lipid mediators, ATLa, RvD1, and RvE1, regulate VEGF-A/-C and VEGFR2 and as a result, significantly reduce the development of NV in the inflamed cornea, in addition to their resolving effects on innate immunity. We also report that corneal tissues and infiltrating innate immune cells express Fpr-rs2 (the LXA4 receptor) and ChemR23 (the RvE1 receptor); the ligation of which directly suppress angiogenesis. In the aggregate, these results in a model of surgically induced corneal inflammation and angiogenesis confirm the functions of ATLa, RvD1, and RvE1 as potent dual anti-inflammatory and pro-resolution molecules that can also effectively stop angiogenesis.

The neutrophil is the most prominent and earliest cell to migrate into the cornea in the early stages of inflammation, and anti-inflammatory lipid mediators can promote resolution by shortening the duration of neutrophil tissue infiltration.²⁵ In line with this current understanding, we show here that the administration of ATLa, RvD1, and RvE1, indeed blocked neutrophil infiltration of the cornea at 24 h and 72 h post insult—a time point which coincided with the down-regulation of proinflammatory cytokine (e.g., TNF- α , IL-1- α , and IL-1- β) expression known to be secreted by innate immunocytes, in particular neutrophils. Moreover, our data also show that macrophage infiltration is also reduced significantly after local administration of ATLa, RvD1, and RvE1. These results highlight the importance of neutrophil infiltration in the local chemotaxis of subsequent immunocyte populations such as the macrophage. It has been shown that ATLa and RvE1 can also increase macrophage phagocytosis (e.g. of apoptotic neutrophils) and this may also contribute to the resolution of inflammation following treatments.²⁶ Taken together, local administration of these lipid mediators to the cornea control local innate immune cell infiltration and enhance the resolution of inflammation.

While the healthy or normal cornea is avascular, local inflammation can stimulate the ingrowth of neovessels from the surrounding limbal and conjunctival areas through secretion of pro-angiogenesis factors by local vascular endothelial and inflammatory cells. Cytokines such as IL-1 β , IL-1 α , and TNF- α , are known to enhance the expression of angiogenic factors.²⁷⁻²⁹ Among all the angiogenic factors, the VEGF species play a pivotal role in vascular development. Ligation of VEGFR2 by VEGF-A is critical in vascular EC proliferation and differentiation in hemangiogenesis. On the other hand, binding of VEGF-C/-D to VEGFR3 stimulates the development of lymphatic vessels. In addition, VEGF-C can also bind and activate VEGFR2,^{30,31} and thereby contribute to HA, despite having a weaker binding affinity than VEGF-A.³² Interestingly, we found that treatments with ATLa, RvD1, or RvE1 significantly reduced the gene expression levels of VEGF-A, VEGF-C and their receptor VEGFR2, but not VEGF-D or VEGFR3. This indicates that such treatments selectively regulate HA, rather than LA, and this was further supported by immunohistochemistry. Future work will have to further delineate why ATLa, RvD1, or RvE1 lipid mediators differentially regulate VEGF species/receptors.

While IL-1 secretion can stimulate VEGF expression and thereby promote angiogenesis, administration of exogenous VEGF-A can achieve a similar effect (possibly independent of other downstream effectors resulting from IL-1 signaling). We therefore questioned whether the suppression of corneal NV by ATLa, RvD1, and RvE1 is due to suppression of IL-1 β and/or VEGF-A stimulation. Very recently, the contribution of RvD1 and RvE1 to the regulation of angiogenesis through the suppression of TNF- α expression has been reported based on a hypoxia-induced pathological retinal NV mouse model.³³ Here, we show that RvD1 and RvE1 efficiently reduce IL-1 β -induced, but not VEGF-A-induced, corneal angiogenesis. This suggests that the anti-angiogenesis function of RvD1 and RvE1 largely depends on their anti-inflammatory/pro-resolution function, rather than direct regulation of VEGF-A function. This

is further supported by a reduction of macrophage infiltration observed in IL-1 β -induced corneal angiogenesis with RvE1 and RvD1 treatment.³⁴

Interestingly, however, our results indicated that ATLa treatment not only reduced IL-1 β induced corneal angiogenesis, but also that induced by VEGF-stimulation. The exclusive ability to suppress VEGF-A-induced angiogenesis by ATLa treatment, could be in part, result from impairment in the early stage of EC migration.³⁵ It is also noteworthy that relative to the other treatments, ATLa is thought to be active for a longer duration as it is a stable analog of LXA4/ATL which resists local inactivation.³⁶ Moreover, ATLa exerts its inhibitory effects on multiple steps of the VEGF-A-induced angiogenesis, such as inhibition of EC adhesion,^{37,38} and suppression of VEGF-A-induced EC proliferation.³⁹ These factors explain the actions of ATLa and its demonstrable higher potency in suppressing angiogenesis compared to the synthetic forms of RvD1 and RvE1.

LXA4's anti-inflammation and pro-resolution functions are related to the receptor ALX/FPRL1, which has been identified on neutrophils, monocytes, macrophages, dendritic cells, epithelial cells and keratocyte in humans. This function is subserved by multiple receptors in the murine system, including Lxa4r/Fprl1 and Fpr-rs2 which share 89% and 83% homology at the nucleotide and protein levels, respectively.³⁷ ChemR23, a receptor for RvE1 involved in attenuation of TNF- α activated NF- κ B, is abundantly expressed in macrophages and dendritic cells, but less so in neutrophils.^{18,40} Our present findings first demonstrate that both Fpr-rs2 and ChemR23, but not Lxa4r/Fprl1, are expressed by the infiltrating CD11b⁺ cells (which include macrophages, and monocytic dendritic cells, and a subset of neutrophils) in the cornea. These results suggest that ATLa activation of Fpr-rs2, and RvE1 activation of ChemR23, on these CD11b⁺ immunocytes stops their local migration and cytokine production into cornea.

Finally, it is noteworthy that while the expression of ALX in the cornea has been implicated in epithelial cell proliferation in a wound healing model,^{41,42} the location of ALX in the cornea had not been established to date. Here, for the first time, we distinguished the distributions of Fpr-rs2 and ChemR23 in the cornea, which include epithelial cells and stromal keratocytes in both normal and inflammatory conditions. In conclusion, ATLa, RvD1, and RvE1 effectively resolve corneal inflammation and angiogenesis by controlling innate inflammation via marked reduction of proinflammatory cytokine secretion and inhibiting VEGF/VEGFR expression. These novel lipid mediators offer a potentially new therapeutic strategy in controlling corneal angiogenesis, a leading cause of visual blindness worldwide.

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Nonstandard abbreviations used

ATLa, 15-epi-16-(*p*-fluorophenoxy)-lipoxin A4 methyl ester)
 BV, blood vessels
 DHA, docosahexaenoic acid
 EC, endothelial cells
 EPA, eicosapentaenoic acid
 HA, hemoangiogenesis

LA, lymphangiogenesis
 LV, lymphatic vessels
 LXA4, lipoxin A4
 NV, neovascularization
 Rv, resolvin, resolution phase interaction product
 RvD1, 7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid
 RvE1, 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-EPA
 PUFA, polyunsaturated fatty acids

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Figure 1. Expression of receptors: Fpr-rs2 and ChemR23 in Inflamed Corneas

A. RT-PCR was used to analyze expression of Fpr11, Fpr-rs2 (ALX, LXA4 receptors), and ChemR23 (RvE1 receptor). Corneas of normal or inflamed eyes (10 corneas pooled per group) were collected and the epithelium was subsequently separated from the subjacent stroma-endothelium in the respective groups. RNA was isolated from these tissues, as well as from MK/T-1 cells (corneal keratocyte cell line) with or without TNF- α and IL-1 β stimulation, or FACS-sorted CD11b⁺ cells from inflamed corneas. In addition, RNA was isolated from lymph nodes as a positive control. This experiment was repeated 3 times. **B.** The mean density of each band was measured by using NIH image J software. The densities of Fpr-rs2 and ChemR23

band were normalized with the density of the corresponding GAPDH band. Data is shown as a mean of 3 experiments, and error bars represent SEM.

Figure 2. Resolvins and Lipoxin Reduce Neutrophil and Macrophage Infiltration in Inflamed Corneas

ATLa, RvD1, RvE1, or vehicle was subconjunctivally injected at 0 h and 48 h after suture placement. For each compound treatment, eyes were enucleated from a group of mice at 24 h, and another group at 72 h after suture placement (3 eyes per group). Cross-sections were stained with anti-neutrophil (NIMP-R14) or anti-macrophage (F4/80) Ab, and 12 sections were used to enumerate the respective leukocyte populations. Results represent the mean ($\pm$ SEM) of 3 eyes per group (* $P < 0.05$, ** $P < 0.001$ vs vehicle-treated group, t-test), and data are representative of two independent experiments.

Figure 3. Resolvins and Lipoxin Reduce Cytokine mRNA Expressions in Inflamed Corneas
ATLa, RvD1, RvE1, or vehicle was subconjunctivally injected at 0 h and 48 h after suture placement. For each compound treatment, corneas were harvested from a group of mice at 24 h, and another group at 72 h after suture placement, as well as from normal untreated control corneas (6 corneas per group). mRNA levels of inflammatory cytokines (including IL-1 α , IL-1 β , and TNF- α) were determined by real-time PCR. Data were normalized to GAPDH mRNA and values were expressed as the fold change over normal control corneas. Results represent the mean ($\pm$ SEM) of three samples per group (each sample consisted of 2 pooled corneas), and data are representative of two independent experiments (* P <0.05, ** P <0.001 vs vehicle-treated group, t-test).

Figure 4. The Impact of Resolvins and Lipoxin on the mRNA Expression of VEGFs and VEGFRs in Inflamed Corneas

ATLa, RvD1, RvE1, or vehicle was subconjunctivally injected at 0 h and 48 h after suture placement. For each compound treatment, corneas were harvested from a group of mice at 24 h, and another group at 72 h after suture placement, as well as from normal untreated control corneas (6 corneas per group). (A) VEGF ligand species (VEGF-A, VEGF-C, VEGF-D) and (B) VEGFRs (VEGFR2 and VEGFR3) were tested by real-time PCR and normalized to GAPDH mRNA. Values are expressed as fold change over the normal control cornea. Results represent the mean ($\pm$ SEM) of three samples per group (each sample consisted of 2 pooled corneas), and the data are representative of two independent experiments (* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0005$, vs vehicle-treated group, t-test).

Figure 5. Suture induced Corneal HA is Reduced with Resolvins and Lipoxin

ATLa, RvD1, RvE1, or vehicle was subconjunctivally injected every 48 h from 0 to 14 days after suture placement. **A.** In a masked fashion, corneal NV was scored biomicroscopically with a slit-lamp using a grid system. Values are expressed as the mean ($\pm$ SEM) of 6 corneas. **B.** Whole corneal tissues were harvested on day 14 and double-stained with anti-CD31 (green) and anti-LYVE-1 (red) for epifluorescence microscopy (20x magnification). **C.** The density of blood vessels ($CD31^{high}/LYVE-1^{-}$) or lymphatic vessels ($CD31^{low}/LYVE-1^{high}$) covering the cornea was analyzed. Values are expressed as the mean ($\pm$ SEM) of 6 corneas of per treatment group (** $P < 0.001$ vs vehicle-treated group, t-test), and the data are representative of two independent experiments.

Figure 6. ATLa, RvD1, and RvE1 Regulate IL-1 β and VEGF-A-induced Corneal HA and LA
Hydron pellets containing IL-1 β or VEGF-A were implanted into the corneas on day 0. ATLa, RvD1, RvE1, or vehicle was subconjunctivally injected every 48 h from 0 to 7 days after pellet implantation (4 corneas per treatment, for each pellet-type). **A.** Slit-lamp examination was performed on day 7 and representative images are shown. **B.** The density of blood vessels (CD31^{high}/LYVE-1^{low}) or lymphatic vessels (CD31^{low}/LYVE-1^{high}) covering each cornea was analyzed. Values are expressed as the mean ($\pm$ SEM) of each treatment group (4 corneas per group) and the data are representative of two independent experiments (* $P < 0.05$ vs vehicle-treated group, t-test).