

Anti-angiogenesis Effect of the Novel Anti-inflammatory and Pro-resolving Lipid Mediators

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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 the authors 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 (CNV) 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-hour intervals. Infiltration of neutrophils and macrophages was quantified after immunofluorescence staining. The mRNA expression levels of inflammatory cytokines, VEGFs, and VEGFRs were analyzed by real-time PCR. CNV was evaluated intravitally 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. Compared to the vehicle-treated eye, ATLa, RvD1-, and RvE1-treated eyes had reduced numbers of infiltrating neutrophils and macrophages and 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 hemangiogenesis (HA) but not lymphangiogenesis. Interestingly, only the application of ATLa significantly suppressed VEGF-A-induced HA.

CONCLUSIONS. ATLa, RvE1, and RvD1 all 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. (*Invest Ophthalmol Vis Sci.* 2009;50:4743–4752) DOI:10.1167/iovs.08-2462

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, CNV (CNV) as a result of viral or chlamydial (trachoma) infection is a leading cause of visual impairment worldwide.

CNV 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 (ECs), 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 (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is known to be beneficial in a wide range of inflammatory disorders. More recently, the potential efficacy of omega-3 PUFA in the inhibition of tumor angiogenesis has been demonstrated.⁴ A novel genus of potent anti-inflammatory and pro-resolving lipid mediators biosynthesized from PUFA includes 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, the endogenous natural compounds, promote the resolution of exudates and display potent anti-inflammatory and immunoregulatory functions.⁵ These actions include reducing neutrophil trafficking and regulating reactive oxygen species.^{6,7} Although the contribution of these lipid mediators in the resolution of inflammation and maintenance of homeostasis has been established in several disease models,^{8–13} their relative efficacy in the modulation of angiogenesis has not been investigated systematically. In this study, we report that resolvin D1 (RvD1), resolvin E1 (RvE1), and a stable analog of aspirin-triggered lipoxin A₄ (ATLa) significantly downregulate the expression of angiogenic growth factors and their receptors and the infiltration of neutrophils and macrophages concomitant with the suppression of inflammatory cytokines. These changes have a significantly greater effect in reducing hemangiogenesis (HA) than lymphangiogenesis (LA).

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METHODS

Animals

Six- to 8-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 ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Suture-Induced Inflammatory Corneal Angiogenesis

Our standard model for the induction of inflammatory CNV is associated with the 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 to induce inflammatory CNV, which is also associated with significant LA, as described.¹⁴ The corneas were followed by slit lamp biomicroscopy for CNV 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 were then summed to derive the NV index (range, 0–12) 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 μ L hydron pellets (IFN Sciences, New Brunswick, NJ) containing 30 ng murine IL-1 β (R&D Systems, Minneapolis, MN) or 200 ng 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 killed 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/10 μ L per mouse every 48 hours after suture or pellet placement. For these experiments, RvD1 and RvE1 were prepared by total organic synthesis, as reported previously,^{18,19} in the total organic synthesis core for NIH P50 DE0169191. ATLa was synthesized as described previously.²⁰ Physical properties were monitored routinely by liquid chromatography coupled to tandem mass spectrometry matching the reported biological and physical properties before analysis in the present experiments.

RNA Isolation and 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 μ L RNA stabilization reagent (RNAlater; Qiagen, Valencia, CA) at 4°C overnight and then were stored at –30°C for 2 to 3 days. After they were incubated in 250 μ L of 20 mM EDTA (sterile, pH 7.4) at 37°C for 30 minutes, the epithelial layers were peeled from 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 was used as a positive control.

A combined-method for total RNA isolation was used with reagent (Trizol; Invitrogen Corp., Carlsbad, CA) and spin columns (RNeasy MinElute; Qiagen, Valencia, CA), as described previously.²¹ Reverse transcription of total RNA was conducted using oligo(dT)₂₀ primer and reverse transcriptase (Superscript III; Invitrogen, Carlsbad, CA). PCR was

conducted using primer pairs for Fpr1 (sense, GATGCTAGAGGGGATGT-GCAC; antisense, TCTTCAGGAAGTGAAGCC; 530 bp), Fpr-rs2 (sense, TGCTGTCAAGATCAACAGAAG; antisense, TGCCAGGAGGTGAAGTA-GAAC; 359 bp), ChemR23 (sense, ACCACACCCCTCTACCTGCTG; antisense, TGGTGAAGCTCCTGTGACTG; 237 bp), and GAPDH (sense, GAAGGGCATCTTGGGCTACAC; antisense, GCAGCGAAGCTTATTGATG-GTATT; 373 bp). 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 with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). 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

One microliter total cDNA, synthesized from 400 ng total RNA with random hexamers using reverse transcriptase (Superscript III; Invitrogen, Carlsbad, CA), was loaded in each well, and assays were performed in triplicate. Quantitative PCR was performed with PCR master mix (TaqMan Universal; Applied Biosystems, Foster City, CA) and dye-labeled pre-designed primers (FAM-MGB; Applied Biosystems) for IL-1 α (Mm9999060_ml), TNF- α (Mm99999068_ml), IL-1 β (Mm00434228_ml), VEGFR2 (Mm00440099_ml), VEGFR3 (Mm00433337_ml), VEGFA (Mm00437304_ml), and VEGFC (Mm00437313_ml). PCR conditions were 2 minutes at 50°C, 10 minutes at 95°C, followed by 35 cycles of 15 seconds at 95°C and then 1 minute 60°C (ABI PRISM 7900 HT; Applied Biosystems). PCR amplification of the housekeeping gene encoding GAPDH (Mm999999915_g1) 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. 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 (11088874103; Roche Applied Science, Indianapolis, IN) at 37°C for 1 hour 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 they were passed through a steel mesh. On 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 with a high-performance cell sorter (MoFlo; 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 Robert 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 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) and 10 ng/ μ L IL-1 β were added to 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 nonspecific 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 room temperature. Each step was followed by three thorough washings in PBS for 5 to 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). Secondary antibodies were rhodamine-conjugated donkey anti-rabbit IgG and rhodamine-conjugated goat anti-rat IgG (Santa Cruz Biotechnology). Isotype controls included FITC-conjugated rat IgG2a, purified rat IgG2b, and purified rabbit IgG (Santa Cruz Biotechnology).

To quantify corneal angiogenesis, digital pictures of corneal flat-mounts were taken using an image analysis system (Spot Image Analysis; Diagnostic Instruments, Sterling Heights, MD). The areas covered by CD31^{high}/LYVE-1^{low} vessels (blood vessels) or CD31^{low}/LYVE-1^{high} vessels (lymphatics) were measured morphometrically with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). The total corneal area was outlined using the innermost vessel of the limbal arcade as the border. Vessel density was calculated by the proportion of neovascularized area to the whole corneal area.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical significance between the vehicle and each lipid mediator group was analyzed by the two-tailed *t*-test (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 Fprl1 (another murine LXA4 receptor), were present in the epithelial and stroma-endothelial layers (Fig. 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-1β 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 (Fig. 1).

Reduction of Neutrophil and Macrophage Infiltration by ATLa, RvD1, or RvE1

Next, we treated inflamed corneas with ATLa, RvD1, or RvE1 to assess the action of these lipid mediators on the infiltration

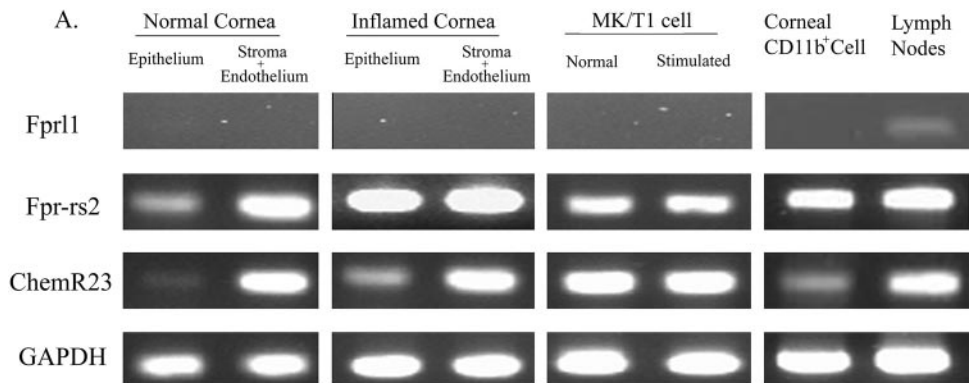
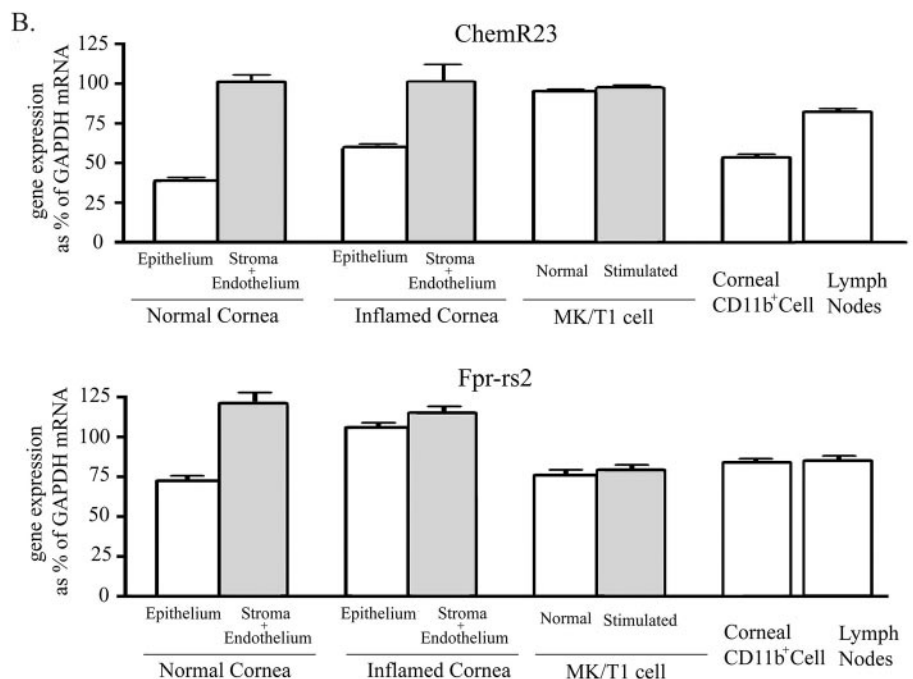


FIGURE 1. Expression of receptors: Fpr-rs2 and ChemR23 in inflamed corneas. (A) RT-PCR was used to analyze the expression of Fprl1, Fpr-rs2 (ALX, LXA4 receptors), and ChemR23 (RvE1 receptor). Corneas of normal or inflamed eyes (10 corneas pooled per group) were collected, and the epithelium was subsequently separated from the subjacent stroma-endothelium in the respective groups. RNA was isolated from these tissues and from MK/T-1 cells (corneal keratocyte cell line) with or without TNF-α and IL-1β 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 three times. (B) Mean density of each band was measured by using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). Densities of the Fpr-rs2 and ChemR23 band were normalized with the density of the corresponding GAPDH band. Data are shown as a mean of three experiments, and error bars represent SEM.



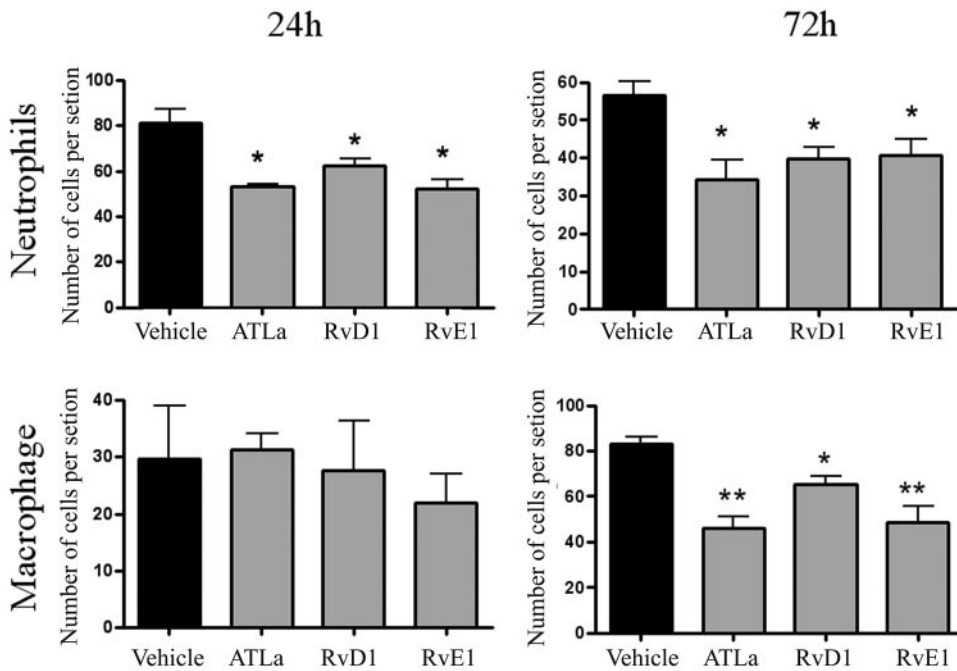


FIGURE 2. Resolvins and lipoxin reduce neutrophil and macrophage infiltration in inflamed corneas. ATLa, RvD1, RvE1, or vehicle was subconjunctivally injected at 0 hour and 48 hours after suture placement. For each compound treatment, eyes were enucleated from a group of mice at 24 hours and from another group at 72 hours after suture placement (three eyes per group). Cross-sections were stained with anti-neutrophil (NIMP-R14) or anti-macrophage (F4/80) antibody, and 12 sections were used to enumerate the respective leukocyte populations. Results represent the mean (\pm SEM) of three eyes per group (* P < 0.05, ** P < 0.001 vs. vehicle-treated group; t -test), and data are representative of two independent experiments.

of neutrophils and macrophages (Fig. 2). Approximately 30% inhibition of neutrophil recruitment (P < 0.05) into the inflamed corneas was observed at 24 and 72 hours with the administration of ATLa (neutrophils: 24 hours, 53 ± 1 cells/section; 72 hours, 34 ± 5 cells/section; $n = 3$), RvD1 (neutrophils: 24 hours, 62 ± 4 cells/section; 72 hours, 40 ± 3 cells/section; $n = 3$), or RvE1 (neutrophils: 24 hours, 52 ± 4 cells/section; 72 hours, 41 ± 4 cells/section; $n = 3$) compared with the vehicle-treated group (neutrophils: 24 hours, 81 ± 6 cells/section; 72 hours, 83 ± 3 cells/section; $n = 3$). Similarly, macrophage infiltration was also reduced, but this was only observed at 72 hours 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% to 40% (P < 0.05) reduction in macrophage infiltration compared with the vehicle control group (macrophage: 83 ± 3 cells/section; $n = 3$).

Reduction of Inflammatory Cytokine Expression by ATLa, RvD1, or RvE1

The mRNA levels of inflammatory cytokines IL-1 α , IL-1 β , and TNF- α were monitored by real-time PCR at 24 hours and 72 hours after suture placement (Fig. 3). Treatment with ATLa, RvD1, or RvE1 led to greater than 50% reduction in the increase of IL-1 β expression levels compared with vehicle-treated controls (P < 0.05) after the induction of corneal inflammation. Increases in TNF- α expression were also significantly suppressed by these three mediators at 24 hours, but not at 72 hours, except for RvE1 application, which led to a significant lowering of TNF- α expression at 72 hours. The expression of IL-1 α was not significantly altered by these lipid mediators (Fig. 3). We also confirmed the protein levels of inflammatory cytokine IL-1 β using ELISA in the different treatment groups. As with RNA levels, the ATLa-treated group showed a 70% decrease in IL-1 β protein level compared with the vehicle-treated group (P < 0.001; data not shown).

Impact of ATLa, RvD1, or RvE1 on the Expression of VEGFs and VEGFRs

To determine the effect of ATLa, RvD1, and RvE1 in the modulation of angiogenesis, we measured mRNA expression

for the critical ligands (VEGF-A, -C, -D) and receptors (VEGFR-2, -3) involved in angiogenesis (Fig. 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, VEGF-C, and their receptor, VEGFR-2, at 24 hours and 72 hours after suture placement. However, mRNA expression for the lymphangiogenic growth factor VEGF-D and its receptor VEGFR-3 were not significantly altered in the ATLa, RvD1, or RvE1 groups compared with vehicle-treated controls.

Evaluation of Clinical CNV and Histologic Assessment of HA and LA

We measured the growth of corneal neovessels over a 2-week period by slit lamp biomicroscopy. Use of corneal sutures in this model induces inflammatory NV within 2 days and peaks approximately 2 weeks after manipulation, as described previously.¹⁴ We observed that application with any of the three lipid mediators led to significant suppression of the angiogenic response compared with the vehicle control (Fig. 5). We further compared the density of blood vessels (BVs) and lymphatic vessels (LVs) using whole-mounted corneas harvested from the different groups, and costained these with anti-CD31 and anti-LYVE-1 (BVs are CD31^{high}/LYVE-1^{low}, whereas LVs 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 ($8.26\% \pm 0.63\%$; $n = 6$), RvD1 ($8.4\% \pm 0.39\%$; $n = 6$), or RvE1 ($10.92\% \pm 0.53\%$; $n = 6$) application compared with vehicle application ($23.18\% \pm 1.12\%$; $n = 6$). Interestingly, we did not observe any significant changes in the density of lymphatic vessels among the lipid mediator groups compared with 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 compared with a more indirect inhibitory effect on angiogenesis by the 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 flatmounts har-

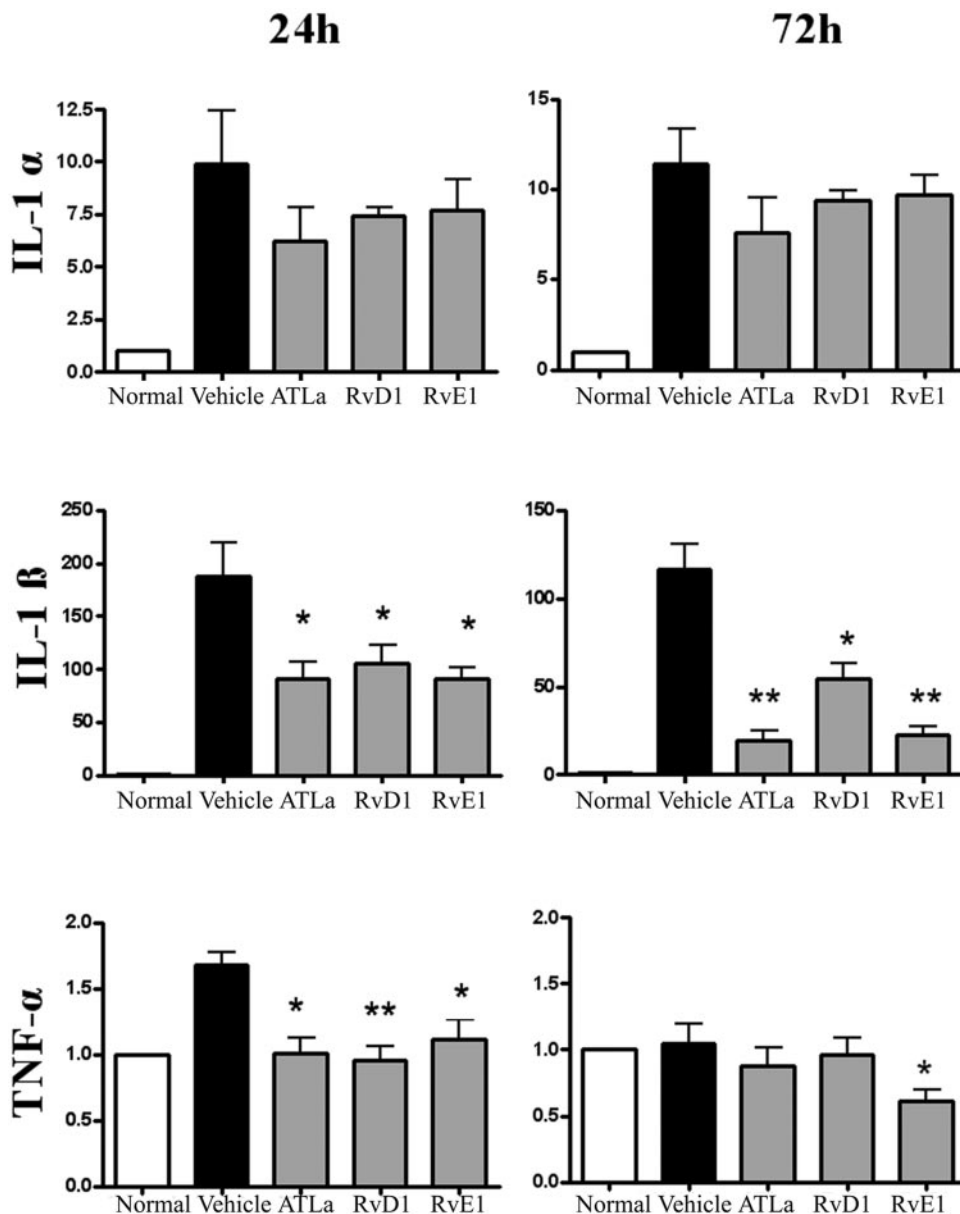


FIGURE 3. Resolvins and lipoxin reduce cytokine mRNA expression in inflamed corneas. ATLa, RvD1, RvE1, or vehicle was subconjunctivally injected at 0 hour and 48 hours after suture placement. For each compound treatment, corneas were harvested from a group of mice at 24 hours, from another group at 72 hours after suture placement, and from normal untreated control corneas (six 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 two pooled corneas), and data are representative of two independent experiments (* P < 0.05, ** P < 0.001 vs. vehicle-treated group; t -test).

vested from VEGF-A micropellet stimulation showed that BV density in the group treated with ATLa ($5.9\% \pm 0.4\%$; $n = 4$), but not in the group treated with RvD1 ($10.9\% \pm 2.3\%$; $n = 4$) or RvE1 ($14.1\% \pm 2.2\%$; $n = 4$), was significantly lower than in the group treated with vehicle ($14.75\% \pm 3.8\%$; $n = 4$). Vessel growth stimulated by IL-1 β micropellets was more marked than vessel growth stimulated by VEGF-A. Nonetheless, treatment with ATLa (BV density: $16.1\% \pm 3.0\%$; $n = 4$), RvD1 (BV density: $17.1\% \pm 2.4\%$; $n = 4$), or RvE1 (BV density: $18.6\% \pm 2.2\%$; $n = 4$) significantly impaired IL-1 β -induced BV growth compared with vehicle treatment (BV density: $26.8\% \pm 2.0\%$; $n = 4$). Interestingly, and corroborating our previous observations in suture-induced CNV, no significant reduction of LA stimulated by either IL-1 β or VEGF-A was observed with any of these mediator treatments (Fig. 6).

DISCUSSION

Here we report that the lipid mediators ATLa, RvD1, and RvE1—in addition to their resolving effects on innate immu-

nity—regulate VEGF-A/C and VEGFR2 and, as a result, significantly reduce the development of NV in the inflamed cornea. 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 suppresses 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 to 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 hours and 72 hours after insult, coinciding with the downregulation of proinflammatory cytokine (e.g., TNF- α , IL-1 α , and IL-1 β) expression known to be secreted by innate immunocytes,

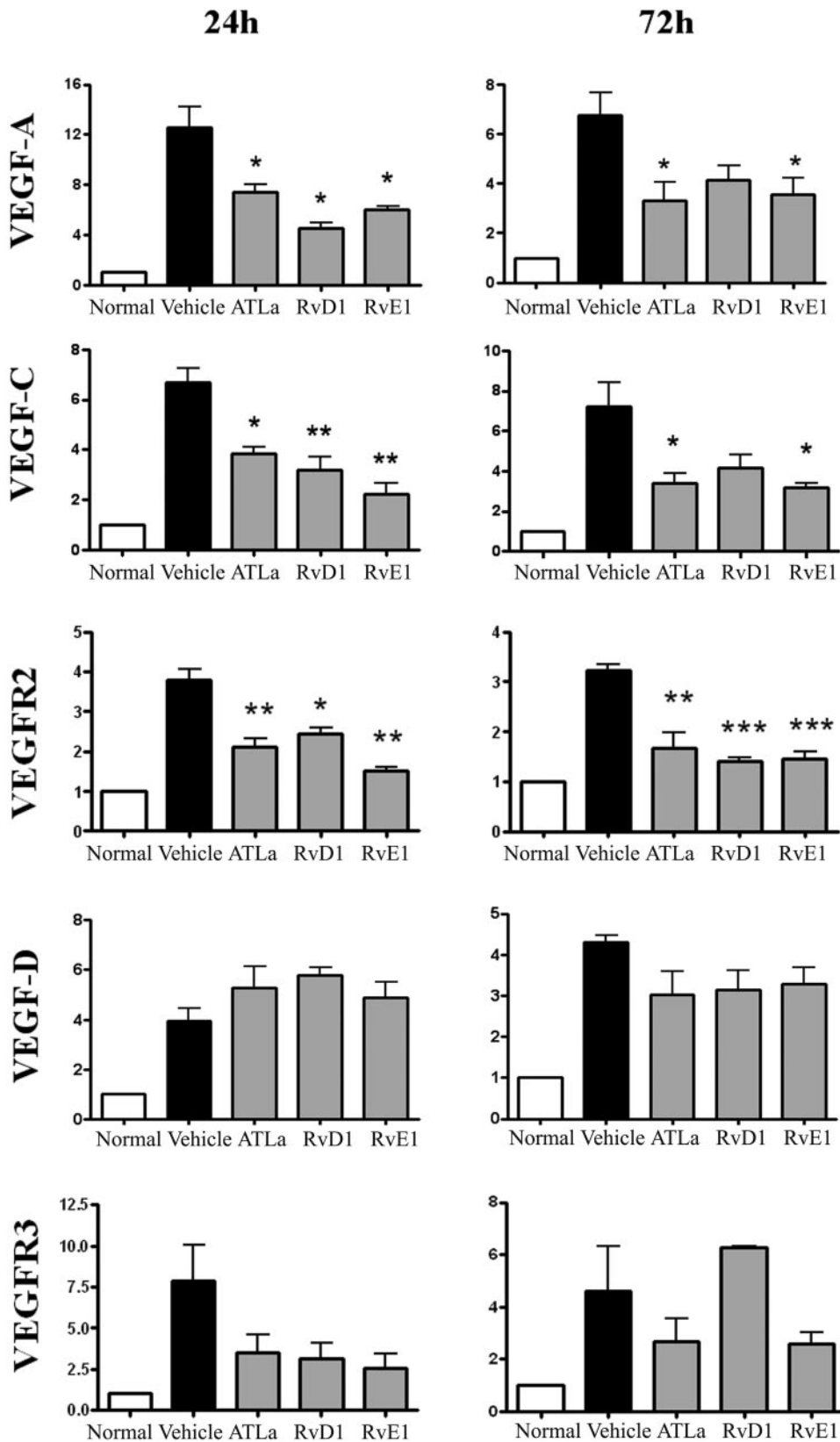


FIGURE 4. The impact of resolvins and lipoxin on mRNA. Expression of VEGFs and VEGFRs in inflamed corneas. ATLa, RvD1, RvE1, or vehicle was subconjunctivally injected at 0 hour and 48 hours after suture placement. For each compound treatment, corneas were harvested from a group of mice at 24 hours, from another group at 72 hours after suture placement, and from normal untreated control corneas (six 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 two 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).

particularly neutrophils. Moreover, our data show that macrophage infiltration is also reduced significantly after the 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 contribute to the resolution of inflammation after treatment.²⁶ Taken together, local administration of these lipid mediators to the cornea

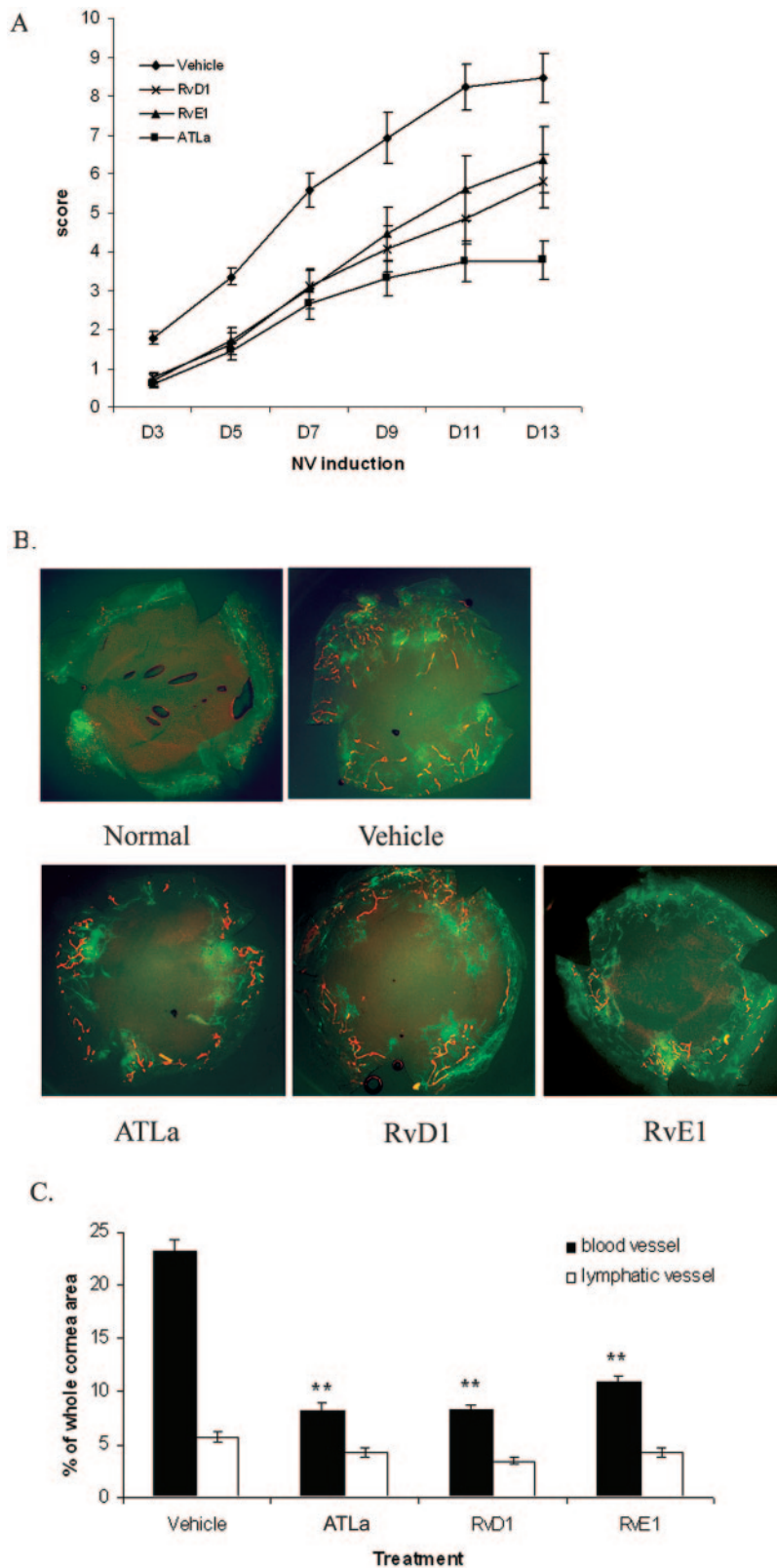


FIGURE 5. Suture-induced corneal HA is reduced with resolvins and lipoxin. ATLa, RvD1, RvE1, or vehicle was subconjunctivally injected every 48 hours from 0 to 14 days after suture placement. **(A)** In a masked fashion, CNV was scored biomicroscopically with a slit lamp using a grid system. Values are expressed as the mean (\pm SEM) of six corneas. **(B)** Whole corneal tissues were harvested on day 14 and were double stained with anti-CD31 (green) and anti-LYVE-1 (red) for epifluorescence microscopy (20 \times magnification). **(C)** The density of blood vessels (CD31^{high}/LYVE-1^{low}) or lymphatic vessels (CD31^{low}/LYVE-1^{high}) covering the cornea was analyzed. Values are expressed as the mean (\pm SEM) of six corneas per treatment group (** P < 0.001 vs. vehicle-treated group; t -test), and the data are representative of two independent experiments.

control local innate immune cell infiltration and enhance the resolution of inflammation.

Although the healthy or normal cornea is avascular, local inflammation can stimulate the ingrowth of neovessels from the surrounding limbal and conjunctival areas through the secretion of proangiogenesis 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

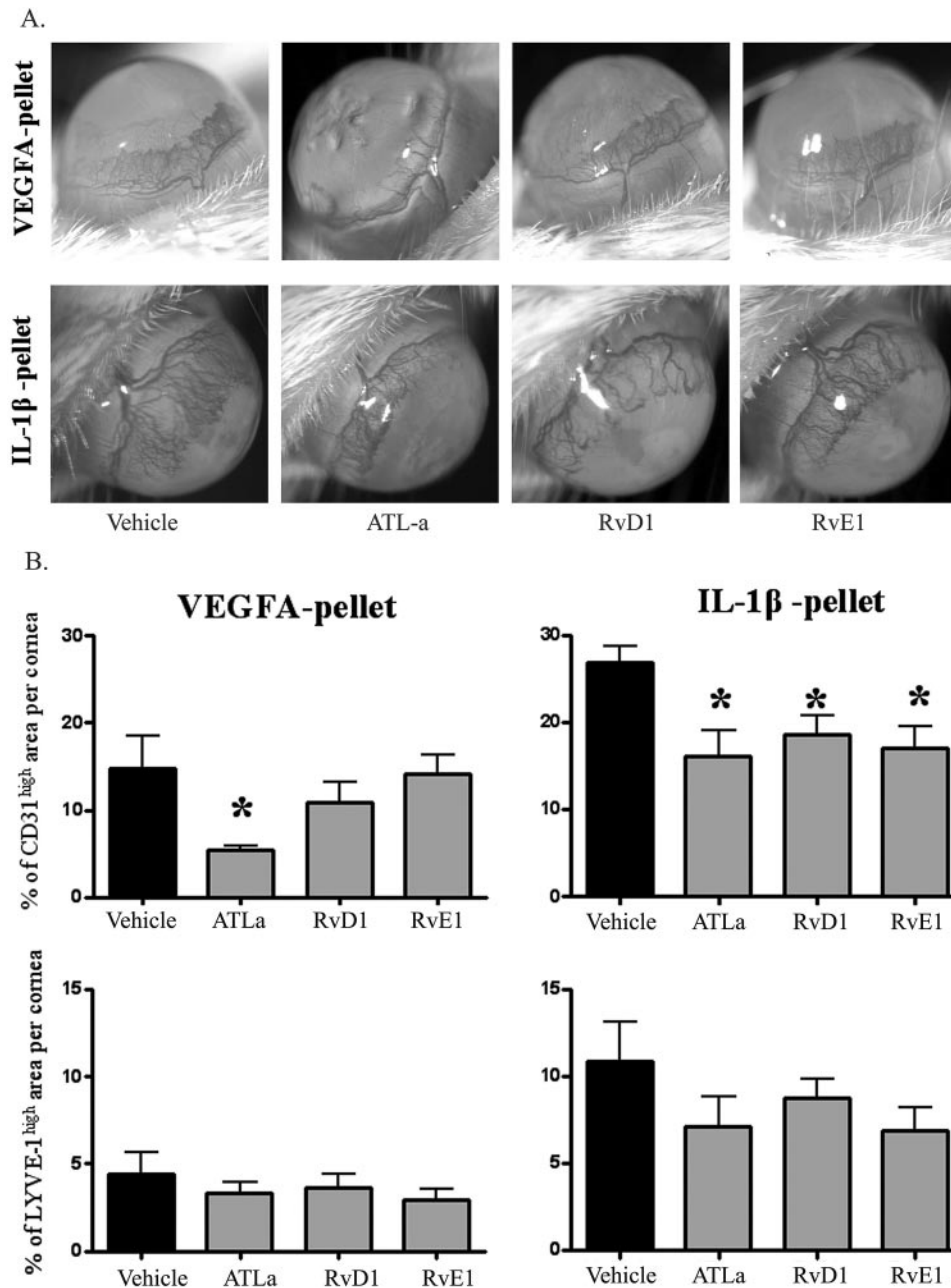


FIGURE 6. ATLa, RvD1, and RvE1 regulate IL-1 β - and VEGF-A-induced corneal HA and LA. Hydrion pellets containing IL-1 β or VEGF-A were implanted in the corneas on day 0. ATLa, RvD1, RvE1, or vehicle was subconjunctivally injected every 48 hours from 0 to 7 days after pellet implantation (four corneas 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 (four corneas per group), and the data are representative of two independent experiments (* $P < 0.05$ vs. vehicle-treated group; *t*-test).

of VEGF-C/D to VEGFR3 stimulates the development of lymphatic vessels. In addition, VEGF-C can bind to and activate VEGFR2^{30,31} and thereby contribute to HA, despite having 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 of VEGF-D or VEGFR3. This indicates that such treatments selectively regulate HA rather than LA, a finding further supported by immunohistochemistry. Future work will have to further delineate why ATLa, RvD1, and RvE1 lipid mediators differentially regulate VEGF species and receptors.

Although IL-1 secretion can stimulate VEGF expression and thereby promote angiogenesis, the administration of exogenous VEGF-A can achieve a similar effect (possibly independently of other downstream effectors resulting from IL-1 signaling). Therefore, we questioned whether the suppression of CNV by ATLa, RvD1, and RvE1 results from the suppression of

IL-1 β or VEGF-A stimulation. 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 pathologic retinal NV mouse model.³⁵ Here we show that RvD1 and RvE1 efficiently reduce IL-1 β -induced, but not VEGF-A-induced, corneal angiogenesis. This suggests that the antiangiogenesis function of RvD1 and RvE1 largely depends on their anti-inflammatory/pro-resolution function rather than on the direct regulation of VEGF-A function. This is further supported by the 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 reduced not only 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, in part, result from impairment in the early stage of EC migration.³⁵ Compared with other treatments, ATLa is thought

to be active for a longer duration because it is a stable analog of LXA4/ATL, which resists local inactivation.³⁶ Moreover, ATLa exerts its inhibitory effects on multiple steps of VEGF-A-induced angiogenesis, such as the inhibition of EC adhesion^{37,38} and the suppression of VEGF-A-induced EC proliferation.³⁹ These factors explain the actions of ATLa and its demonstrably higher potency in suppressing angiogenesis compared with the synthetic forms of RvD1 and RvE1.

The anti-inflammation and pro-resolution functions of LXA4 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 the 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 demonstrate that Fpr-rs2 and ChemR23, but not Lxa4r/Fprl1, are expressed by the infiltrating CD11b⁺ cells (which include macrophages, 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 the cornea.

Finally, though 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 has 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 normal and inflammatory conditions. In conclusion, ATLa, RvD1, and RvE1 effectively resolve corneal inflammation and angiogenesis by controlling innate inflammation by marked reduction of proinflammatory cytokine secretion and inhibiting VEGF/VEGFR expression. These novel lipid mediators offer a potentially new therapeutic strategy for controlling corneal angiogenesis, a leading cause of blindness worldwide.

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