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Submitted: December 2, 2014
Accepted: April 5, 2015

Citation: Lee H-S, Hos D, Blanco T, et al. Involvement of corneal lymphangiogenesis in a mouse model of allergic eye disease. *Invest Ophthalmol Vis Sci*. 2015;56:3140–3148. DOI:10.1167/iovs.14-16186

Purpose. The contribution of lymphangiogenesis (LA) to allergy has received considerable attention and therapeutic inhibition of this process via targeting VEGF has been considered. Likewise, certain inflammatory settings affecting the ocular mucosa can trigger pathogenic LA in the naturally avascular cornea. Chronic inflammation in allergic eye disease (AED) impacts the conjunctiva and cornea, leading to sight threatening conditions. However, whether corneal LA is involved is completely unknown. We addressed this using a validated mouse model of AED.

Methods. Allergic eye disease was induced by ovalbumin (OVA) immunization and chronic OVA exposure. Confocal microscopy of LYVE-1–stained cornea allowed evaluation of corneal LA, and qRT-PCR was used to evaluate expression of VEGF-C, -D, and -R3 in these mice. Administration of VEGF receptor (R) inhibitor was incorporated to inhibit corneal LA in AED. Immune responses were evaluated by in vitro OVA recall responses of T cells, and IgE levels in the serum.

Results. Confocal microscopy of LYVE-1–stained cornea revealed the distinct presence of corneal LA in AED, and corroborated by increased corneal expression of VEGF-C, -D, and -R3. Importantly, prevention of corneal LA in AED via VEGFR inhibition was associated with decreased T helper two responses and IgE production. Furthermore, VEGFR inhibition led a significant reduction in clinical signs of AED.

Conclusions. Collectively, these data reveal that there is a distinct involvement of corneal LA in AED. Furthermore, VEGFR inhibition prevents corneal LA and consequent immune responses in AED.

Keywords: ocular allergy, lymphangiogenesis, Th2, VEGF, RTK inhibitor

Considerable attention has recently been focused on lymphangiogenesis (LA) in human disease.[1,2] This process is defined as new outgrowth of lymphatic vessels from preexisting ones, and can be triggered by inflammation through VEGF.[3] On the one hand, LA supports tissue homeostasis by facilitating absorption of interstitial fluid, as well as promoting antigen (Ag) trafficking to the lymph node (LN) in infection.[4–7] On the other hand, there are a number of settings where such an outgrowth of new lymphatic vessels instead contributes to pathogenesis, such as in tumor metastasis,[8,9] graft rejection,[10] and chronic inflammatory diseases. The latter have been described for example in chronic obstructive pulmonary disease (COPD), inflammatory arthritis, ulcerative colitis, psoriasis, and ocular surface diseases.[11–15] Extensive effort has been directed toward understanding the biology of LA and harnessing such advancements for applications in medicine.[16–19]

The mouse cornea has emerged as an ideal model site for in vivo study of inflammatory LA (as well hemangiogenesis),[20–26] particularly with respect to VEGF ligand/receptor–mediated activity. As opposed to adjacent sites of the ocular surface such as the conjunctiva, the cornea is unique because it is naturally avascular under normal physiologic conditions, with the vasculature restricted to the limbus. However, certain inflammatory conditions impacting the ocular surface tissues can induce pathologic angiogenesis of the cornea.[25,26] Corneal LA has been demonstrated in Herpes Simplex Virus Type 1 keratitis, penetrating keratoplasty, limbal stem cell deficiency, and dry eye disease.[27–31] Vessel invasion of the cornea is undesirable, in part, because it can disturb corneal clarity and visual acuity. Clinical identification of cornea LA is being pursued in preclinical models and in humans,[32,33] as is intervening with the progression of corneal LA as a therapeutic measure.[16]

Allergic eye disease is a type of ocular surface inflammation, and is driven by stereotypical mediators of atopy such as T helper 2 lymphocytes (Th2), mast cells, and eosinophils.[34–36] Furthermore, in addition to affecting the conjunctiva, chronic allergic inflammation can also impact the cornea and become sight threatening[36]; however, whether corneal LA is involved in allergic eye disease (AED) is completely unknown. We addressed this herein using a well-established mouse model of

AED, which is induced by allergen sensitization and subsequent chronic allergen exposure.[37–39] This leads to a robust eosinophil involvement[39] and sequelae consistent with chronic allergic inflammation, such as blepharitis and corneal epitheliopathy.[37] The data presented herein reveal, for the first time, the distinct involvement for corneal LA in AED, and that prevention of corneal LA via VEGF receptor (R) inhibition diminishes AED. These data suggest that corneal LA in human disease should be considered, particularly in severe forms such as vernal and atopic keratoconjunctivitis (AKC and VKC, respectively).

Methods

Animals and Anesthesia

Female C57BL/6 (CD45.2) mice at 8- to 10-weeks old were purchased from Charles River Laboratories (City, State, Country), and CD45.1 (C57BL/6) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). CX3CR1$^{eGFP/eGFP}$ mice were purchased from Jackson Labs, and bred as heterozygotes (CX3CR1$^{eGFP/+}$) by crossing with C57BL/6 mice. All mice were housed in an animal facility of Duke University School of Medicine (Durham, NC, USA) or Schepens Eye Research Institute, Harvard Medical School (Boston, MA, USA). Anesthesia was used for all surgical procedures with intraperitoneal (IP) administered ketamine/xylazine suspensions (120 and 20 mg/kg, respectively). The Institutional Animal Care and Use Committee approved all experimental protocols, and all mice were managed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Euthanasia was performed in accordance with American Veterinary Medical Association Guidelines for Euthanasia of Animals. This was accomplished via carbon dioxide asphyxiation with a precharged chamber and bilateral thoracotomy subsequently to ensure nonrecovery.

Model of AED

This has been previously described.[37–39] Briefly, each immunization consisted of 10 μg of ovalbumin (OVA; Sigma-Aldrich Corp., St. Louis, MO, USA), 1 mg of aluminum hydroxide (Sigma-Aldrich Corp.), and 300 ng of pertussis toxin (Sigma-Aldrich Corp.), which were thoroughly mixed in 100 μL of sterile Hank's balanced salt solution (HBSS). Immunization was administered once via IP route, and mice were subsequently rested for 2 weeks. Mice were then challenged by instillation of OVA eye drops (250 μg/5 μL) once daily for at least 7 days in the same eye, while leaving the contralateral eye unchallenged.

Scoring Clinical Disease

This procedure has been previously described.[37–39] Briefly, scoring was performed 20 minutes post challenge and done once daily for at least 7 days. Mice were examined biomicroscopically based on four independent parameters, which include: (1) lid edema, (2) tearing/discharge, (3) chemosis, and (4) hyperemia. Each parameter was ascribed 0 (i.e., no disease) to 3+ (i.e., maximal disease) and was summed to yield a maximum score of 12+. All scoring was performed in a masked fashion.

VEGFR Inhibition

To inhibit the VEGFR, we used Axitinib, which is a selective second-generation tyrosine kinase (TK) inhibitor of VEGFR. Axitinib (AG 013736; Selleckchem, Houston, TX, USA) was dissolved at 5 mg/ml. Vehicle was prepared with polyethylene glycol 400 (Sigma-Aldrich Corp.) in acidified water (pH 2–3) at a 3:7 (vol/vol) ratio, as previously described.[40] Forty Mice were given a dose of 25 mg/kg once daily, as previously described. Treatment was administered via IP route concurrent with the topical OVA instillation period.

Topical Administration of CCR7 Blocking Antibody

This procedure was adapted from Schlereth et al.[37] A monoclonal blocking antibody against mouse CCR7 or the isotype control was used (R&D Systems, Minneapolis, MN, USA). Each dose consisted of 25 ug of Ab in 5 ul sterile HBSS (0.5%). One instillation was administered topically.

Generation of CD11b+ Bone Marrow–Derived Dendritic Cells (BMDC)

This has been previously described.[38] Briefly, BM cells were collected from femurs and tibiae of freshly euthanized naïve C57BL/6 mice and were seeded at 2 × 10^5/mL in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 20 ng/mL of mouse GM-CSF (Biolegend, San Diego, CA, USA). Medium was changed on day 4, and nonadherent and loosely adherent cells were collected on day 7. Cells were then washed thoroughly and set at the appropriate concentrations for subsequent T cell cocultures described below.

Intrastromal (Cornea) Injection of DCs

CD11b+ BMDC were generated, as described previously.[37,38] Bone marrow–derived dendritic cells were pulsed with OVA (0.2 mg/mL) overnight and thoroughly washed, as previously described.[37,38] Each mouse was injected with 5 × 10^4 CD45.1 BMDCs in sterile HBSS. The procedure for intrastromal injections was adapted from Jin et al.[41] Briefly, a small paracentral and intrastromal tunnel was created using a 33-G needle (Hamilton Company, Reno, NV, USA) in deeply anesthetized mice. Sterile HBSS was then injected, creating a stromal swelling throughout the entire cornea. Carefully the syringe was removed and thereafter cells were engrafted using a 30-G needle. Stromal swelling and proper cell injection were easily observed under a dissecting microscope ensuring homogeneous distribution around the cornea. Stromal swelling was resolved immediately and corneas appeared transparent several minutes after the procedure.

Whole-Mount Immunostaining

Freshly excised corneas were fixed in acetone for 15 minutes or PFA 4% at 4°C overnight. Cornea whole-mounts were immunostained overnight at 4°C with FITC-conjugated rat anti–mouse CD31/PECAM-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and goat anti-rabbit LYVE-1 antibody (Abcam, Cambridge, MA, USA). Specimens were then stained at room temperature for 1 hour with rhodamine-conjugated (Santa Cruz Biotechnology) or Alexa 594-conjugated (Jackson ImmunoResearch, West Grove, PA, USA) secondary antibody and mounted using Vector Shield mounting medium with DAPI. In another experiment, corneas were stained for LYVE-1 in addition to FITC-conjugated CD45.1 antibody (Abcam).

Corneal Vessel Area Quantitation

Micrographs of corneal whole-mounts were captured via fluorescence microscopy (model E800; Nikon, Tokyo, Japan). The areas covered with vessels were then analyzed with an algorithm established in Cell^F software (Olympus, Hamburg,

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Germany) as previously described.[42] Briefly, gray-scale images of micrographs were generated to optimize vessel signal to noise ratio and the corneal area was subsequently defined along the limbus for quantitative analysis of blood and lymphatic vessels. The area covered by blood and lymphatic vessels in the cornea was correlated with the total area of the cornea (vessel ratio).

Confocal Microscopy

Micrographs were captured via with an inverted Leica SP5 laser confocal scanning microscope (Leica Microsystems, Buffalo Grove, IL, USA). A ×20 objective and alternating UV, 488 and 561 lasers was used to generate a Z-stack (0.5 μm each step) with 1024 dpi resolution. Acquisitions were digitally analyzed with FIJI (Advanced Image J, National Institutes of Health; provided in the public domain at http://rsb.info.nih.gov/ij). Files were reconstructed into high quality three-dimensional (3D) images using Imaris Bitplane software (Bitplane, Zurich, Switzerland).

Multiphoton Intravital Microscopy (MP-IVM) and Image Analysis

Mice were maintained under anesthesia using constant infusion administered via IP catheter of ketamine/xylazine (120 mg/kg) at a rate of 0.2 mL/h. Body temperature was controlled at 37°C. Mice were dorsally placed, facilitating perpendicular exposure of the corneal apex to the microscope objective. Objective to cornea space was filled with an ophthalmic gel (GenTeal; Novartis Ophthalmics, East Hanover, NJ, USA) to create a coupling immersion interface with a refractive index ($n = 1.339$) similar to water ($n = 1.333$ at 20°C) as well as to provide eye lubrication. A 25x/1.05 NA water objective of an Olympus BX61WI upright microscope fixed stage was used. The laser used was a Chameleon Vision II single box Ti:Sapphire fsec laser (Coherent, Inc., Santa Clara, CA, USA), permitting pulse compensation in a tunable range of 680 to 1080 nm at 40 nm/s, 80 MHz rep rate, 140 fsec pulse width with a 0 to 47,000 fsec2 units of dispersion compensation. Laser was tuned at 910 nm (BGR cube) or 950 nm (CYR cube) for two-photon excitation and second harmonic generation (SHG). By using a motorized XY stage, the multiarea time-lapse software (Olympus) automates the process for a 3D image acquisition and stitching. Image stacks were analyzed using an Imaris 6.1.3-FIJI bridge (FIJI update version; Imaris update version; Bitplane).

RNA Isolation and Real-Time PCR

Total RNA was extracted using Trizol (Invitrogen, Grand Island, NY, USA) and RNeasy Microkit (Qiagen, Venlow, Lumberg). First strand cDNA was synthesized with random hexamers using SuperScript IIITM reverse transcriptase (Invitrogen), and then quantitative real-time PCR was performed using Taqman PCR Mastermix and FAM dye-labeled predesigned primers (Applied Biosystems, Venlow, Lumberg) for VEGF-C (Mm00437310_m1), VEGF-D (Mm01131929_m1), VEGF-R3 (Mm01292604_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mm99999915_g1). The GAPDH gene was used as the endogenous reference for each reaction. The results were analyzed by the comparative threshold cycle (CT) method with Light Cycler analysis software (Version 3; Roche, Basel, Switzerland) and the relative expression level of each sample was expressed as fold change from normal.

Quantitation of Sera IgE

Blood was collected from submandibular vein of mice 20 minutes following final challenge on Day 7, and serum was collected as previously described.[37] Total IgE was measured via ELISA, as per manufacturer's instructions (Innovative Research, Novi, MI, USA).

In Vitro T-Cell Assay

This has been previously described.[38] Briefly, freshly euthanized mice were dissected to excise cervical and submandibular LN of the side ipsilateral to the challenged eye. Single-cell suspensions were prepared and T cells (CD90) magnetically purified as per manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Viable T cells were counted and plated at 1.25 × 10^6/well and cocultured with 0.625 × 10^6/well of immature BMDCs. RPMI media was supplemented with 10% FBS and OVA (1 mg/mL) for 24 hours in round-bottom 96-wells. Cultures were restimulated with PMA/ionomycin (Sigma-Aldrich Corp.) for 6 hours and supernatants were harvested. Cytokines IL-4, -5, and -13 were measured via ELISA, as per manufacturer's instructions (Ready-set-go ELISA kit; eBioscience, San Diego, CA, USA).

In Vitro Lymphatic Endothelial Cell (LEC) Proliferation Assay

This was method has been previously described.[29] Briefly, human lymphatic microvascular endothelial cells (PromoCell, Heidelberg, Germany) were cultured in EGM2-MV medium containing 5% FCS. Cells were seeded in a 96-well plate at a density of 4 × 10^3 cells per well and cultured overnight before medium was replaced with EGM2-MV medium containing 5% FCS, BrdU, and 100 ng/mL of recombinant human IL-4, -5, or -13 (R&D Systems). After 48 hours cells were fixed and stained as per manufacturer's instructions (Cell Proliferation ELISA; Roche). Colorimetric analysis was performed with an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA). The mean extinction of the control wells was defined as 100%; the extinction of all wells was then related to this value.

Statistical Analysis

Data are expressed as the mean ± SEM of at least three independent trials. The significance of the difference between groups was analyzed with the two-tailed Student's *t*-test using Prism software (version 5.0; GraphPad, San Diego, CA). *P* less than 0.05 was considered statistically significant.

Results

Involvement of Corneal LA in AED

Using the established AED mouse model we herein investigated whether corneal LA occurs in allergic eye disease. Allergic eye disease is induced via systemic OVA immunization, followed 2 weeks after with administration of allergen challenge via multiple instillations of OVA eye drops, as previously described.[37–39] We then excised the corneas from these mice and prepared whole mounts for fluorescence microscopy following staining for lymphatic vessels (LYVE-1; Fig. 1A). Results showed that corneas from AED mice had marked corneal LA, as sprouting of lymphatic vessels into the cornea was readily detected (Fig. 1A). Percent area of corneal lymphatic vessels of the cornea[42] corroborated this observation (Fig. 2B). We further analyzed corneas from AED mice via qRT-PCR for lymphangiogenic VEGF ligands and receptors. Consistent with the presence of corneal lymphangiogenesis, corneas in AED showed a significant upregulation of the lymphangiogenic ligands VEGF-C and VEGF-D, as well as a near

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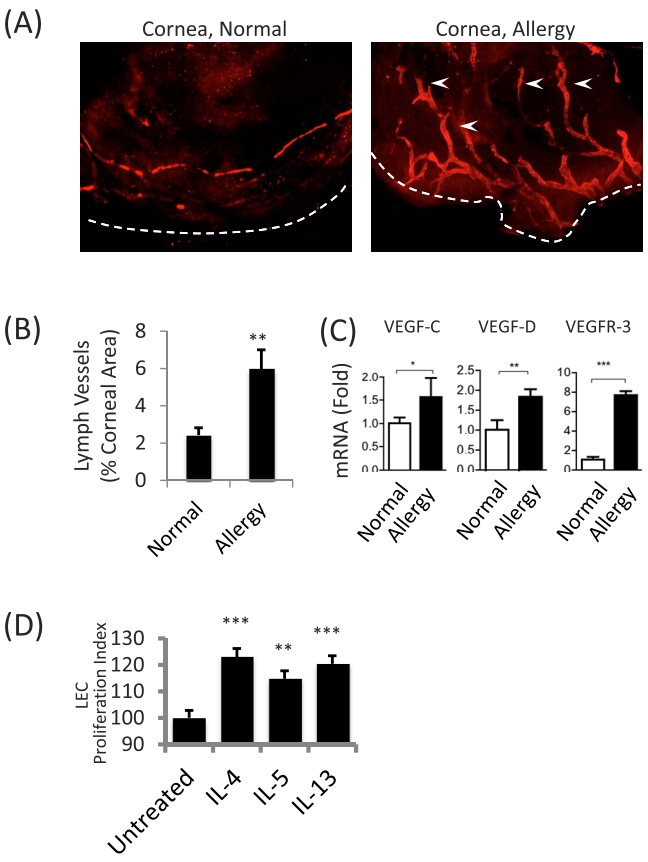

Figure 1. Involvement of corneal lymphangiogenesis (LA) in the allergic eye disease (AED) model. (**A**, **B**) Allergic eye disease was induced via eye drop instillations of OVA, which were administered 2 weeks post OVA sensitization. (**A**, **B**) Identification of corneal lymphangiogenesis in AED. Whole-mounted corneas (n = 4–7/group) were stained for LYVE-1 (*red*) and micrographs captured via fluorescence microscopy. *Dashed lines* indicate the limbal edge of the excised cornea. (**B**) Percent of cornea area covered by lymphatic vessels in normal versus AED cornea. Data is presented as the mean ± SEM of data from two independent experiments. (**C**) Increased corneal VEGF-C, -D, and VEGFR-3 levels detected in AED. Excised corneas (n = 3/group) were analyzed for mRNA expression via qRT-PCR. Data is presented as the mean ± SEM of four experiments. (**D**) In vitro LEC proliferation is by IL-4, -5 or -13. Cultured LECs were stimulated with the indicated factors and proliferation was measured at 48 hours. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

7-fold increase in mRNA levels of VEGFR-3 (Fig. 1C). Lastly, we examined the effect of IL-4, -5, or -13 (i.e., T helper 2 cytokines) on proliferation of lymphatic endothelial cells (LEC) in vitro. Interestingly, the addition of IL-4, -5 or -13 led to a significant increase in LEC proliferation (Fig. 1D). Thus, these data led us to conclude that corneal lymphangiogenesis is involved in the AED model.

Evidence for a Contribution of Corneal LA in AED to the Egress of Allergen-Laden Antigen Presenting Cells

Because lymphatic vessels serve as the conduits for antigen-presenting cell (APC) homing to the lymph node, we also examined whether corneal LA in the AED model contributes to egress of allergen-laden APC. We first determined in the AED setting whether corneal APCs are capable of capturing allergen from ocular surface. This was accomplished by inducing AED in CX3CR1$^{eGFP/+}$ mice, wherein corneal APCs are eGFP+.[43] Mice were challenged with Texas Red-conjugated OVA, as previously described.[37] Thirty minutes thereafter, mice were examined by multiphoton intravital microscopy (MP-IVM; Fig. 2A), as previously described.[44] Strikingly, we found that many corneal eGFP+ APCs colocalized with OVA, as well as some eGFP− APCs (Figs. 2A, 2B). These data indicate that corneal APCs have the capacity to capture instilled OVA from in the AED setting.

We next examined whether corneal LA in the AED model contributes to egress of allergen-laden APCs. We induced AED via immunization and subsequent topical instillation of OVA in C57BL/6 mice, which naturally posses the CD45.2 allele. At the end of the OVA instillation period, the timepoint at which corneal lymphangiogenesis was seen, hosts received OVA pulsed (CD11b+) DCs[37,38] derived from donor C57BL/6 CD45.1 congenic mice, thus, allowing us to identify donor DCs. These donor cells (5 × 10^4 cells/mouse) were administered into the host cornea by intrastromal injection, as previously described.[41] Mice were simultaneously treated with or without the addition of topical CCR7 blocking antibody, which inhibits egress of DCs through lymphatic vessels.[34,37,45] Corneas were harvested 6 hours post injection and examined for DC distribution by CD45.1 staining, and the presence of lymphatic vessels was confirmed by LYVE-1 staining. In AED corneas, injected DCs were seen heavily concentrated around lymphatic sprouts adjacent to the limbus, as well as around lymphatic vessels in limbal arcade (Fig. 2C). Colocalization of CD45.1 DCs with lymphatic vessel sprouts was readily detectable (Fig. 2C), suggesting egress of DCs via neolymphatics. This was in stark contrast with injected DCs in AED corneas treated topically with CCR7 blocking antibody. We observed strikingly high numbers of injected DCs interspersed throughout the width of the entire cornea of these mice, including the central region (Fig. 2D), suggesting retention of injected DCs in the cornea. Furthermore, colocalization of CD45.1 DCs with lymphatic vessels was not readily detectable (Fig. 2D). Taken together, these data suggest that corneal LA in the AED setting contributes to the egress of allergen-laden APCs.

VEGFR Inhibition Impairs Corneal LA in AED

We next investigated the effect of VEGFR inhibition corneal LA in AED. As previously described, we incorporated a tyrosine kinase (TK) inhibitor of VEGFR to accomplish this.[46] We used Axitinib, which is a selective second-generation TK inhibitor of all VEGFRs.[47] Allergic eye disease was induced via multiple instillations of OVA eye drops 2 weeks post systemic OVA immunization, and VEGFR inhibitor (or vehicle control) was administered concurrent with the OVA eye drop instillation period. Corneas were then excised and prepared for whole-mount fluorescence microscopy of LYVE-1 staining. Results showed that VEGFR inhibition decreased lymphatic vessel presence in the cornea, as compared with vehicle treated controls in AED (Fig. 3A). Percent area of corneal lymphatic vessels of the cornea[42] demonstrated that the lymphangiogenic response in allergic mice was almost completely abrogated after VEGFR inhibition and comparable with normal, healthy mice (Fig. 3B).

VEGFR Inhibition Blunts Type-2 Immune Responses in AED

As previously mentioned, lymphatics are the conduits for antigen-presenting cell homing to the lymph node (LN) and consequent T-cell activation. Given our finding that corneal LA occurs in AED and that VEGFR inhibition can successfully prevent this response, we next sought to examine the effect of VEGFR inhibitor on type-2 immune responses. We accom-

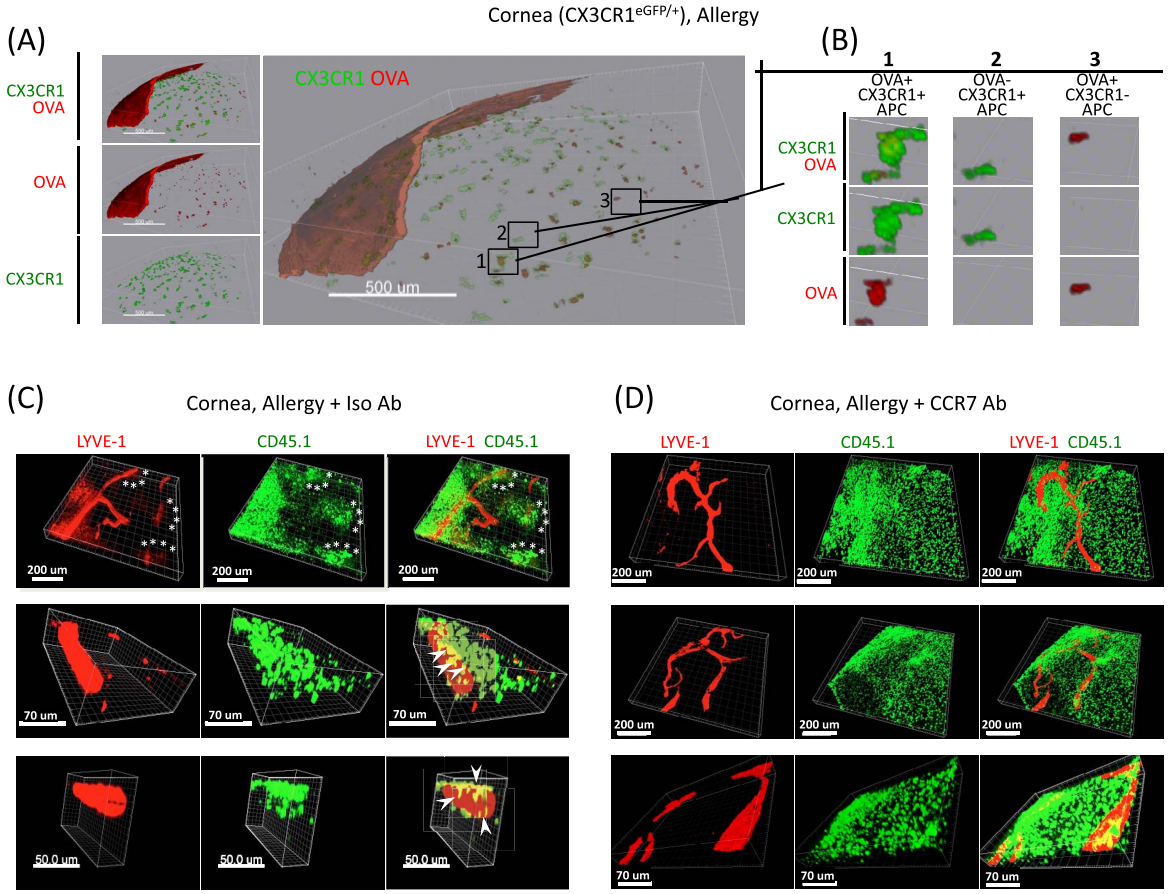

Figure 2. Corneal LA in AED contributes to egress of allergen-laden APCs. (**A**, **B**) Cornea APCs (CX3CR1+) capture instilled OVA in the AED setting. Two weeks post OVA sensitization, CX3CR1$^{eGFP/+}$ mice were instilled with OVA (Texas-Red) onto the ocular surface and imaged 30 minutes thereafter via multiphoton intravital microscopy (MP-IVM). (**B**) Colocalization of OVA (*red*) with eGFP+ APCs (and few eGFP– APCs) was readily detected. (**C**, **D**) Corneal LA in AED contributes to egress of allergen-laden APCs. Congenic CD45.2 mice with AED received an injection of OVA-pulsed CD45.1 BMDCs (5 × 10^4 cells) into the corneal stroma. Immediately thereafter, mice were topically administered with isotype control (**C**) or CCR7 blocking Ab (**D**). Corneas ($n = 6$/group) were excised 6 hours post injection and stained for LYVE-1 (*red*), CD45.1 (*green*), and DAPI (*blue*) staining. *Asterisks* (*) indicate CD45.1+ cells concentrated around lymphatic sprouts. *Arrows* indicate colocalization (*yellow*) of CD45.1 cells with lymphatic sprouts.

plished this by examining T-cell production of Th2 cytokines (IL-4, -5, and -13). In addition, we measured serum IgE levels, as Th2 cells are required for IgE production from B cells. Allergic eye disease was therefore induced via multiple instillations of OVA eye drops 2 weeks post systemic OVA immunization, and VEGFR inhibitor or vehicle control was administered concurrent with the OVA eye drop instillation period. Afterwards, purified T cells from eye draining LNs (i.e., cervical and submandibular nodes) were prepared and cocultured with OVA-pulsed DCs.[38] Culture supernatants were collected and assayed for IL-4, -5, and -13 via ELISA.[37–39] We found that T cells from VEGFR inhibitor treated mice produced significantly less levels of IL-4, -5, and -13, relative to vehicle treated AED mice (Fig. 4). Consistent with this, we observed a significant decrease in total IgE levels in the serum after VEGFR inhibition (Fig. 5). These data taken together allow us to conclude that VEGFR inhibition leads to a decrease in type-2 immune responses in AED.

VEGFR Inhibition Reduces Clinical Responses of AED

Clinical disease in allergy is mediated by type-2 immune responses, and given our finding that VEGFR inhibitor blunts these responses in AED, we next wanted to examine the clinical effect of VEGFR inhibition in this model. Allergic eye disease was induced and VEGFR inhibitor or vehicle control was administered concurrent with the OVA eye drop instillation period. Hyperemia, edema, discharge, and chemosis were clinical scored in a masked fashion at 20 minutes post challenge on days 1 through 7, as previously described.[37–39] We observed that the VEGFR inhibitor–treated group had significantly reduced clinical scores on days 4 through 10, as compared with vehicle-treated and treated groups (Fig. 6). These data allow us to conclude that clinical disease is reduced by VEGFR inhibition in AED.

Discussion

The current study has revealed the distinct involvement of corneal LA in AED, using the AED mouse model.[37–39] We further showed that VEGFR inhibition significantly blunted corneal LA, which associated with a significant decrease in type-2 immune responses and clinical disease. Corneal LA may similarly occur in human allergic eye disease as well. Unfortunately, lymphatic vessels are difficult to identify because they are not clinically detectable, which could explain why corneal LA in allergic eye disease may have gone unnoticed in the clinic. Efforts in preclinical models and in

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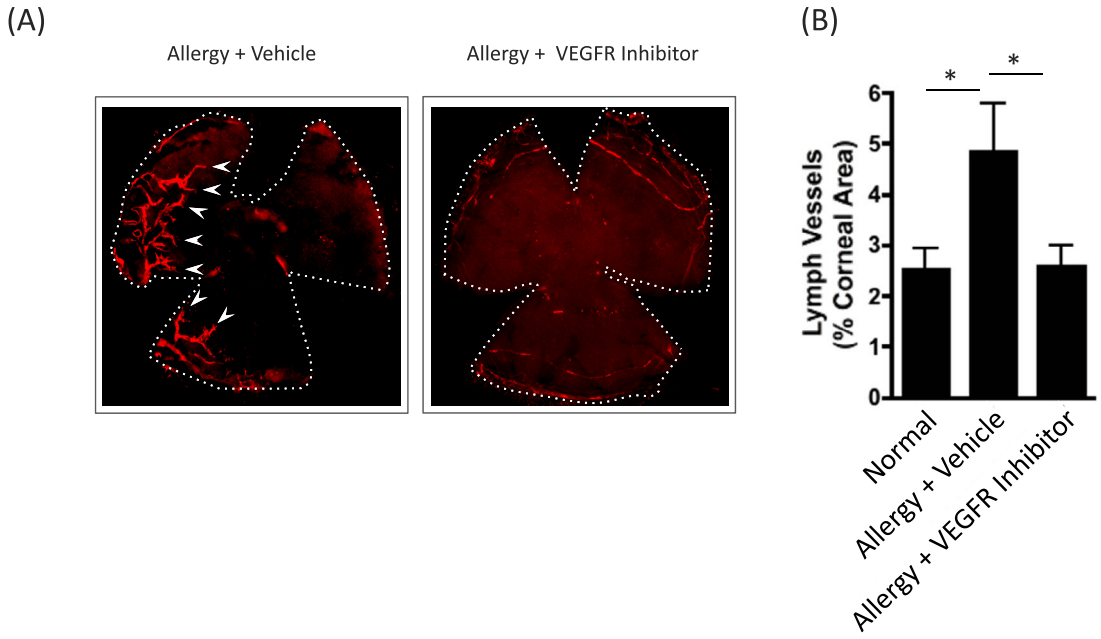

Figure 3. Vascular endothelial growth factor receptor inhibition prevents corneal LA in AED. (**A**, **B**) Vascular endothelial growth factor receptor inhibition reduces corneal lymphangiogenesis. Allergic eye disease was induced and VEGFR inhibitor or vehicle control was administered concurrent with the topical OVA instillation period. Excised corneas ($n = 6$–11/group) were stained for LYVE-1 (*red*) and micrographs captured via fluorescence microscopy. *Dashed lines* in the micrographs show the edge of the excised and radially cut cornea; *arrows* indicate lymphatic sprouting into the cornea. (**B**) Percent of cornea area covered by lymphatic vessels in AED mice treated with VEGFR inhibitor was significantly decreased relative to vehicle control treated mice. Data is presented as the mean ± SEM of data from two independent experiments (*$P < 0.05$).

humans to identify corneal LA are currently being pursued.[32,33] Nevertheless, there is some indirect evidence in the literature to support our current findings. For example, patients who suffer from ocular allergy are at an elevated risk for immune rejection in corneal transplantation, which has been proven in mice as well.[48,49] Furthermore, in the mouse model of corneal allotransplantation, Flynn et al.[49] showed that perisurgical ocular allergy was associated with augmented corneal LA,[49] and Dietrich et al.[10] reported that in mice the presence of corneal LA was a risk factor for corneal allograft rejection. Thus, further studies in humans are warranted to validate the presence of corneal LA in ocular and whether such activity

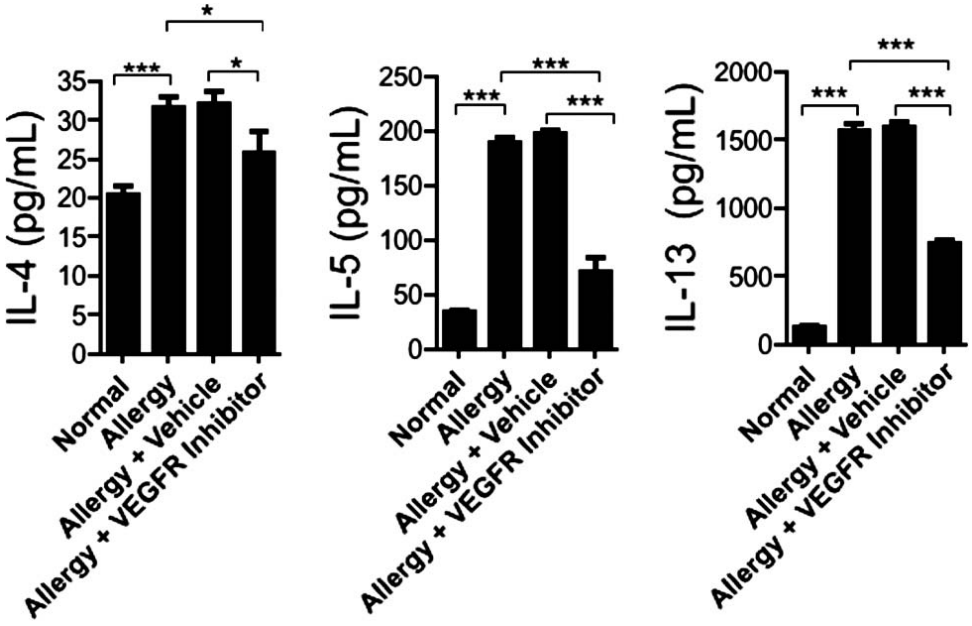

Figure 4. Vascular endothelial growth factor receptor inhibition inhibits T helper 2 cells in AED. Allergic eye disease was induced and VEGFR inhibitor or vehicle control was administered concurrent with the topical OVA instillation period. Purified T cells from eye draining LN were cocultured with OVA-pulsed BMDCs, and culture supernatants were collected for ELISA analysis of IL-4, -5, and -13 levels. Data ($n = 4$/group, total $n = 12$/group) are representative of three independent experiments and presented here as the mean and SEM (*$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$).

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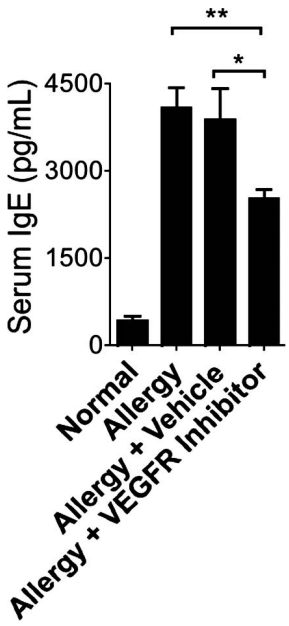

Figure 5. Vascular endothelial growth factor receptor inhibition inhibits IgE levels in AED. Allergic eye disease was induced and VEGFR inhibitor or vehicle control was administered concurrent with the topical OVA instillation period. Peripheral blood was drawn, and sera was prepared to measure IgE levels via ELISA. Data ($n = 4$/group, total $n = 12$/group) are representative of three independent experiments and presented here as the mean and SEM ($^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$).

occurs solely in more severe forms of allergic eye disease (e.g., AKC, and VKC), or can also be involved in mild to moderate disease as well (e.g., seasonal/perennial allergic conjunctivitis).[36]

In mice, our comparison of LYVE-1+ staining in confocal micrographs of whole-mounted corneas was our first piece of evidence that showed presence of corneal LA in AED mice, and our finding that the administration of VEGFR inhibitor prevented the appearance of these lymphatics strongly supported this conclusion. This was also confirmed quantitatively by measuring percent area of cornea covered by lymphatic vessels, a method that was previously described by Bock et al.[42] In addition, these corneas showed significantly increased VEGF-C, -D, and -R3 expression, which triggers the key signaling pathway in the lymphangiogenic process.[50] Furthermore our in vitro data suggest that LEC proliferation is stimulated by Th2 cytokines, IL-4, -5, or -13. Our data is consistent with Lee et al.,[51] who also reported a link between lymphangiogenesis and type-2 immune responses, as demonstrated in a mouse model of allergic airway inflammation. Corneal hemangiogenesis (HA) can also occur in ocular surface inflammation. However, we observed that corneal LA was dominant in AED mice (data not shown), as similarly reported in the desiccating stress induced dry eye disease model in mice.[27] Future work is required to validate in AED the possibility of an isolated corneal LA response.

The expression of VEGF ligands has multiple potential cellular sources in the context of corneal LA in AED. A likely source is mast cells, as their ability to express multiple VEGF species is well documented, as are eosinophils.[52–56] Both in the mouse model and in humans the contribution of mast cells in immediate hypersensitivity responses, and the contribution of eosinophils in late phase responses are well established.[36] It is therefore conceivable that a contribution to lymphangiogenic processes in the cornea seen here may have come from such cells by depositing VEGF into the tear film, as previously described.[57] Alternatively, VEGF ligands could also be produced within the cornea, as Wuest et al.[31] showed that corneal epithelial cells in inflammation express VEGF-A. Macrophages[20] and neutrophils[58] could similarly express VEGF ligands, and both cell types are involved in the AED model. Lastly, our data have revealed an in vitro role in LEC proliferation for IL-4, -5, and -13 cytokines. In addition to the production by mast cells and eosinophils, a main source of such cytokines is Th2 cells.

Lymphangiogenesis is intended to support the host by facilitating increased absorption of inflammatory edema,[5–7] which would be important in the maintenance of corneal clarity and visual acuity. Lymphangiogenesis is also intended to maintain an activated adaptive immune response to promote expulsion of parasitic helminthes, which is in line with the current thinking that the immune system in atopic hosts misperceives innocuous allergen as an extracellular pathogen.[59] Consistent with this, our study revealed several lines of evidence that associate corneal LA with allergic immune responses and consequent clinical disease, which was similarly shown by Huggenberger et al.[6] For example, our MP-IVM experiments using instillation of Texas Red-conjugated OVA in CX3CR1$^{eGFP/+}$ mice with AED provide direct evidence that corneal APCs capture allergen. Furthermore, our confocal data demonstrate a role for corneal LA in the egress of allergen-laden

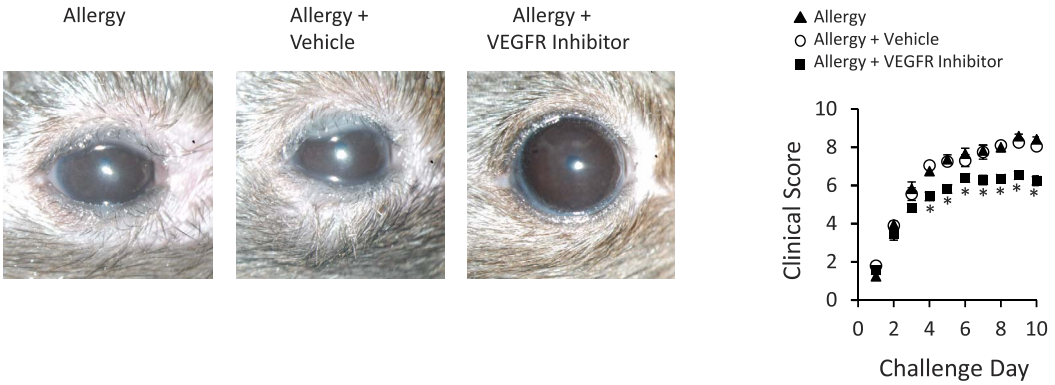

Figure 6. Vascular endothelial growth factor receptor inhibition dampens clinical signs of AED. Allergic eye disease was induced and VEGFR inhibitor or vehicle control was administered concurrent with the topical OVA instillation period. Representative clinical images are included. Clinical scoring was performed once per day at 20 minutes post OVA instillation. Mice were scored for tearing/discharge, eyelid edema, chemosis, and hyperemia. Data ($n = 4$/group, total $n = 12$/group) are representative of three independent experiments and presented here as the mean and SEM ($^{*}P < 0.05$).

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corneal DCs and suggest that this occurs in a CCR7-mediated fashion.[37] Lastly, we showed that administration of VEGFR inhibitor blunted corneal LA significantly and this resulted in decreased Th2 responses in the LN and IgE levels in the serum, as well as decreased clinical signs at the ocular surface.

It is important to note that homing of allergen-laden DCs and consequent activation of Th2 responses in this mouse model is not solely attributed to corneal LA. Indeed, our group has previously reported using the AED model that conjunctival DCs, specifically the CD11b+ subset, play a key role in activation of Th2.[34,35,37,38] Furthermore, we cannot rule out in the current study the possibility that VEGFR inhibition may also act at the level of conjunctival lymphatics, which in turn contributed to decreased Th2 and clinical disease, or directly at the level of immune cells, which are also known to express VEGF receptors.

With respect to the role of corneal LA on clinical disease, it is noteworthy that the clinical scoring method used herein quantifies clinical signs of the immediate hypersensitivity response (i.e., hyperemia, chemosis, tearing/discharge, and lid swelling), which is largely mediated by mast cell degranulation. Our data suggests that corneal LA in AED is contributing at the level of T-cell responses, which play a more direct role in late phase allergic activity. In agreement with this, the AED model manifests significant late phase disease, as shown by the eosinophil recruitment.[39] Thus, the quantitative clinical scoring used herein is probably underestimating the therapeutic effect of VEGFR inhibitor, which is appreciated by the representative clinical slit-lamp images provided. Thus, future work will be necessary to accurately quantify clinical signs of late phase allergy as well, which may include onset/progression of punctate keratopathy, thick mucoid discharge, and blepharitis.

In summary, our results indicate that corneal LA is involved in the AED model. Furthermore, we show that VEGFR inhibition significantly reduced corneal LA, which this was associated with decreased Th2 responses, IgE levels, and clinical responses in AED. Results of this study warrant further examination in humans, and consideration of targeting LA processes clinically, particularly in severe forms of allergic eye disease that involves the cornea, such as in VKC and AKC.

Acknowledgments

Supported by grants from R01EY021798 (DRS; National Eye Institute, Bethesda, MD, USA), Research to Prevent Blindness (DRS; New York, NY, USA), and P30EY005722 (Arshavsky; National Eye Institute).

Disclosure: **H.-S. Lee**, None; **D. Hos**, None; **T. Blanco**, None; **F. Bock**, None; **N.J. Reyes**, None; **R. Mathew**, None; **C. Cursiefen**, None; **R. Dana**, None; **D.R. Saban**, None

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