

ONLINE FIRST

Blockade of Prolymphangiogenic Vascular Endothelial Growth Factor C in Dry Eye Disease

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Objective: To determine whether blocking prolymphangiogenic factors such as vascular endothelial growth factor C (VEGF-C) would suppress alloimmunity in dry eye disease using a murine model.

Methods: The effects of intraperitoneal injections of 400 µg of anti-VEGF-C antibody (treated group) and intraperitoneal normal saline (untreated group) were studied in murine dry eyes induced by exposing mice to high-flow desiccated air in a controlled-environment chamber. Growth of lymphatic vessels and infiltration of macrophages were evaluated by immunohistochemistry using CD31 (panendothelial marker), lymphatic vessel endothelial receptor 1 (lymphatic endothelial marker), and CD11b (monocyte and macrophage marker). Real-time polymerase chain reaction was performed to quantify expression of different inflammatory cytokine transcripts in the conjunctiva and lymph nodes as well as vascular endothelial growth factors and their receptors (VEGF-A, VEGF-C, VEGF-D, VEGFR-2, and VEGFR-3) in the cornea.

Results: Blocking VEGF-C led to significant reductions in lymphatic caliber ($P=.02$) and lymphatic area ($P=.006$) in the corneas of mice with dry eye disease. In

addition to significantly decreasing CD11b⁺ cells ($P=.005$), anti-VEGF-C treatment significantly decreased transcript levels of VEGF-C ($P=.002$), VEGF-D ($P=.01$), and VEGFR-3 ($P=.02$) in the corneas of the treated group. A significant decrease in expression of inflammatory cytokines in the conjunctiva (interleukin 1 α , $P=.003$; interleukin 1 β , $P=.02$; interleukin 6, $P=.005$) and lymph nodes (interferon γ , $P=.008$; interleukin 17, $P=.003$) was also seen with anti-VEGF-C treatment.

Conclusion: Treatment with anti-VEGF-C led to significant improvement in dry eye disease, reflected by a decrease in inflammation at the clinical, molecular, and cellular levels.

Clinical Relevance: Targeting prolymphangiogenic growth factors or their receptors could inhibit the trafficking of antigen-presenting cells to the draining lymph nodes and hence prove to be a potential therapeutic target for dry eye disease.

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DRY EYE DISEASE (DED) IS A complex, multifactorial, immune-mediated disorder of the ocular surface affecting about 5 million Americans older than 50 years.^{1,2} Millions more have manifestations precipitated under adverse environmental conditions such as low humidity. Dry eye disease severely affects the vision-related quality of life, and the symptoms can be both psychologically and physically debilitating.¹ The current therapeutic options for DED are limited, mostly palliative, and costly.³ Currently, topical cyclosporine is the only approved treatment for this disease.¹

The ocular surface inflammation in DED is sustained by ongoing activation and infiltration of pathogenic immune cells primarily of the CD4⁺ T-cell compart-

ment.^{4,5} Recently, we demonstrated that lymphangiogenesis, without concurrent growth of blood vessels (hemangiogenesis), occurs in the DED cornea.⁶ Interestingly, these lymphatics not only grow toward the central cornea but also show significantly increased caliber compared with those restricted to the limbal areas of normal corneas. Furthermore, DED corneas show a significant upregulation of prolymphangiogenic specific vascular endothelial growth factor C (VEGF-C), VEGF-D, and their receptor VEGFR-3, confirming that the low-grade inflammation seen in DED is selectively conducive for lymphangiogenesis.⁶

Lymphangiogenesis is linked to a number of pathological conditions, including lymphedema and cancer.⁷ Correlative studies with human tumors and functional

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studies using animal tumor models show that increased levels of VEGF-C or VEGF-D in tumors promote metastasis to regional lymph nodes (LNs).⁸ Also, it is now well established that VEGF is accountable for many ocular pathologies involving angiogenesis, including age-related macular degeneration, diabetic retinopathy, neovascular glaucoma, and corneal transplantation.⁹ During the past several years, agents targeting VEGF-A have been developed for intraocular use and have revolutionized ophthalmological care for many of these potentially blinding conditions.¹⁰ Here, we tested the hypothesis that a strategy targeting the primarily prolymphangiogenic VEGF-C may suppress the inflammation and epitheliopathy associated with DED and could thus provide therapeutic value for the treatment of dry eyes.

METHODS

EXPERIMENTAL DRY EYE MURINE MODEL

Eight- to ten-week-old female C57BL/6 mice (Charles River Laboratory, Wilmington, Massachusetts) were used in accordance with the standards in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The research protocol was approved by the Schepens Eye Research Institute Animal Care and Use Committee. As described previously,¹¹ dry eye was induced in the mice by placing them in a controlled-environment chamber (CEC). To achieve maximum ocular surface dryness, the conditions in the CEC were supplemented with topical application of atropine sulfate, 1% (Falcon Pharma, Fort Worth, Texas), twice for the first 48 hours and subcutaneous injections of 0.1 mL of 5-mg/mL scopolamine hydrobromide (Sigma-Aldrich, St Louis, Missouri) 3 times a day for the entire duration of the experiment.¹²

CORNEAL SURFACE STAINING

Fluorescein staining of the corneal epithelium was used as a diagnostic tool to study the effect of desiccating stress on the ocular surface of the mice. Corneal fluorescein staining was performed at baseline (day -1, before placing the mice in the CEC) and subsequently at days 2, 5, 9, and 13. We applied 0.7 μ L of fluorescein, 2.5% (Sigma-Aldrich), using a micropipette into the inferior conjunctival sac of the mice's eye as previously described.^{11,12} After 5 minutes, punctate staining on the corneal surface was evaluated in a masked fashion with a slitlamp biomicroscope using the National Eye Institute grading system.^{11,12}

ANTI-VEGF-C ANTIBODY AND TREATMENT REGIMEN

A day before placing the mice in the CEC, they were randomly divided into 2 groups. The treatment group ($n=5$) received daily intraperitoneal injections of 400 μ g of anti-VEGF-C antibody (VGX-100; Vegenics, Circadian Technologies, Coorparoo, Queensland, Australia) in 100 μ L of normal saline from day -1 to day 13. According to the manufacturer, VGX-100 binds to and precipitates all forms of VEGF-C including full-length, partially processed, and mature forms, thus inhibiting VEGF-C binding and activation of VEGFR-2 and VEGFR-3. Another group (untreated group) placed in the CEC ($n=5$) received 100 μ L of intraperitoneal normal saline for the same duration of the experiment. Age- and sex-matched mice not placed in the CEC served as normal controls. Mice were then killed on day 14 for cellular and molecular studies. All experiments were repeated 3 times.

IMMUNOHISTOCHEMISTRY

The following primary antibodies were used for immunohistochemical staining: fluorescein isothiocyanate-conjugated rat antimouse CD11b as a marker for monocytes and macrophages (isotype fluorescein isothiocyanate-conjugated rat antimouse IgG2bk; BD Pharmingen, San Diego, California), fluorescein isothiocyanate-conjugated goat antimouse CD31 as a panendothelial marker (isotype fluorescein isothiocyanate-conjugated rat IgG2A; Santa Cruz Biotechnology, Santa Cruz, California), and purified rabbit antimouse lymphatic vessel endothelial receptor 1 (LYVE-1) as a lymphatic endothelial marker (isotype rabbit IgG; Abcam, Cambridge, Massachusetts). Rhodamine-conjugated goat antirabbit (titer of 1:100; BD Pharmingen) was the secondary antibody used.

Freshly excised corneas were washed in phosphate-buffered saline, fixed in acetone for 15 minutes, and then double stained with CD31 and LYVE-1 as described before.¹³ To analyze infiltration of CD11b⁺/LYVE-1 cells, corneas from 3 mice from each group were taken and cells were counted in 5 to 6 areas in the periphery (0.5- μ m area from the limbus) using an epifluorescence microscope (model E800; Nikon, Melville, New York) at $\times 40$ magnification. The mean number of cells was obtained by averaging the total number of cells in all of the areas studied, and the result was expressed as the number of positive cells per square millimeter.

QUANTIFICATION OF LYMPHATICS IN THE CORNEA

Lymphatics were quantified by an automated image analysis program written using Matlab as described previously (MathWorks, Inc, Natick, Massachusetts).⁶ In brief, lymphatics were isolated from digitized immunofluorescent micrographs and subsequently analyzed for lymphatic area and lymphatic caliber. Lymphatic area represents the total surface area of the lymphatic vessels, and lymphatic caliber is a summary measure of the diameters of the lymphatic vessels present.

RNA ISOLATION AND MOLECULAR ANALYSIS USING REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was isolated from the cornea, conjunctiva, and draining LNs of mice from normal, untreated, and treated groups using the RNeasy microkit (Qiagen, Valencia, California). Equal amounts of RNA were used to synthesize complementary DNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, California) according to the manufacturer's recommendations. Real-time polymerase chain reaction was performed using FAM-MGB dye-labeled predesigned primers (Applied Biosystems, Foster City, California) for glyceraldehyde-3-phosphate dehydrogenase, interleukin 1 α (IL-1 α), IL-1 β , IL-6, interferon γ , IL-17A, VEGF-A, VEGF-C, VEGF-D, VEGFR-2, and VEGFR-3. Complementary DNA (2.5 μ L) was loaded in each well, and assays were performed in duplicates. A nontemplate control was included in all of the experiments to evaluate DNA contamination of the reagent used. The results were normalized by the cycle threshold of glyceraldehyde-3-phosphate dehydrogenase as an internal control.

STATISTICAL ANALYSIS

A 2-tailed paired *t* test was performed. $P < .05$ was deemed statistically significant. Results are presented as the mean (SEM) of at least 3 experiments.

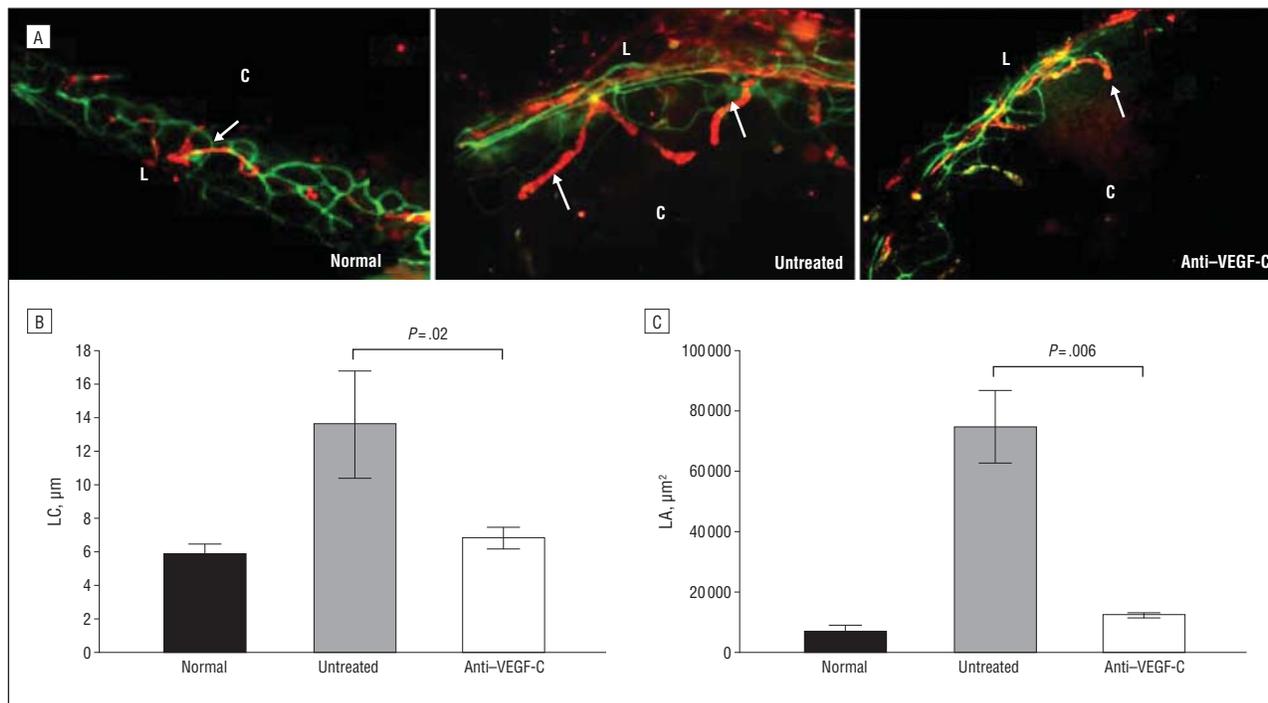


Figure 1. Effect of in vivo blockade of anti-vascular endothelial growth factor C (anti-VEGF-C) on corneal lymphatics in dry eye disease. A, Representative micrographs of corneal whole mounts immunostained for CD31 (green) and lymphatic vessel endothelial receptor 1 (red) expressions showing CD31^{lo} lymphatic vessel endothelial receptor 1^{hi} lymphatic vessels (orange) in normal, untreated, and anti-VEGF-C treated corneas (original magnification $\times 100$). Arrows indicate lymphatics; C, cornea; and L, limbus. Morphometric evaluation showing significant decrease in lymphatic caliber (LC) (B) and lymphatic area (LA) (C) in anti-VEGF-C treated corneas compared with normal and untreated corneas. Data from a representative experiment of 3 performed experiments are shown as mean (SEM); each group consists of 4 or 5 mice.

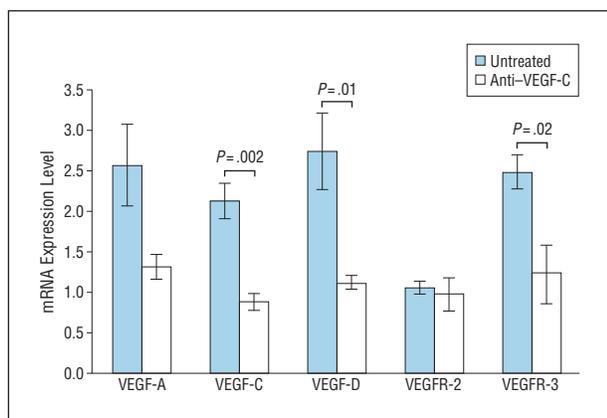


Figure 2. Effect of in vivo blockade of anti-vascular endothelial growth factor C (anti-VEGF-C) on angiogenic markers in cornea. Real-time polymerase chain reaction analysis showing transcript levels of VEGF-A, VEGF-C, VEGF-D, VEGF receptor 2 (VEGFR-2), and VEGFR-3 in corneas of different groups. Among-group comparisons showed significant decreased expression levels of VEGF-C, VEGF-D, and VEGFR-3 in the anti-VEGF-C treated group compared with the untreated group. mRNA indicates messenger RNA. Data are shown as mean (SEM).

RESULTS

EFFECT OF VEGF-C NEUTRALIZATION ON LYMPHATICS IN DRY EYE CORNEAS

There is ingrowth of lymphatics from the limbus toward the center of the cornea with progression of DED.⁶ Corneal whole mounts were double stained for CD31 and LYVE-1 and quantified for lymphangiogen-

esis. Blood vessels were identified as CD31^{hi}/LYVE-1⁻, and lymph vessels were identified as CD31^{lo}/LYVE-1^{hi} (Figure 1A). The dry eye group treated with anti-VEGF-C antibody showed a significant (nearly 2-fold) reduction in lymphatic caliber (Figure 1B; $P = .02$) and a nearly 5-fold reduction in total lymphatic area (Figure 1C; $P = .006$) of corneal lymphatic vessels compared with the untreated group.

EFFECT OF VEGF-C NEUTRALIZATION ON ANGIOGENIC MOLECULES IN THE CORNEA

The characteristic lymphangiogenic factors are VEGF-C and VEGF-D, which act by binding to their receptor VEGFR-3.⁷ The VEGF-A indirectly contributes to lymphangiogenesis by recruiting VEGF-C- and VEGF-D-secreting macrophages.^{14,15} Expression of different VEGFs and their receptors were quantified in the cornea using real-time polymerase chain reaction. Anti-VEGF-C treatment significantly decreased transcript levels of VEGF-C ($P = .002$), VEGF-D ($P = .01$), and VEGFR-3 ($P = .02$) in the corneas of treated mice (Figure 2). Although decreased levels of VEGF-A were seen in the anti-VEGF-C treated group, the changes were not statistically significant ($P = .05$). No change in the expression levels (from the baseline levels in normal corneas) of the prohemangiogenic receptor VEGFR-2 was observed in the anti-VEGF-C treated dry eye group or the untreated dry eye group.

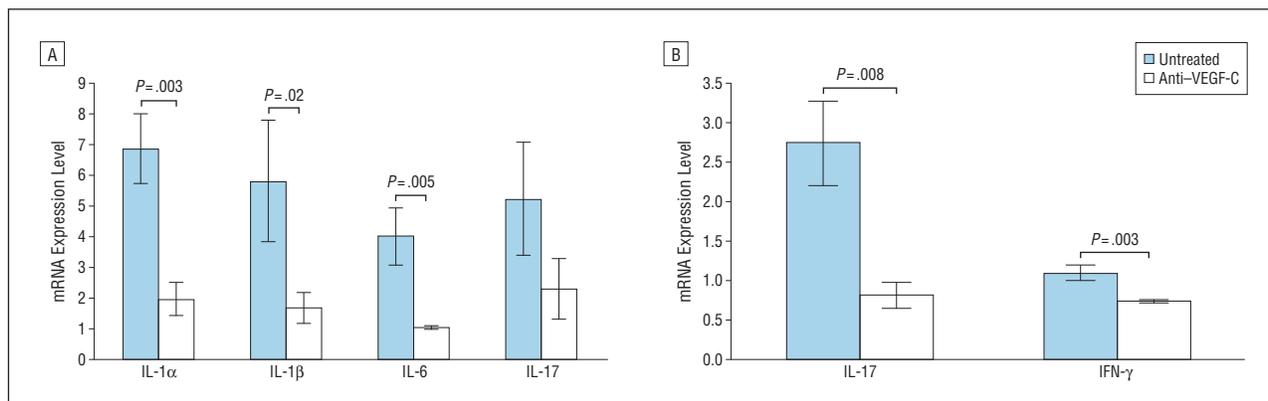


Figure 3. Effect of in vivo blockade of anti-vascular endothelial growth factor C (anti-VEGF-C) on inflammatory cytokines in the conjunctiva and lymph nodes. Real-time polymerase chain reaction analysis showing transcript levels of inflammatory cytokines interleukin 1 α (IL-1 α), IL-1 β , IL-6, and IL-17 in the conjunctiva (A) and inflammatory cytokines IL-17 and interferon γ (IFN- γ) in the lymph nodes (B). Anti-VEGF-C significantly decreased levels of cytokines in the ocular surface and lymph nodes compared with the untreated group, suppressing levels close to those observed in normal (nondry) eyes and normal lymph nodes (messenger RNA [mRNA] expression level at 1 on the y-axis represents levels in normals). Data from a representative experiment of 3 performed experiments are shown as mean (SEM); each group consists of 4 or 5 mice.

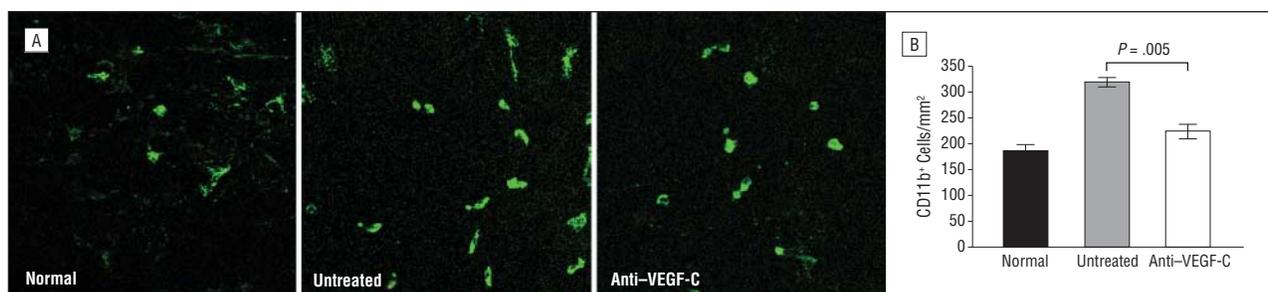


Figure 4. Effect of in vivo blockade of anti-vascular endothelial growth factor C (anti-VEGF-C) on infiltration of CD11b⁺ cells in corneas with dry eye disease. A, Representative confocal images of whole-mounted corneas showing CD11b⁺ cells (green) in normal, untreated, and anti-VEGF-C treated eyes. B, Anti-VEGF-C treatment significantly decreased the number of CD11b⁺ cells in the corneas with dry eye disease compared with the normal and untreated groups. Data from a representative experiment of 3 performed experiments are shown as mean (SEM); each group consists of 4 or 5 mice.

EFFECT OF VEGF-C NEUTRALIZATION ON IMMUNOINFLAMMATORY MARKERS IN THE CONJUNCTIVA AND DRAINING LNs

There is ample evidence for increased levels of proinflammatory cytokines in the conjunctiva in DED.^{5,16} Real-time polymerase chain reaction was used to quantify messenger RNA expression levels of IL-1 α , IL-1 β , IL-6, and IL-17 in the conjunctiva (**Figure 3A**). The group receiving anti-VEGF-C treatment showed significant decreases in the levels of IL-1 α (approximately 5-fold; $P = .003$), IL-1 β (approximately 4-fold; $P = .02$), and IL-6 (approximately 3-fold; $P = .005$). Although a similar downward trend (approximately 3-fold) was seen in the levels of IL-17, statistical significance was not achieved due to high variance.

In DED, antigen-presenting cells migrate from the ocular surface to draining LNs where they stimulate T cells, leading to expansion of IL-17-secreting Th17 cells and interferon γ -secreting Th1 cells.¹⁷ Interestingly, treatment with anti-VEGF-C was paralleled by significant reductions in levels of IL-17 ($P = .008$) and interferon γ ($P = .003$) in the draining LNs (**Figure 3B**).

EFFECT OF VEGF-C NEUTRALIZATION ON CD11b⁺ CELLS IN DRY EYE CORNEAS

Recruitment and activation of monocytic (CD11b⁺) cells to the cornea is an important hallmark of DED.¹⁸

To evaluate changes to the CD11b⁺ cells as a result of VEGF-C blockade, at the end of the study period (day 14), corneal whole mounts were immunostained for CD11b and the CD11b⁺ cells were quantified under the confocal microscope. Therapy with anti-VEGF-C significantly decreased CD11b⁺ cells in the treated corneas compared with the untreated corneas ($P = .005$); an overall 30% reduction in CD11b⁺ cells was seen in the treated group (**Figure 4**), suggesting that immune activation in the cornea is blunted as a result of VEGF blockade.

EFFECT OF VEGF-C NEUTRALIZATION ON CLINICAL SIGNS OF DED

To determine whether treatment with anti-VEGF-C decreases the corneal signs of DED (surface epitheliopathy), mice were assessed clinically using fluorescein dye (**Figure 5**). Two days after induction of DED, no difference was seen among the untreated and anti-VEGF-C treated groups in the corneal fluorescein staining scores. However, starting at day 5, mice treated with anti-VEGF-C antibody began to show a significant decrease in corneal fluorescein staining compared with the untreated group ($P = .005$). This difference was significantly maintained for the entire duration of the experiment (day 14).

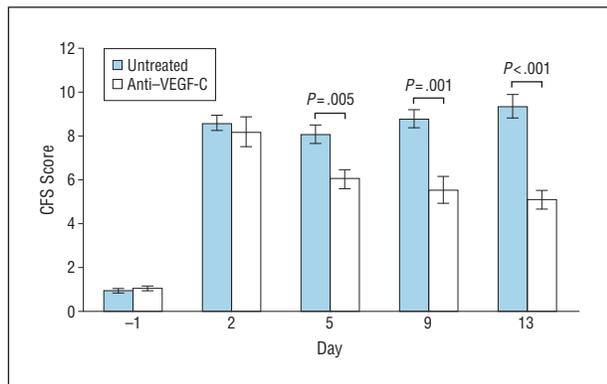


Figure 5. Effect of in vivo blockade of anti-vascular endothelial growth factor C (anti-VEGF-C) on corneal epitheliopathy in dry eye disease. Corneal fluorescein staining (CFS) scores were significantly decreased in the group treated with anti-VEGF-C at days 5, 9, and 13 compared with the untreated group. Data from a representative experiment of 3 performed experiments are shown as mean (SEM); each group consists of 5 mice.

COMMENT

Lymphatics play an important role in generating immunoinflammatory responses in peripheral tissues by directing antigen-presenting cells from the periphery to the draining LNs, where T cells are primed and expanded.^{7,19,20} The archetypal lymphangiogenic factors are VEGF-C and VEGF-D, which preferentially bind VEGFR-3, a tyrosine kinase receptor expressed by lymphatic endothelial cells and activated macrophages, to induce lymphangiogenesis.^{7,8} Targeting the VEGF-C/VEGF-D/VEGFR-3 pathway has proven beneficial in preclinical models of lymphedema and tumor metastasis.²¹ In our recent work where we provide evidence of selective lymphangiogenesis (but not blood vessel ingrowth) occurring in DED corneas, we demonstrate upregulation of VEGF-C, VEGF-D, and VEGFR-3 in DED corneas.⁶

Accordingly, in our study, systemic anti-VEGF-C treatment was initiated before induction of DED. Although lymphangiogenesis still occurred in the treated corneas in the setting of a desiccating environment, there were significant reductions in both lymphatic area and lymphatic caliber compared with the untreated group. Lymphatics are potential conduits that provide access to antigen-presenting cells and antigens that migrate from the cornea to the draining LNs.²² Studies have shown that VEGF-A/VEGFR-2-induced lymphatic vessels differ in structure and function from those induced by VEGFR-3 ligands, exhibiting a relatively dilated, leaky, and poorly functional phenotype.²³⁻²⁵ Hence, in this study it is possible not only that interrupting VEGF-C decreased the number and area of lymphatics but also that the residual lymphatics could be functionally different and less efficient as carriers of antigen-presenting cells.

Inflammation is the main stimulus leading to the expression of VEGF-C and VEGF-D.²⁶ Corneas with DED are characterized by high levels of proinflammatory cytokines such as IL-1 β and IL-17, which are also known to have strong angiogenic potential, in part by regulating the gene expression of various VEGF ligands and receptors.²⁶⁻²⁸ We have previously illustrated T-cell activation in the regional LNs along with induction of

autoimmunity in the draining LNs of mice with DED due to generation of pathogenic Th17 cells.^{29,30} Current data regarding the LNs of anti-VEGF-C treated mice demonstrate decreased expression of IL-17 (Th17 cells) and interferon γ (Th1 cells). Moreover, the inflammatory milieu in DED induces the recruitment and mobilization of activated CD11b⁺ cells in the cornea.¹⁸ In this study, the decrease in the inflammatory attractants at the ocular surface led to decreased recruitment of CD11b⁺ cells in the anti-VEGF-C treated corneas. This is significant as various studies have suggested that the CD11b⁺ macrophages play an important role in inflammation-induced lymphangiogenesis and induction of immunity and that depletion of these macrophages suppresses these processes.^{14,19} Thus, disrupting the VEGF-C/VEGFR-3 axis appears to interrupt the afferent arm of the immune cycle as was evident by decreased levels of proinflammatory cytokines in both the ocular and lymphoid tissues.

In addition, anti-VEGF-C treatment led to suppression of epithelial disease, as a result of desiccating stress, as seen by a significant decrease in corneal fluorescein uptake. This may be the result of a decrease in expression of proinflammatory cytokines, as there is evidence that elevated cytokine levels in the tear film create an environment in which terminal differentiation of the ocular surface epithelium is impaired, thereby impairing the epithelial surface production of mature surface protective molecules.³¹

In conclusion, this study provides evidence that blocking VEGF-C helps to suppress inflammation and corneal epitheliopathy as a result of DED. This may form the basis for novel treatments targeting the lymphatic system in ocular surface disease.

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Financial Disclosure: The Schepens Eye Research Institute has filed for intellectual property rights for regulation of lymphatic growth in dry eye using an antilymphangiogenic agent that includes an inhibitor of VEGF-D- or VEGF-C-mediated signal transduction but not the specific antibody VGX-100 used in this study. Dr Dana is listed as an inventor on this application.

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