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IL-36γ Augments Ocular Angiogenesis by Promoting the Vascular Endothelial Growth Factor—Vascular Endothelial Growth Factor Receptor Axis

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Prevention of inflammatory angiogenesis is critical for suppressing chronic inflammation and inhibiting inflammatory tissue damage. Angiogenesis is particularly detrimental to the cornea because pathologic growth of new blood vessels can lead to marked vision impairment and even loss of vision. The expression of proinflammatory cytokines by injured tissues exacerbates the inflammatory cascade, including angiogenesis. IL-36 cytokine, a subfamily of the IL-1 superfamily, consists of three proinflammatory agonists, IL-36α, IL-36β, and IL-36γ, and an IL-36 receptor antagonist (IL-36Ra). Data from the current study indicate that human vascular endothelial cells constitutively expressed the cognate IL-36 receptor. The current investigation, for the first time, characterized the direct contribution of IL-36γ to various angiogenic processes. IL-36γ up-regulated the expression of vascular endothelial growth factors (VEGFs) and their receptors VEGFR2 and VEGFR3 by human vascular endothelial cells, suggesting that IL-36γ mediates the VEGF-VEGFR signaling by endothelial cells. Moreover, by using a naturally occurring antagonist IL-36Ra in a murine model of inflammatory angiogenesis, this study demonstrated that blockade of endogenous IL-36γ signaling results in significant retardation of inflammatory angiogenesis. The current investigation on the proangiogenic function of IL-36γ provides novel evidence of the development of IL-36γ—targeting strategies to hamper inflammatory angiogenesis. *(Am J Pathol 2023, 193: 1740—1749; https://doi.org/10.1016/j.ajpath.2023.01.003)*

The cornea exhibits a unique quality of being completely avascular in nature, which is maintained by a dynamic balance between proangiogenic and antiangiogenic factors.[1] Vascular endothelial growth factors (VEGFs) and receptors (VEGFRs) act as key mediators of angiogenesis. Interestingly, a healthy cornea constitutively expresses soluble VEGFR1 and VEGFR3, which bind to VEGF-C and -D as decoy receptors to maintain corneal avascularity.[2–4] In inflammatory or traumatic disorders, this homeostatic equilibrium is disrupted as VEGF-A is significantly up-regulated and overwhelms the antiangiogenic receptors.[5] Angiogenesis, a growth of new vascular structures, plays a critical role in sustaining inflammatory response.[6] Given its central role in the pathogenesis of graft rejection, tumorigenesis, and autoimmune flares,[7–9] considerable effort has been made to characterize the underlying mechanisms that regulate angiogenesis and develop novel therapeutic modalities.

IL-36, a subfamily of the IL-1 cytokine, promotes inflammatory response by binding to the IL-36 receptor (IL-36R) IL1RL2.[10] Three agonist isoforms, IL-36α, IL-36β, and IL-36γ, promote pathogenesis of various autoimmune disorders.[11] Specifically, elevated expression of IL-36γ has been observed in the skin of patients with psoriasis and the synovial fluid of patients with psoriatic arthritis.[12,13] Both pathologic conditions are ridden with aberrant angiogenesis[14]; however, no study has investigated the direct contribution of IL-36γ on inflammatory angiogenesis.[15]

IL-36γ promotes activation of infiltrating immune cells and hampers corneal epithelial closure following injury.[16] This study investigated whether IL-36γ directly interacts

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with vascular endothelial cells to modulate angiogenesis. Real-time assessment of neovascularization was performed in a widely used and well-standardized murine model of inflammatory angiogenesis.[17] The paucity of blood vessels in the naive state makes the cornea an excellent *in vivo* system to investigate the mechanisms of angiogenesis.

The current study evaluated the expression of IL-36γ in the naive cornea and during inflammatory angiogenesis and found that IL-36γ is highly up-regulated following an inflammatory insult. Moreover, expression of IL-36R was seen in corneal vascular endothelial cells, underscoring the capacity of IL-36γ to exert direct effects on endothelial cells. These data demonstrate that endogenous IL-36γ directly up-regulates the expression of VEGFs and their receptors (VEGFR2 and 3) to promote angiogenesis. Moreover, topical blockade of IL-36γ significantly prevented inflammatory angiogenesis, suggesting a critical contribution of IL-36γ in ocular surface neovascularization.

Materials and Methods

Animals

Six- to eight-week–old male BALB/c wild-type mice were used (Charles River Laboratories, Wilmington, MA) for the described *in vivo* experiments. The study protocol was approved by the Animal Care and Use Committee of Schepens Eye Research Institute. The mice were housed and treated according to the Association for Research in Vision and Ophthalmology guidelines for use of animals in ophthalmic and vision research.

Corneal Angiogenesis

Corneal angiogenesis was induced by placing a single intrastromal suture on anesthetized mice, as previously described.[18,19] An 11.0 nylon suture (MANI, Tochigi, Japan) was used to place a single figure-of-eight intrastromal suture on the nasal side of the cornea, 1.0 mm from the limbus. Triple antibiotic ointment (Neomycin and Polymyxin B Sulfates and Bacitracin Zinc Ophthalmic Ointment USP, Bausch + Lomb, Wilmington, MA) was topically applied to the cornea. To minimize suture-induced pain, buprenorphine was administered via subcutaneous injection following the suture placement.

Topical IL-36γ Blockade

To block endogenous IL-36γ, 2.5 μL of soluble recombinant mouse IL-36 receptor antagonist (IL-36Ra) (0.2% in phosphate-buffered saline; Biolegend, San Diego, CA) was administered topically five times a day for 5 days after suture placement.[16] Mouse serum albumin (0.2% in phosphate-buffered saline; Thermo Fisher Scientific, Waltham, MA) served as a control protein.

Slit-Lamp Microscopy

Inflammatory angiogenesis was clinically assessed using slit-lamp biomicroscopy. Slit-lamp micrographs were captured on days 3 and 5 after suture placement. Slit-lamp micrographs were converted into binary images, and vascular density as percentage area of the whole cornea was calculated using the "Vessel Analysis" plugin in ImageJ software version 1.52v (NIH, Bethesda, MD; *https://imagej.nih.gov/ij*) as described previously.[18]

Immunofluorescence Staining

Harvested corneas were immunostained, as described previously.[20] Corneas were fixed in 4% paraformaldehyde and blocked with 10% fetal bovine serum and immunostained with fluorescein isothiocyanate–conjugated CD31 antibody (1:100; Biolegend) or mouse IL-1 receptor (IL-1R) rp2/IL-1R6 antibody (1:50; catalog number AF2354; R&D Systems, Minneapolis, MN) overnight at 4 °C. Corneas stained with IL-1Rrp2 antibody were incubated with donkey anti-goat IgG tetramethylrhodamine-isothiocyanate antibody (1:250; catalog number A16004; Thermo Fisher Scientific) for 1 hour. Corneas were whole-mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Micrographs of immunostained corneas were captured using a confocal microscope (TCS-SP8; Leica, Buffalo Grove, IL). The area covered by $CD31^+$ blood vessels was calculated using ImageJ software version 1.52v.

Enzyme-Linked Immunosorbent Assay

Levels of IL-36γ in the murine cornea were analyzed using an in-house enzyme-linked immunosorbent assay (ELISA).[16] Corneal lysates were prepared by allowing corneal tissue (in 0.1% Triton-X100) to undergo three freeze-thaw cycles at −180°C/37°C, followed by homogenization with a motorized pestle and centrifugation at 180 × *g* for 10 minutes. Lysates were diluted with coating buffer (50:50) and plated (100 μL per well) in a 96-well ELISA plate, which was incubated overnight at 4°C. The wells were washed with washing buffer (DuoSet ELISA kit; R&D Systems) and blocked with 2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) for 1 hour. To detect IL-36γ, the plates were incubated with IL-1F9/IL-36γ antibody (dilution 1:50; LSBio, Seattle, WA) for 3 hours followed by incubation with horseradish peroxidase–conjugated anti-rabbit IgG antibody (Biolegend) for 1 hour under continuous rocking. The wells were washed with washing buffer, incubated with substrate solution for 10 minutes in the dark to allow for color development, followed by the addition of stop solution (DuoSet ELISA kit; R&D Systems). The results were analyzed using a SpectraMax Plus 384 microplate reader (Molecular Devices, San Jose, CA).

Vascular Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Thermo Fisher Scientific. The cells were cultured under standard culture conditions (37 °C and 5% carbon dioxide) in endothelial cell basal medium 2 (EGFM-2MV media) supplemented with growth factors (5% fetal bovine serum, VEGF, fibroblast growth factor, epidermal growth factor, and insulin-like growth factor) (Lonza, Basel, Switzerland).

Tube Formation Assay

Tube formation assay was set up in a flat-bottom, 96-well plate in quadruplicates as previously described.[21,22] In brief, 50 μL of Matrigel basement membrane (Millipore, Burlington, MA) was plated into each well and incubated for 1 hour at 37 °C to achieve adequate polymerization. A total of 2×10^4 HUVECs were cultured in basal media alone (negative control), with growth factors (5% fetal bovine serum, VEGF, fibroblast growth factor, epidermal growth factor, and insulin-like growth factor) (positive control) or with recombinant human IL-36γ (100 ng/mL; R&D Systems) for 24 hours. Tube formation was assessed at 12 hours, and micrographs were captured using an inverted brightfield microscope (Leica DMi, Buffalo Grove, IL). Measures of tube formation [number of branches, total branch length (in pixels), and number of nodes) were calculated using the "Angiogenesis Analzyer" plugin in ImageJ software version 1.52.

Proliferation Assay

Proliferation of HUVECs was measured using the BrdU cell proliferation assay (Sigma-Aldrich). In a flat-bottom, 96-well plate, 1×10^4 HUVECs were cultured alone in basal media (negative control), with growth factors (positive control), or with recombinant human IL-36γ (100 ng/mL; R&D Systems) for 12 hours under standard cell culture conditions. BrdU label was added, and cells were incubated for an additional 12 hours to measure cumulative cell proliferation. Subsequently, the culture plate was processed according to the manufacturer's protocol. A SpectraMax Plus 384 microplate reader (Molecular Devices) was used to measure absorbance at 450/550 nm.

Flow Cytometry

Single-cell suspensions of HUVECs were stained with anti−IL-36R/IL1RL2 polyclonal antibody (Proteintech, Rosemont, IL) or with the respective isotype (purified rabbit IgG; Thermo Fisher Scientific). Cells were further processed by staining with secondary goat−anti-rabbit IgG (Alexa Fluor 555, Thermo Fisher Scientific). The stained cells were acquired using an LSR II flow cytometer (BD Biosciences, San Jose, CA) and analyzed on Summit 4.3 software (Dako Colorado Inc., Fort Collins, CO).[23]

RNA Isolation and Real-Time PCR

RNA isolation was performed using the RNeasy Micro Kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol.[24,25] cDNA was synthesized using oligo(dT) primer and SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). Real-time PCR was performed using TaqMan Universal PCR Mastermix and preformulated TaqMan primers (Thermo Fisher Scientific) for *Il36g* (IL1f9; Mm00463327_m1, Hs00219742_m1), *Vegfa* (Mm00437304_m1, Hs00900055_m1), *Vegfc* (Mm00437313_m1, Hs00153458_m1), *Vegfd* (Mm00438965_m1, Hs01128659_m1), *Vegfr2* (Mm00440099_m1, Hs00911700_m1), *Vegfr3* (Mm00433337_m1, Hs01047679_m1), and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; Mm99999915_g1, Hs99999905_m1) in the Mastercycler RealPlex[2] platform (Eppendorf, Germany). The results were normalized to *Gapdh* (internal control) and analyzed using the comparative threshold cycle method.

Statistical Analysis

Unpaired two-tailed *t*-tests were used to compare means between two groups, and one-way analysis of variance was used to compare means of multiple groups. The significance level was set at $P < 0.05$. Results are presented as the means ± SD (*in vitro*) experiments and means ± SEM (*in vivo*) of at least three independent experiments. Sample sizes were estimated on the basis of previous experimental studies on corneal angiogenesis.[18−20]

Results

IL-36γ, Up-Regulated during Inflammatory Angiogenesis, Enhances the Expression of VEGFs and VEGRFs by Vascular Endothelial Cells

Given the recent findings on IL-36γ as the primary IL-36 agonist up-regulated following corneal injury,[16] the current study investigated the expression of IL-36γ in the cornea in the naive state and during inflammatory angiogenesis. Corneas were harvested at 24 hours following inflammatory angiogenesis induction for real-time PCR and ELISA analysis. Similar to previous findings,[16] IL-36γ, which was constitutively expressed in the naive cornea, was significantly up-regulated during inflammatory angiogenesis at mRNA ($P = 0.01$) and protein ($P = 0.004$) levels compared with that of naive controls (Figure 1, A and B). To further identify the source of IL-36γ in the inflamed microenvironment, expression of IL-36γ was assessed in the corneal epithelium and stroma, which was significantly up-regulated in both tissue layers (Figure 1C).

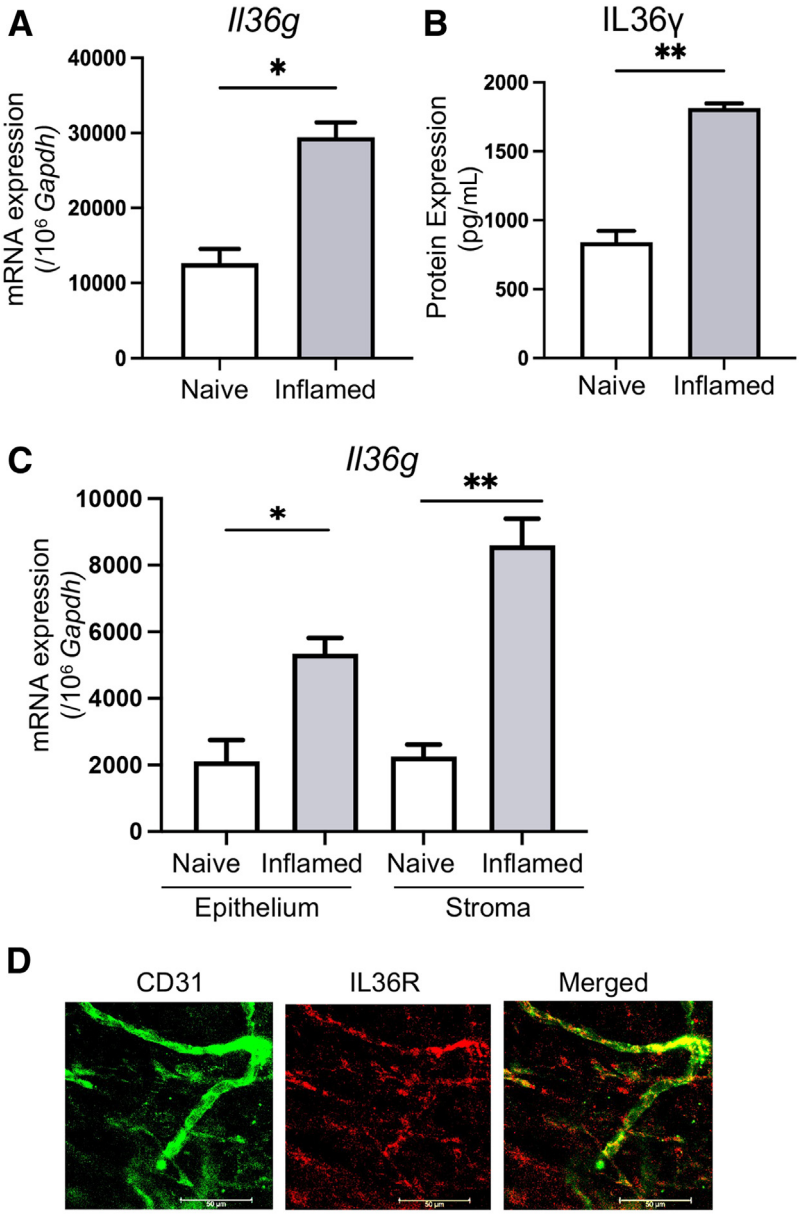

Figure 1 IL-36γ is significantly up-regulated during inflammatory angiogenesis. Inflammatory angiogenesis was induced by placing a single figure-of-eight intrastromal suture 1.0 mm from the limbal vessels in Balb/c mice. Twenty-four hours following suture placement, corneas were harvested. Corneas harvested from naive mice served as control. **A:** mRNA expression of IL-36γ in naive and inflamed corneas, as quantified by real-time PCR analysis. **B:** Protein levels of IL-36γ in naive and inflamed corneas, as quantified by enzyme-linked immunosorbent assay analysis. **C:** mRNA expression of IL-36γ in naive and inflamed corneal epithelium and stroma, as quantified by real-time PCR analysis. **D:** Representative immunofluorescent micrographs showing expression of CD31 and IL-36 receptor (IL-36R) by corneal vessels. Data from three independent experiments are shown. Data are expressed as means ± SD. $n = 4$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ (*t*-test). Scale bars = 50 μm (**D**).

IL-36γ has been reported to exert proinflammatory functions by binding to IL-36R expressed on target cells in autoimmune disorders.[26] Immunofluorescent analysis of corneas showed co-expression of IL-36R on $CD31^{+}$ vascular endothelial cells (Figure 1D). Having confirmed that IL-36γ is up-regulated during corneal angiogenesis and that corneal vascular endothelial cells express IL-36R, the study next sought to investigate the direct effect of IL-36γ on the formation of new blood vessels. Flow cytometry analysis demonstrated that primary HUVECs expressed substantial levels of IL-36R (Figure 2A). To study the molecular change IL-36γ exerts on vascular endothelial cells, HUVECs were stimulated with recombinant human IL-36γ (100 ng/mL) for 24 hours, and expression of VEGF-A, -C, and -D and their receptors was evaluated using real-time PCR (Figure 2, B and C). IL-36γ significantly up-regulated the expression of VEGF-A ($P = 0.03$), VEGF-C ($P = 0.006$), and VEGF-D ($P = 0.04$) (Figure 2B). Moreover, the addition of IL-36γ up-regulated the receptors VEGFR2 ($P = 0.01$) and VEGFR3 ($P = 0.006$) (Figure 2C). Interestingly, IL-36γ was not expressed by HUVECs at baseline and following stimulation (Supplemental Figure S1), suggesting that IL-36γ is secreted from epithelial cells and infiltrating immune cells in the corneal microenvironment. Together, these data indicate that IL-36γ exerts proangiogenic function on vascular endothelial cells by up-regulating both the vascular growth factors and their receptor expression.

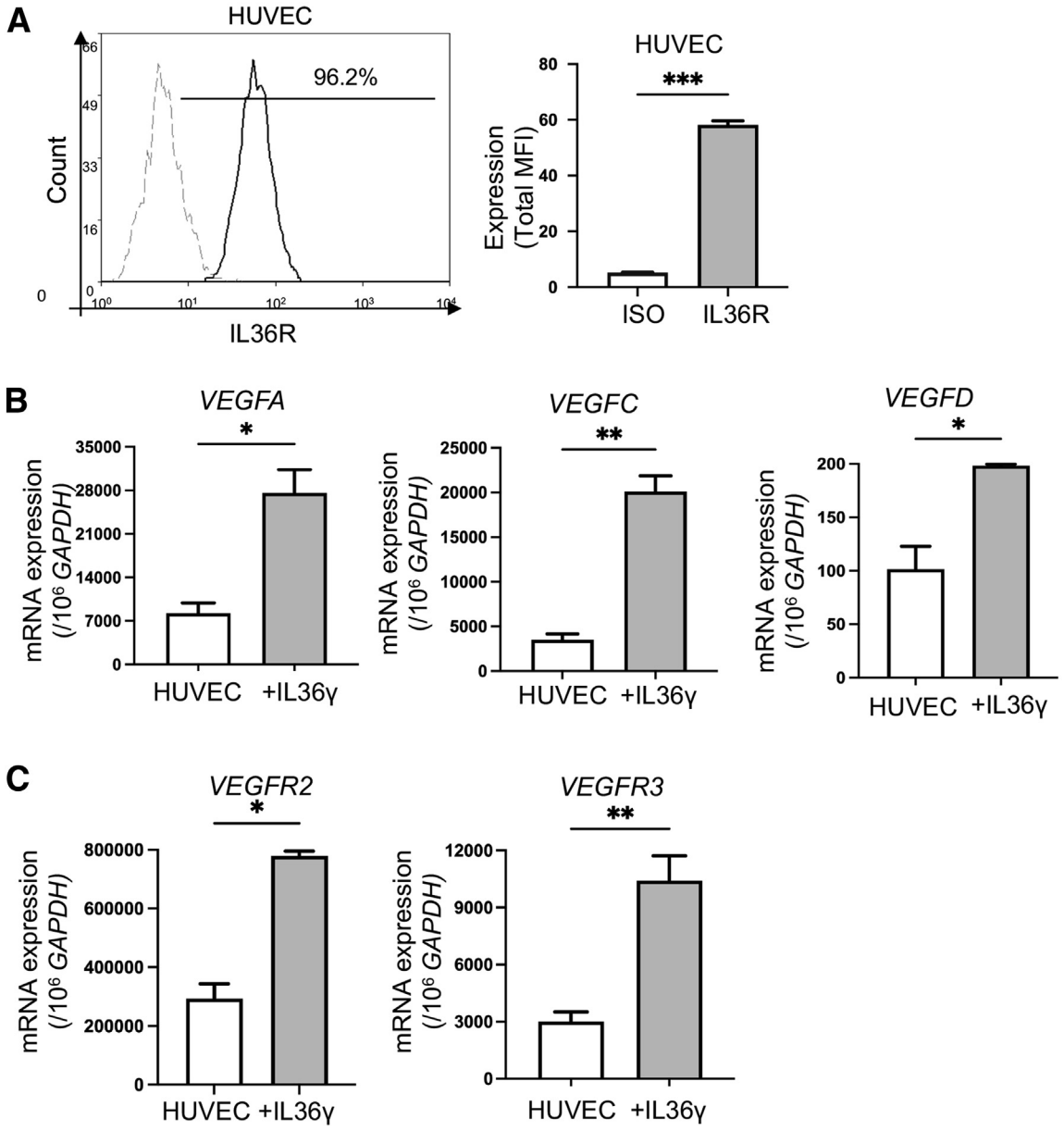

Figure 2 IL-36γ induces expression of vascular endothelial growth factors (VEGFs) and VEGF receptors (VEGFR) on vascular endothelial cells. **A:** Representative histogram (**left**) and bar chart (**right**) quantify the expression of IL-36 receptor (IL-36R) on human vascular endothelial cells (HUVECs). **B** and **C:** Primary HUVECs were cultured alone in basal media or with recombinant human IL-36γ (100 ng/mL) for 24 hours at 37 °C. The bar chart quantifies mRNA expression of VEGF-A, -C, and -D (**B**) and VEGFR2 and VEGFR3 (**C**) by HUVECs following IL-36γ stimulation. Representative data from 4 independent experiments are shown. Data are expressed as means ± SD. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ (*t*-test).

IL-36γ Directly Promotes Proliferation and Tube Formation of HUVECs

Given the high level of IL-36γ in vascularized corneas and its effect in up-regulating expression of vascular growth factors, the study next investigated the effect of IL-36γ on the cellular function of HUVECs. Vascular endothelial cells proliferate and construct capillaries to form new blood vessels[27]; thus, the study assessed whether IL-36γ can directly induce proliferation and tube formation by HUVECs. HUVECs were cultured in basal media alone or with recombinant human IL-36γ (100 ng/mL) on Matrigel for 24 hours. HUVECs cultured with endothelial growth factors served as a positive control. Tube formation was visualized under the bright-field microscopy at 12 hours of culture and quantified (Figure 3, A and B). HUVECs cultured in the presence of IL-36γ formed substantial vascular networks (Figure 3A). HUVECs in the presence of IL-36γ formed a higher number of vascular branches ($P = 0.003$), longer branches ($P = 0.04$), and nodes ($P = 0.003$) compared with those cultured in media alone (Figure 3B). To assess the effect of IL-36γ on vascular

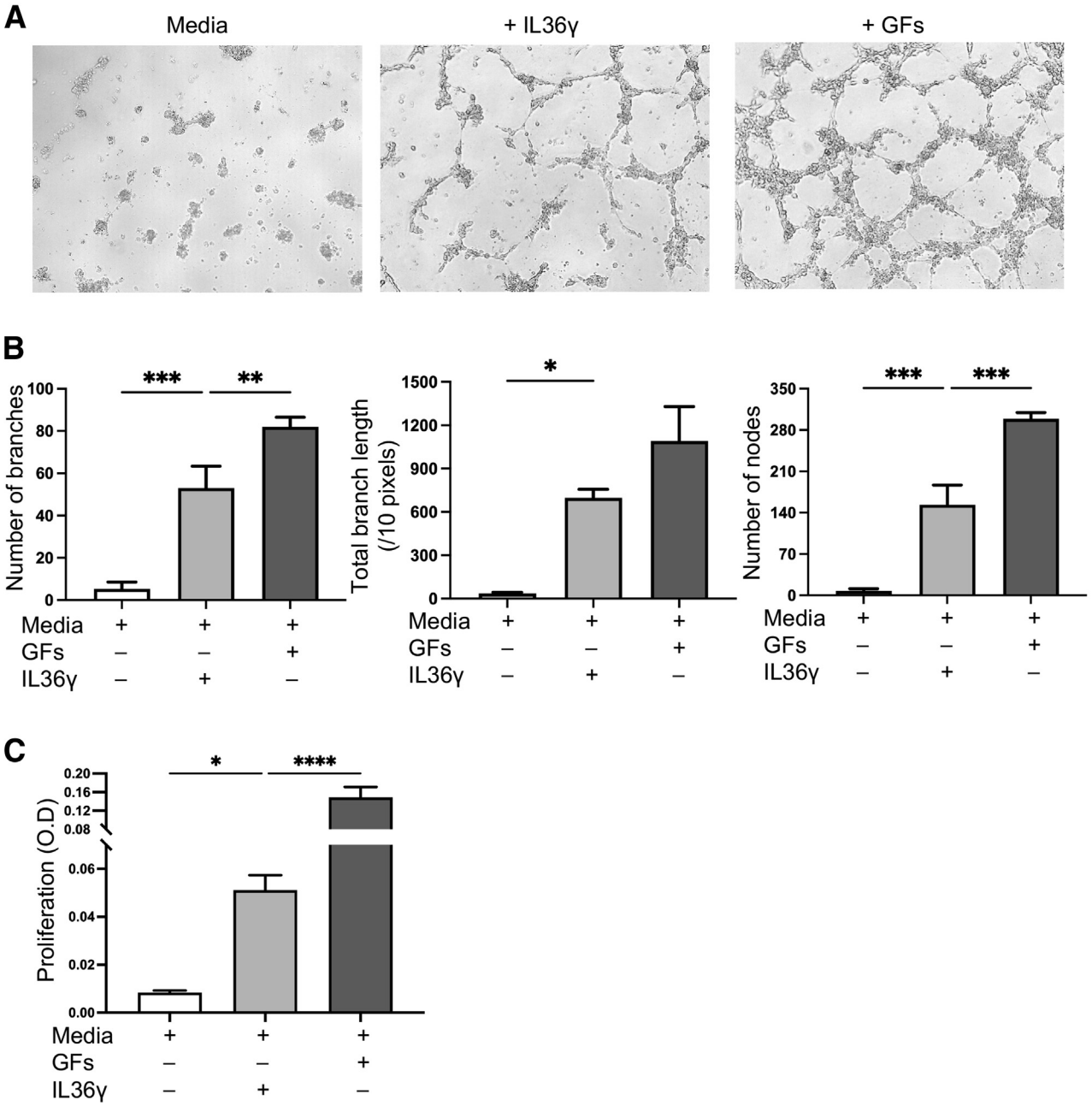

Figure 3 IL-36γ directly promotes vascular endothelial tube formation and proliferation. Primary human vascular endothelial cells were cultured alone in basal media, with growth factor (GFs), or with recombinant human IL-36γ (100 ng/mL) for 24 hours at 37 °C. **A:** Representative phase-contrast micrographs showing tube formation by vascular endothelial cells of indicated groups at 12 hours. GFs served as control. **B:** Bar chart showing measures of tube formation (number of branches, total branch length, and number of nodes) in the indicated cultures. Measures were quantified by assessing micrographs captured at 12 hours of cultures using the "Angiogenesis Analyzer" plugin in ImageJ software version 1.52v (NIH, Bethesda, MD; *http://imagej.nih.gov/ij*). **C:** Cumulative bar chart showing proliferation of vascular endothelial cells in the indicated groups, measured using BrdU incorporation assays. Representative data from four independent experiments are shown. Data are expressed as means ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ (one-way analysis of variance). Original magnification, ×10 (**A**). O.D., optical density.

endothelial cell proliferation, BrdU incorporation assay was used. IL-36γ promoted HUVEC proliferation in a dose-dependent manner (Supplemental Figure S2). In the presence of 100 ng/mL of IL-36γ, a sixfold increase in HUVECs was observed compared with those cultured in media alone ($P = 0.03$) (Figure 3C). These data demonstrate that IL-36γ directly promotes *in vitro* angiogenesis by inducing tube formation and proliferation.

In Vivo Suppression of Endogenous IL-36γ Results in Reduced Expression of Angiogenic Factors

To evaluate the effect of blocking endogenous IL-36γ in suppressing inflammatory angiogenesis *in vivo*, recombinant IL-36Ra, a naturally occurring antagonist,[28] was administered topically (0.2% in phosphate-buffered saline) five times a day for 5 days following suture placement (Figure 4A). Mouse

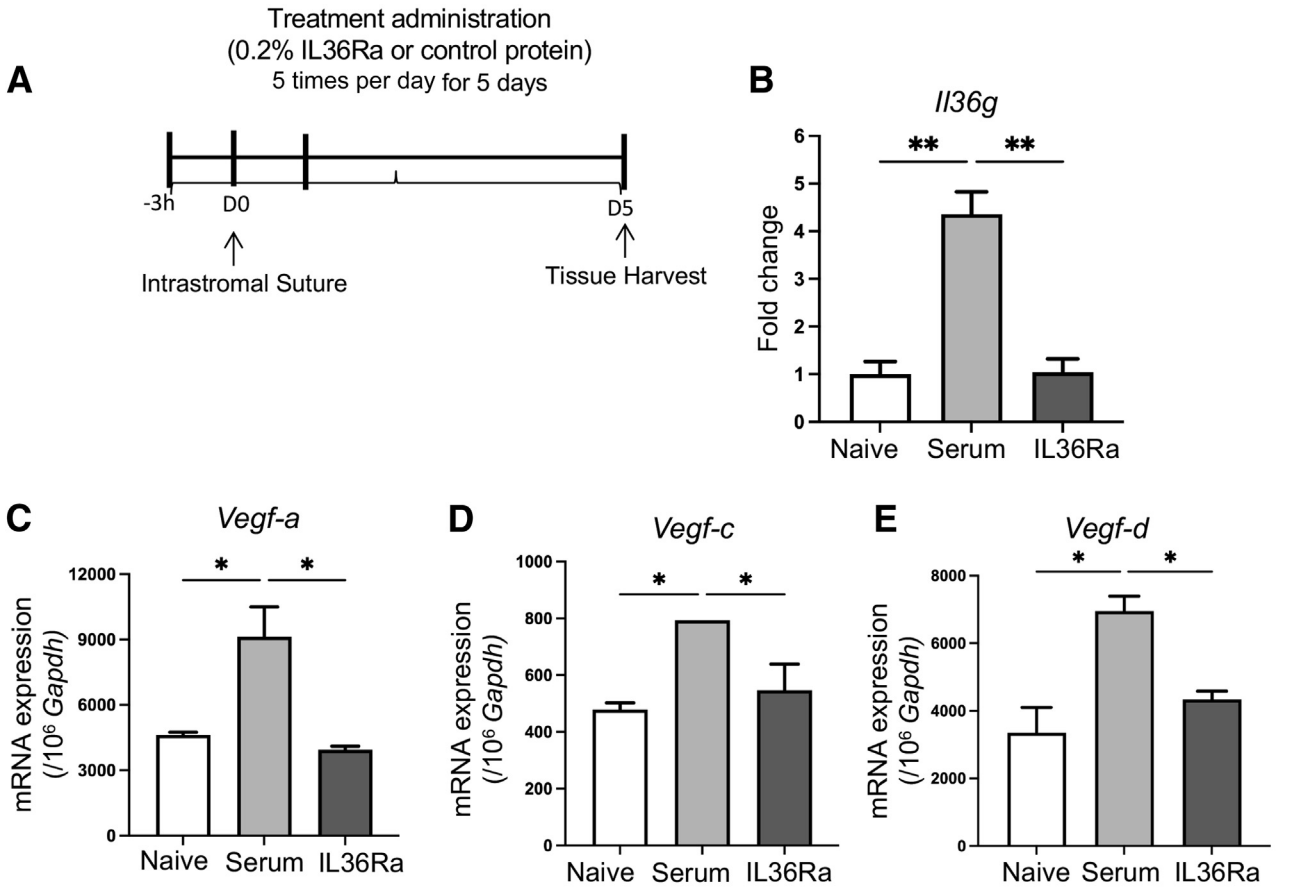

Figure 4 Blockade of IL-36γ suppresses expression of vascular endothelial growth factors (VEGFs) *in vivo*. **A:** Schematic experimental design depicting frequencies of IL-36 receptor antagonist (IL-36Ra) (0.2%) and control protein (0.2%) administration. Corneas were harvested on day 5 after suture placement for real-time PCR analysis and immunohistochemistry analysis. **B:** Bar chart showing expression of IL-36γ in fold change from a naive cornea following topical IL-36Ra treatment and quantifying the expression of VEGF-A (**C**), VEGF-C (**D**), and VEGF-D (**E**) in corneas of indicated treatment groups, as quantified by real-time PCR. Data from two independent experiments are shown. Data are expressed as means ± SEM. $n = 5$ mice per group. $^*P < 0.05$, $^{**}P < 0.01$ (one-way analysis of variance).

serum albumin served as a control. On day 5 after suture placement, the efficacy of IL-36Ra treatment was confirmed by evaluating the expression of IL-36γ in the harvested corneas. IL-36Ra treatment resulted in 80% suppression of IL-36γ, to near-homeostatic levels ($P = 0.005$; Figure 4B). To assess the effect of blocking IL-36γ on the expression of VEGFs in the inflammatory milieu, corneas were harvested on day 5 after suture placement, and expression of VEGF-A, -C, and -D was evaluated using real-time PCR. Consistent with the controlled co-culture system, corneas, in the absence of IL-36γ up-regulation, expressed significantly lower VEGF-A ($P = 0.01$; Figure 4C), VEGF-C ($P = 0.04$; Figure 4D), and VEGF-D ($P = 0.03$; Figure 4E) compared with control-treated corneas. In fact, the expression of VEGFs was comparable to that in naive mice, suggesting IL-36γ is a critical cytokine in up-regulating VEGFs during inflammatory angiogenesis.

Pharmacologic Blockade of IL-36γ Prevents Corneal Angiogenesis

Finally, to assess whether inhibition of endogenous IL-36γ function prevents corneal angiogenesis, IL-36Ra— or control-treated corneas were clinically assessed using a slit-lamp biomicroscope. Corneas were treated as outlined in the experimental design (Figure 4A), and slit-lamp micrographs were captured on days 0, 3, and 5 (Figure 5A). IL-36Ra—treated corneas showed significantly less angiogenic density compared with those treated with control protein ($P = 0.0001$; Figure 5B). To assess the extent of vascularization at the microscopic level, corneas were harvested and stained with anti-CD31 antibody for immunofluorescence analysis. Consistent with the clinical assessment, IL-36Ra treatment resulted in a significantly less vascularized area compared with control treatment ($P = 0.01$; Figure 5C). Moreover, significant suppression of VEGFR2 ($P = 0.01$) and VEGFR3 ($P = 0.001$) was observed following IL-36Ra treatment, as quantified by real-time PCR analysis (Figure 5, D and E). In sum, these data indicate that IL-36γ exerts a proangiogenic effect during angiogenesis and that blockade of this endogenous cytokine prevents inflammatory corneal neovascularization.

Discussion

This study highlights the function of endogenous IL-36γ in promoting angiogenesis following an inflammatory insult to

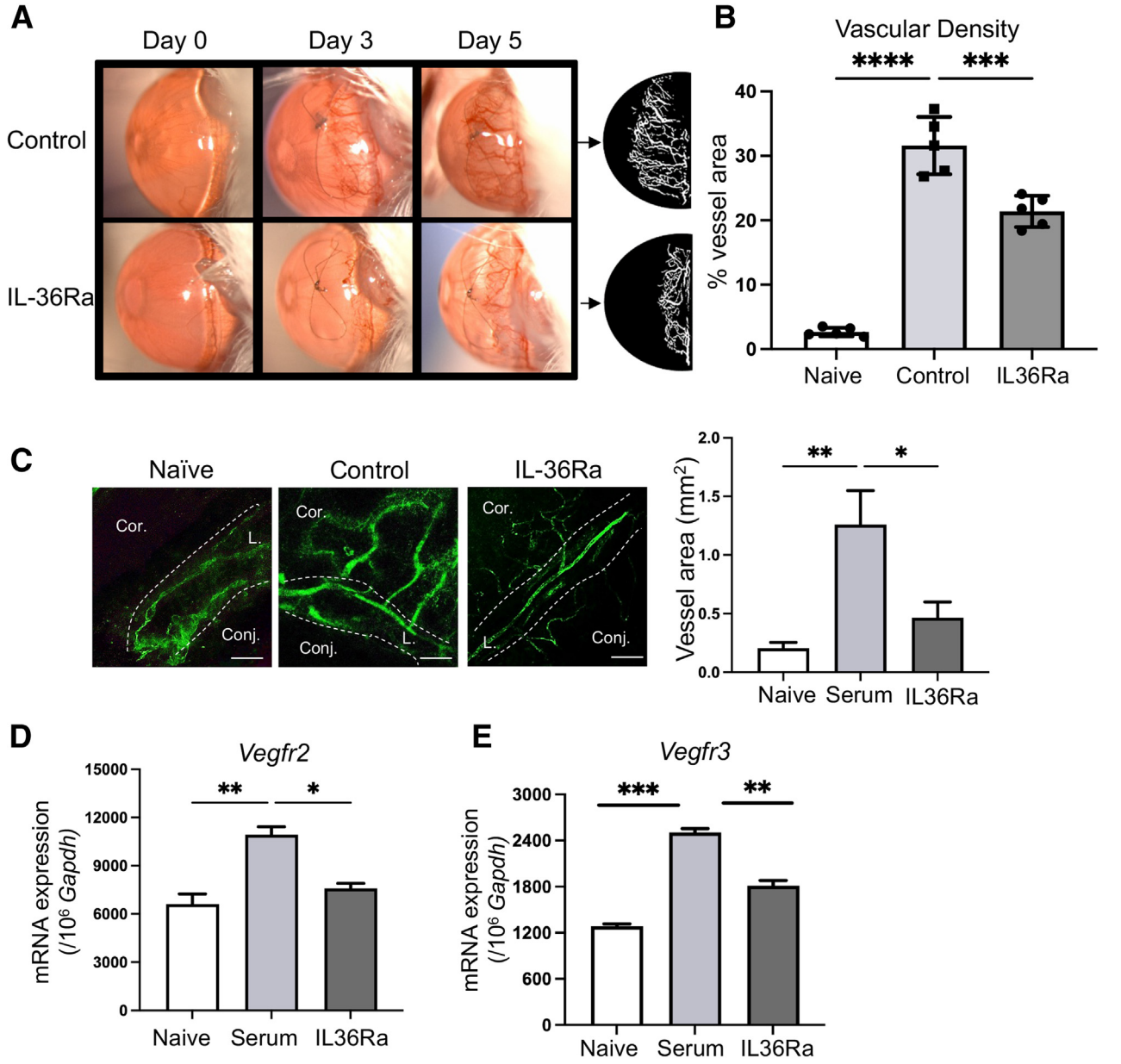

Figure 5 Blockade of IL-36γ prevents inflammatory corneal angiogenesis. **A:** Representative slit-lamp biomicroscope micrographs of corneas on day 0 (before suture placement) and days 3 and 5 after suture placement. **B:** Binary images (**left**) and bar chart (**right**) quantifying corneal neovascularization. Slit-lamp micrographs were converted into binary images, and vascular density, as the percentage area of the cornea covered by blood vessels, was calculated using the "Vessel Analysis" plugin in ImageJ software version 1.52v (NIH, Bethesda, MD; http://imagej.nih.gov/ij). Corneas were harvested on day 5 after suture placement for immunofluoresence analysis. **C:** Representative immunofluorescent micrographs (**left**) and quantitative bar chart (**right**) showing the area covered by $CD31^+$ vascular endothelial cells in indicated treatment groups. Limbal vessels are demarked by the dashed white lines. **D** and **E:** Expression of vascular endothelial growth factors receptors (VEGFR) 2 (**D**) and 3 (**E**) in the corneas of indicated treatment groups, as quantified by real-time PCR. Representative data from two independent experiments are shown. One dot represents one mouse. Data are expressed as means ± SEM. $n = 5$ mice per group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ (one-way analysis of variance). Scale bar = 100 μm (**C**). Conj, conjunctiva; Cor, cornea; IL-36Ra, IL-36 receptor antagonist; L, limbus.

the cornea. It demonstrated that IL-36γ is significantly up-regulated following corneal insult in a murine model of inflammatory angiogenesis. IL-36γ up-regulated the expression of VEGFs and their cognate receptors by vascular endothelial cells. Furthermore, IL-36γ directly promoted proliferation and tube formation by vascular endothelial cells and *in vivo* blockade of endogenous IL-36γ prevented inflammatory angiogenesis.

IL-36γ is an agonist of IL-36, a subfamily of the IL-1 cytokine, that mediates inflammatory response by binding to IL-36R and IL-1R to activate NF-κB and mitogen-activated protein kinase.[29] IL-36 cytokines, especially IL-36γ, are

markedly up-regulated in psoriasis lesions ridden with pathologic angiogenesis.[12,13] Angiogenesis, also termed neovascularization, is the formation of new blood vessels from parental vessels. Although angiogenesis can be physiologic, amounting evidence has demonstrated that angiogenesis plays a central role in sustaining inflammatory response and tumor growth.[30,31] Studies have reported high levels of IL-36γ in pathologies of aberrant blood vessel formation and have demonstrated various proinflammatory functions of IL-36γ on innate immune cells and epithelial cells.[16,32,33] However, no study has investigated the direct contribution of IL-36γ on angiogenesis. A recent study examined the indirect role of IL-36γ on vascular cell activation by co-culturing vascular endothelial cells and fibroblasts in the presence or absence of IL-36γ.[15] The report shows that IL-36γ induces VEGF-A expression by fibroblasts to promote tube formation by vascular endothelial cells.[15] The current study demonstrated, for the first time, the direct contribution of IL-36γ on vascular endothelial cell proliferation and tube formation, highlighting IL-36γ as a potential target to prevent inflammatory angiogenesis.

Epithelial cells and innate immune cells, such as macrophages, express IL-36R.[16,34] Herein, HUVECs constitutively expressed high levels of IL-36R, indicating that IL-36γ can directly bind and activate vascular endothelial cells. The in-depth analysis of the effect of IL-36γ signaling on vascular endothelial cells demonstrates that IL-36γ up-regulates the expression of not only VEGF-A, -C, and -D but also their receptors VEGFR2 and VEGFR3. VEGFs and their receptors play major roles in pathologic angiogenesis by promoting vascular permeability, cell proliferation, and migration.[35−37] VEGF-A, a potent proangiogenic factor, promotes hemangiogenesis by binding to its receptors VEGFR1 and VEGFR2.[35] However, VEGFR2 has been studied more extensively as the primary signaling receptor that directly promotes angiogenesis.[38] VEGFR1indirectly contributes to hemangiogenesis, in conjunction with VEGFR2, by recruiting VEGF-A−secreting immune cells.[39,40] VEGFR3 has historically been thought to be restricted to the lymphatic endothelium; however, several reports have demonstrated the VEGFR3 is up-regulated in microvasculature of tumors[41] and wounds[42] and that VEGFR3 is highly expressed in angiogenic sprouts to mediate vascular density, vessel branching, and endothelial cell function.[43,44] Thus, the current observation of up-regulation of VEGFs and both VEGFR2 and VEGFR3 suggests that IL-36γ mediates VEGF and its receptor system to modulate angiogenesis.

Angiogenesis occurs in sequential steps *in vivo*: migration of endothelial cells to sprouting vessel ends, proliferation of endothelial cells, and morphogenesis of the cells into tubelike structures.[45] Herein, IL-36γ directly promoted two critical steps of angiogenesis using *in vitro* proliferation and tube formation assays. Vascular endothelial cells underwent significant proliferation and tube formation in the presence of IL-36γ, suggesting IL-36γ alone is sufficient to induce neovascularization in the absence of other proangiogenic signals from immune cells or fibroblasts.

The cornea serves as an ideal site for angiogenic studies due to its transparent nature and avascularity.[46] Vessels are restricted only to the limbal area and angiogenesis occurs toward the central cornea in response to an angiogenic trigger.[47] Using a well-established murine model of inflammatory angiogenesis and topical IL-36Ra treatment, we demonstrate that topical blockade of IL-36γ prevents inflammatory angiogenesis compared with control treatment. IL-36Ra, a naturally occurring IL-36 antagonist, competitively binds to IL-36R to prevent IL-36γ signaling.[28] Our molecular analysis of corneal VEGF expression demonstrates that inhibition of IL-36γ signaling hinders the expression of VEGF-A, -C, and -D.

Taken together, these data demonstrate that IL-36γ promotes inflammatory angiogenesis and that inhibition of endogenous IL-36γ prevents neovascularization following inflammatory insult. The findings of this study provide novel insights into IL-36γ−induced signaling of VEGF-VEGFR and its orchestration of angiogenesis by inducing proliferation and tube formation of vascular endothelial cells.

Author Contributions

W.J.C. designed the study, performed experiments, analyzed data, and wrote the manuscript; E.E. and A.S., performed *in vivo* experiments; and S.K.M. and S.K.C. contributed to the underlying hypothesis, designed the study, analyzed data, and wrote the manuscript.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2023.01.003*.

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