Repeat Corneal Neovascularization is Characterized by More Aggressive Inflammation and Vessel Invasion Than in the Initial Phase

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Purpose. Treatment of corneal neovascularization can lead to vessel regression and recovery of corneal transparency. Here, we examined the response of the cornea to a repeated stimulus after initial vessel regression comparing the second wave of neovascularization with the first.

Methods. Corneal neovascularization was induced by surgical suture placement in the rat cornea for 7 days, followed by suture removal and a 30-day regression period. Corneas were then re-sutured and examined for an additional 4 days. Longitudinal slit-lamp imaging, in vivo confocal microscopy, and microarray analysis of global gene expression was conducted to assess the inflammatory and neovascularization response. Inhibitory effect of topical dexamethasone for repeat neovascularization was assessed.

Results. After initial robust neovascularization, 30 days of regression resulted in the recovery of corneal transparency; however, a population of barely functional persistent vessels remained at the microscopic level. Upon re-stimulation, inflammatory cell invasion, persistent vessel dilation, vascular invasion, and gene expression of Vegfa, Il1β, Il6, Ccl2, Ccl3, and Cxcl2 all doubled relative to initial neovascularization. Repeat neovascularization occurred twice as rapidly as initially, with activation of nitric oxide and reactive oxygen species, matrix metalloproteinase, and leukocyte extravasation signaling pathways, and suppression of anti-inflammatory LXR/RXR signaling. While inhibiting initial neovascularization, a similar treatment course of dexamethasone did not suppress repeat neovascularization.

Conclusions. Persistent vessels remaining after the initial resolution of neovascularization can rapidly reactivate to facilitate more aggressive inflammation and repeat neovascularization, highlighting the importance of achieving and confirming complete vessel regression after an initial episode of corneal neovascularization.

Keywords: corneal neovascularization, neovascularization, cornea, angiogenesis, inflammation

Corneal neovascularization is a sight-threatening condition that can cause blindness, and may result from inflammatory, infectious, and other causes, such as trauma, contact lens wear, and acquired or congenital limbal stem cell deficiency.1,2 Although to date there is no approved treatment specific for corneal neovascularization,1 typically broad immunosuppression (corticosteroids)3 and off-label antiangiogenic agents4–7 are used in the cornea, although newer therapies are undergoing clinical trials.8

These treatments, however, typically administered prophylactically after corneal transplantation or upon presentation with a vascularized cornea, diminish corneal neovessels, but do not achieve complete vascular regression.8–10 Because regression is not complete, some corneal neovessels inevitably remain, and these could precipitate repeat neovascularization if the underlying stimulus remains or where treatment is discontinued or loses efficacy. This repeated neovascular effect may mimic the situation in the treatment of neovascular age-related macular degeneration (nAMD), where a single treatment with intravitreally injected anti-VEGF agents has only limited effect and is followed by robust repeat leakage and continued choroidal neovascularization necessitating repeated injections.10 nAMD is a major disease, with global prevalence of 0.37% of all persons over the age of 40.11 In addition, some study estimates show an approximate 30% recurrence of choroidal neovascularization in nAMD patients treated with anti-VEGF12,13

Corneal transplantation is the most common transplantation procedure performed worldwide, with over 180,000 transplantations performed annually.14 Of these, typically 10% experi-
ence an immune reaction leading to graft failure. In cases of graft failure with corneal inflammation and neovascularization, the survival rate of subsequent transplants (termed ‘high risk’) is below 35% due to recurrent neovascularization. In a prior study describing a single case of corneal graft neovascularization, it was observed that a single subconjunctival injection of anti-VEGF antibody led to initial reduction of vessel caliber after 1 week, but vessel caliber increased again by 3 weeks, indicating a potential rebound effect.

In addition to the cessation of antiangiogenic treatment, repeat corneal injury, inflammation, or persisting pathology could present a risk of repeat episodes of corneal neovascularization. It is well known, for example, that rejected, failed corneal grafts carry a high risk of subsequent failure after retransplantation due to repeat neovascularization despite the original invading vessels being physically removed during transplantation. It is also well known that repeat neovascularization episodes can develop rapidly and are more difficult to treat. Given the continued development of improved antiangiogenic treatments and alternative treatment strategies, such as photodynamic therapy and UV light corneal crosslinking, that aim to regress initial neovascularization and substantially regain corneal transparency, it is unclear how the cornea would respond to repeated injury/stimulus or removal of such antiangiogenic treatment. Specifically, it is not known whether repeat neovascularization and the accompanying inflammation would occur in the same manner as the initial neovascularization episode, or if it would differ in phenotype and aggressiveness. Knowledge of the nature and aggressiveness of repeat neovascularization could potentially guide decisions regarding type, dosage, and timing of treatment.

To investigate these questions, and given the importance of inflammation as a driving force and modulator of corneal neovascularization, we used a model of suture-induced inflammatory neovascularization in the rat cornea to investigate the process of repeat neovascularization after a spontaneous and incomplete regression. The analysis was conducted at the phenotypic, and gene expression level, comparing the second episode of neovascularization with the first, by longitudinal examinations conducted in the same corneas.

**Methods**

**Human Data**

Anonymized human photographic data were extracted from an institutional database of clinical slit-lamp photographs and was used for illustrative, nonquantitative purposes only. Patient consent was obtained as per routine clinical practice for the acquisition and storage of slit-lamp photographs, following the tenets of the Declaration of Helsinki.

**Animal Experiments**

Animal experiments were conducted after receiving approval from the Linköping Regional Animal Ethics Committee under ethical permit number #585 and in line with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Wistar rats 5 to 6 weeks of age (Janvier Labs, France) were used. Two 10-0 nylon sutures placed in the temporal cornea 1.5 mm from the limbus were used to induce neovascularization, as previously described. Corneal neovascularization was allowed to proceed for seven days following suture placement (initial neovascularization). Both sutures were then removed from the cornea on day 7 to induce spontaneous vessel regression, which lasted for 30 days. On day 30, the same cornea was re-sutured as described above to induce repeat neovascularization. Following re-suture, the animals were examined at 24, 72, and 96 hours (Fig. 1). For experiments involving treatment, dexamethasone (1 mg/mL Opnol; Clean Chemical Sweden AB, Borlange, Sweden) was given topically to the re-sutured eye immediately after suture placement and after that three times daily for 3 days. The control group was not treated.

**In Vivo Imaging**

In vivo confocal microscopy (IVCM) with the laser-scanning HRT3-RCM system (Heidelberg Retinal Tomograph 3 with Rostock Corneal Module; Heidelberg Engineering, Heidelberg, Germany) was used to image inflammatory cells and vessels in the cornea, as previously described. To determine vessel diameter, IVCM images showing distinct perfused blood vessels were selected from four animals. From each animal, three image sequences were selected, and used to measure the diameter of vessels using the measuring tool in ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA), as previously described. For inflammatory cells, images from four animals per experimental group were analyzed. From each animal, three image sequences were selected, and the number of cells was counted manually using the cell counter tool in ImageJ. Analysis of cell count and vessel diameter was performed by two observers masked to the treatment and time point, and averaged values of both observers were reported.

A rodent slit-lamp microscopy imaging system (Micron III; Phoenix Research Laboratories, Pleasanton, CA, USA) was used to monitor the overall neovascularization response. Digital slit-lamp images were acquired from all experimental animals and analyzed by two observers masked to the treatment and time point. Vessel length (from the limbus to the tip of the vessels) was measured using the measuring tool in ImageJ. The number of vessels extending from the limbus toward the central cornea was counted manually. Using ImageJ, the vascularized area was defined by the area of the polygon with vertices defined by the
furthest invading vessel tips in slit-lamp photographs of the cornea and by the limbal border.

**Hematoxylin and Eosin (H&E) Staining**

Harvested corneal tissue was fixed in 4% (wt/vol) paraformaldehyde in PBS for 24 hours. Fixed samples were embedded in paraffin and sectioned into 5-μm thick sections. The sections were placed on a glass slide and deparaffinized, stained, rehydrated, rinsed in water, and imaged.

**Microarray Whole Transcriptome Analysis**

Whole transcriptome analysis was performed using GeneChip 2.0 ST microarrays (Affymetrix, Santa Clara, CA, USA). Fresh harvested corneal tissue was stored in RNA stabilization solution (RNa Later; ThermoFisher, Waltham, MA, USA) at 4°C until use. Total RNA was extracted from a single cornea per animal as one biological sample, with groups consisting of four independent biological samples per time point without pooling of RNA samples within any group. The RNA integrity was determined using the bioanalyzer (Agilent bioanalyzer 2000; Agilent, Palo Alto, CA, USA), with a RNA integrity number (RIN) value of 7 or more used as a cut-off for sample inclusion for downstream analysis. RNA samples were prepared for microarray hybridization according to the manufacturer’s guidelines (GeneChip WT PLUS Reagent Kit, P/N 703174 Rev. 2; Affymetrix Inc.). Following hybridization in a GeneChip Hybridization Oven 645 (Affymetrix, Inc.), the microarray chips were washed and stained in a Fluidics station 450 (Affymetrix, Inc.). The microarray chips were scanned using the scanner 30007G (Affymetrix, Inc.), and the raw CEL files were normalized to the log2 using the Ranking Analysis of Microarray data (RAM) method. Hierarchical cluster analysis was performed using the Affymetrix expression console (Affymetrix, Inc.). Fold change expression was obtained during initial angiogenesis and revascularization by normalizing to the naive cornea, using transcriptome analysis console (TAC; Affymetrix, Inc.). Differentially expressed genes were defined using filters: one-way ANOVA P value < 0.05, and false discovery rate (FDR) < 0.05.

**Pathway Enrichment and Upstream Regulatory Analysis**

Using the obtained differentially expressed genes, pathway enrichment analysis was performed using QIAGEN’s Ingenuity Pathway Analysis (IPA; QIAGEN, Valencia, CA, USA). Core analysis was initially performed using default parameters, and then pathway analysis was performed to identify activated/inhibited pathways, based on the z-score. Positive and negative z-score values indicated pathway activation and inhibition, respectively. The resultant list of canonical pathways was compared between initial neovascularization and repeat neovascularization. The upstream regulatory analysis was performed following the core analysis. The upstream regulatory analysis was performed to identify activated/inhibited upstream regulators. The obtained upstream regulators were compared between initial neovascularization and repeat neovascularization.

**Gene Expression Analysis by qPCR**

Rat corneas were harvested, and total RNA was extracted from each rat cornea as described above. Gene expression analysis was performed using TaqMan fast advanced master mix (PN:4444554; ThermoFisher) using custom primers for VEGF (PN4351370), Ccl3 (PN4448892), Cxcl2 (PN4453320), Hif1α (1209639 C7), Timp1 (1454605 H5), Hif6 (PN4453320), and Gapdh (PN4351370). Using Power SYBR Green PCR Master mix (Applied Biosystems) gene expression for Ccl2 with primer sequences (F-ATGCGATTATGCCCACCCTG and R-TTCCTATTTGGGGGTCAAGCAC) and Gapdh (F-ATGGTTGAAGGTCCGTTGAA and RTGACTCTGCGTTCGACCTGTG) was performed. Threshold cycle (Ct) values were normalized to Gapdh, and fold change was calculated relative to the naïve cornea.

**Statistical Analysis**

The Student’s t-test was used when comparing two sample means from normally distributed data, while the nonparametric Mann-Whitney U test was used where data were not normally distributed. One-way ANOVA with Tukey’s post hoc multiple comparisons of normally distributed data was used for group analysis. A P value < 0.05 was considered significant. Statistical tests were performed using GraphPad Prism 8 for Windows, GraphPad Software (La Jolla, CA, USA) and data error bars are presented as the standard error of measurement (SEM).

**RESULTS**

**Human Case of Corneal Neovascularization and Ghost Vessels Following Regression**

Clinically, corneal neovascularization (Fig. 2A) can result from physical or chemical injury, surgical procedures, foreign body reactions, localized hypoxia, limbal stem cell deficiency, or from infections.1,27-28 When successfully treated, the regression of pathologic vessels leaves behind threadlike strands in the regressed region after antiangiogenic treatment (Fig. 2B), but the structural and flow characteristics of these features, and their fate during re-stimulation of neovascularization are unknown.

**Revascularization is Characterized by Rapid Hyperdilation of Persistent Vessels Without Vascular Leakage**

Sutures initially placed in the rat cornea induced neovascularization after 7 days (Figs. 3A, 3F), with live imaging by IVCN revealing inflammatory cell infiltration and neovessel invasion (Figs. 3F-J). Subsequent removal of the suture led to clinical regression with approximately 50% of original vessels lacking flow after a regression period of 30 days, and the remaining vessels being severely constricted with minimal flow, resulting in a dramatic restoration of corneal transparency as observed in the slit lamp (Fig. 3B). A population of persistent vessels remained, however, and appeared as sparse, thin thread-like structures evident by IVCN (Fig. 3G) and detailed slit-lamp observation, with markedly constricted lumen supporting mainly plasma flow, with only intermittent serial erythrocyte passage observed (Supplementary Video S1). By IVCN, extravascular inflammatory cells were almost completely absent in this regressed state (Fig. 3G). Following re-suture of the same cornea after 30 days of regression, revascularization was characterized by an influx of inflammatory cells and perfusion of the persistent vessels, starting as soon as 1 day after re-suture (Figs. 3C, 3H). By day 3, a dramatic hyperdilation of the same vessels was observed, with vessel dilation increasing daily (Figs. 3D, 3E, 3I-K). The sequence of events observed in vivo was similarly observed in fixed specimens by H&E staining (Fig. 3L). Notably, the vessels observed during repeat neovascularization did not appear leaky, with the
FIGURE 2. Human case with active corneal neovascularization (arrow) before vessel regression (A) and a human case after regression of corneal neovessels with thin remnant vascular structures indicated by the arrows (B). The latter was a case of Herpes Simplex keratitis with neovascularization resolving after antiviral treatment combined with corticosteroids.

FIGURE 3. Longitudinal imaging of the same cornea indicating more rapid and aggressive neovascular phenotype during repeat corneal neovascularization. (A–E) Time series slit-lamp images are taken from the same cornea during initial, regression, and revascularization phases, indicating more rapid and aggressive neovascular response after re-suturing. (F–J) Corresponding IVCM images at each time point in the same eye, indicating the absence of inflammatory cells in the tissue after regression, but the presence of thin persistent vessels. Inflammation and hyperdilation of persistent vessels were observed during the revascularization phase. (K) The diameter of perfused, persistent vessels measured from IVCM images indicated increased vessel diameter with time (ANOVA \( P < 0.0001 \)) (black asterisks indicate pairwise comparisons relative to regressed state on day 30). During repeat neovascularization, the vessel diameter increased with time (asterisks in green indicate pairwise comparisons relative to the prior time point during repeat neovascularization). (L) H&E sections indicated small sprouting vessels visible on day 4 of initial neovascularization (arrows). By day 7 vessels were larger with increased perfusion indicated by increased erythrocyte presence (arrows). After 30 days of regression, narrow vessels (arrows) with few erythrocytes (mainly plasma flow) were evident. By day 4 of repeat neovascularization, vessels became enlarged and hyperperfused with many erythrocytes (arrow). Note also the dense infiltration of inflammatory cells under the epithelium (asterisk). Scale bars in (L) are 20 \( \mu m \). The error bars in (K) represent SEM, \( n = 5 \), and asterisks indicate \( P \) value < 0.05. Scale bars in IVCM images are 50 \( \mu m \).
corneal tissue in nonvascularized regions remaining transparent and relatively free of edema.

Inflammation is Enhanced During Repeat Neovascularization

Further morphologic changes were noted during repeat neovascularization. Relative to 4 days after the original neovascular response, the hyperdilated persistent vessels 4 days after induction of repeat neovascularization extended twice the distance from the limbus into the cornea (P = 0.02, Figs. 4A, 4B). Vessel caliber additionally doubled (from a mean of 24.1 to 52.2 μm, P = 0.001, Figs. 4C, 4D), and the density of inflammatory cells invading the cornea also doubled (P = 0.03, Figs. 4E, 4F).

Repeat Neovascularization has a Unique Transcriptomic Signature and is Characterized by Enhanced Inflammatory Gene Expression

To investigate gene regulation of the observed inflammatory response, comparative whole-transcriptome expression analysis was performed. Using gene chip microarrays in initial and repeat neovascularization phases normalized to naïve corneas, differentially expressed genes (DEG) were compared. Principle component analysis and hierarchical cluster analysis revealed clustering within groups and separation between groups, indicating a unique overall transcriptomic signature of repeat neovascularization, distinct from the initial phase (Figs. 5A, 5B). Of the DEG in the initial phase, inflammatory genes Ccl2, S100a9, and Cxcl6 were among the most upregulated with an average fold change of 26.6 among them (Fig. 5C). In the repeat phase, Ccl2, S100a9, and Cxcl6 were still among the most upregulated genes but with an average fold change of 55 among them (Fig. 5E). Expression of genes with fold change 50 or more, 20 or more, and 10 or more yielded higher numbers of genes in each category during the repeat relative to initial phase (Fig. 5D). Gene expression was stronger during repeat neovascularization compared with the initial phase for numerous inflammatory and angiogenesis-related genes, including Timp1, Il6, Mmp9, Socs3, Icam, Vegfa, and Cxcr4 (P < 0.05, Fig. 5F).

Inflammation-Related Pathways and Genes and Their Putative Upstream Regulators are More Strongly Activated During Repeat Neovascularization

To further investigate regulatory control of repeat neovascularization, pathway enrichment and upstream regulatory analysis were performed using the microarray data. Pathway enrichment analysis revealed a differing pattern of pathway activation and inhibition during the repeat relative to the initial phase (Figs. 6A, 6B). During the repeat phase, a higher number of inflammatory pathways were enriched and to a higher degree. Notably, the ‘leukocyte extravasation’ pathway activation increased, while the dual pathways ‘LPS/IL-1-mediated inhibition of RXR function’ and ‘LXR/RXR activation’ had a
stronger proinflammatory profile. Specific to the repeat phase, both innate and adaptive immune pathways were activated, as well as the production of nitric oxide (NO) and reactive oxygen species (ROS) pathway. The upstream regulatory analysis revealed that in initial neovascularization, \( \text{IL1b} \) was the most active upstream regulator with activation score 3.8, and with a \( P \) value of overlap (between the DEG and QIAGEN knowledge base) of \( 7.5 \times 10^{-4} \) (Fig. 6C). During repeat neovascularization, \( \text{IL1b} \) remained the most active regulator, but with increased activation score 7.4 and \( P \) value \( 1.1 \times 10^{-16} \) (Fig. 6D). Other upstream regulators activated strongly during repeat neovascularization were \( \text{Vegfa} \) and \( \text{IL6} \) with activation scores 4.5 and 6.25, respectively, and \( P \) values \( 1.5 \times 10^{-10} \) and \( 4.1 \times 10^{-9} \), respectively. To corroborate these results, gene expression of several inflammatory regulators in the corneal tissue was examined by qPCR. Genes \( \text{Vegfa} \), \( \text{IL6} \), \( \text{IL1b} \), \( \text{Ccl2} \), \( \text{Ccl3} \), and \( \text{Cxcl2} \) were more strongly upregulated during repeat neovascularization compared with the initial phase, with the general trend of gene expression by qPCR confirming that obtained by microarray analysis (Fig. 6E).

Dexamethasone Inhibits Initial Neovascularization But is Ineffective in Suppressing Vasodilation and Inflammation During Repeat Neovascularization

Previously, we showed that dexamethasone, a corticosteroid broadly targeting inflammation, vasodilation, and angiogenesis, potently suppresses initial corneal neovascularization. To determine whether corticosteroid treatment could also suppress the repeat neovascular response, dexamethasone was administered topically to the re-sutured eye immediately following re-suture (referred to here as day 0) and for 3 days. Analysis of slit-lamp images at days 1, 3, and 4 revealed that repeat neovascularization still occurred in both nontreated control and dexamethasone-treated groups (Fig. 7), and was therefore not suppressed by the treatment. In vivo imaging in the treated groups (Figs. 8A–C) revealed a similar inflammatory cell infiltration occurred in both groups (Fig. 8 D) and persistent vessels dilated similarly in both groups on day 1 but dexamethasone treatment suppressed dilation on day 3 relative to controls (Fig. 8E). Despite this, dexamethasone
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**FIGURE 6.** Whole transcriptome pathway and upstream regulatory gene expression analysis of repeat corneal neovascularization. (A, B) Analysis of enriched pathways based on whole transcriptome DEG during initial and repeat phases of corneal neovascularization. Blue bars indicate inhibited pathways while orange bars indicate activated pathways, with corresponding inhibition and activation z-scores, respectively. Pathway activation and z-scores increased during repeat neovascularization and consisted primarily of innate and adaptive immune pathways. (C, D) Volcano plots of upstream regulators of the identified pathways indicated stronger activation of regulators during repeat neovascularization. (E) Fold change expression of several key inflammatory genes and upstream regulators as determined by microarray and separate qPCR analysis, compared across naive, day 4 initial and day 4 repeat neovascularization groups. ANOVA with multiple comparisons was used to compare fold change expression. \(N = 4\) samples per group. Error bars represent SEM. \(* * * * P < 0.0001, ** P < 0.001, * * P < 0.01, * P < 0.05.\)
treatment did not suppress repeat neovascularization of the tissue as indicated by the presence of neovessels, resulting in a larger vascularized area relative to suppressed initial neovascularization after treatment (Figs. 8E, 8G). Also, dexamethasone treatment did not reduce vascular area relative to untreated controls. While dexamethasone was 91.2% effective in suppressing initial neovascularization, with the same treatment regimen, it was only 23.7% effective in reducing the vascularized area during repeat neovascularization (Fig. 8H).

In summary, inflammation and the neovascular response are enhanced during repeat neovascularization of the cornea owing to multiple pathways and factor expression (Fig. 9).

**DISCUSSION**

A stronger inflammatory and neovascular response in the tissue was evident, and numerous genes were more strongly expressed during repeat neovascularization. Vegfr1, a potent vasodilator, was upregulated to a significantly greater degree during repeat neovascularization, along with other proinflammatory and proangiogenic genes, such as Il1b, Il-6, Ccl2, Ccl3, Cxcr2, Mmp9, Icam-1, and Cxcr4. Previous studies showed that inhibition of Ccl2 and Il1b suppresses corneal neovascularization by inhibiting inflammatory cell recruitment in the mouse cornea. Also, Ccl3 acting through Ccr5 has been shown to result in macrophage infiltration in the cornea to promote neovascularization. Cxcr2, a key receptor for chemokine signaling, promotes angiogenesis by facilitating chemotaxis of microvascular endothelial cells in a mouse corneal neovascularization model.

Pathway enrichment analysis revealed NO and ROS production pathways activated during repeat neovascularization but not during the initial phase. NO is a strong vasodilator, and ROS is reported to regulate vasodilation and vascular permeability promoting vessel remodeling in response to high-flow conditions. ROS additionally functions as an endothelial cell (EC) activator leading to EC phenotype change and increase in permeability and plays a vital role in tissue reperfusion. Moreover, ROS produced by inflammatory cells at the site of inflammation has been shown to promote leukocyte migration across the endothelial barrier. The function of NO, ROS, and VEGFA signaling provides strong evidence corroborating the rapid reconfiguration of persistent vessels and strong vasodilation responses observed in this study. The observed inflammatory response is also likely to be regulated by enhanced activation of leukocyte extravasation signaling pathway. It is important here to note that the repeat neovascularization occurred in an incompletely regressed vascular bed containing persistent, mature vessels. Although very narrow and barely visible upon slit-lamp examination, these mature vessels responded rapidly and dramatically to hyperdilate, but without the leakage normally associated with new sprouting vessels. Repeat neovascularization, acting on established and mature vessels, may differ fundamentally from initial neovascularization, where new, leaky sprouts invade the cornea and result in edema. At the pathway level, NO and ROS signaling (among other pathways and genes) were not activated in the initial but only in the repeat phase, suggesting differential regulatory control of initial versus repeat episodes that could be driven by the vessel phenotype.

Anti-inflammatory pathways were also modulated during the repeat phase. LXR/RXR signaling was doubly inhibited, directly through inhibition of anti-inflammatory pathway LXR/RXR activation and indirectly by activation of the LPS/IL-1–mediated inhibition of RXR function pathway. This is the opposite of the LXR/RXR activation profile observed during vessel regression in this model, indicating an upregulation of inflammation to reverse the regression phenotype during the repeat phase of neovascularization. Activation of these and other inflammatory pathways may partially be attributed to upstream regulators, such as Hif1a, Vegfa, and Il6 that were more strongly activated during repeat neovascularization. Interestingly, Th1, Th2, and interferon signaling pathways were additionally activated, suggesting a possible role of the adaptive immune response in priming the tissue for faster and more aggressive repeat neovascularization. In line with this, CDB+ T cells were shown to be important for corneal neovascularization in HSV-1 infected mice. Notably, stimulated T helper cells are a source of VEGF, while adaptive immune activation has been linked to the pathogenesis of nAMD. The involvement of other vessel types, such as lymphatics during revascularization, is open for discussion, given that it has been shown that lymphangiogenesis occurs alongside hemangiogenesis, for example during inflammation in penetrating keratoplasty. Using our rat model of suture-induced corneal angiogenesis however, we observed previously that lymphangiogenesis occurs typically at 14 days after induction of angiogenesis, and lymphatics were therefore not observed during initial angiogenic stimulation in the present...
study. Interestingly, however, recurrent inflammation is thought to accelerate the development of functional lymphatic vessels, in conjunction with enhanced inflammation as observed here during revascularization. In addition, macrophages, cells we have previously shown to dominate a regressing corneal vascular bed, are known to support lymphangiogenesis in many ways, including by transdifferentiation into lymphatic endothelial cells. A recent study showed that targeting lymphatics promotes graft survival via a reduced activation of the immune system, suggesting lymphatics as mediators of immunological memory, that may impact rapid corneal revascularization.

Although highly effective in suppressing initial neovascularization, the broad-acting corticosteroid dexamethasone was ineffective in inhibiting repeat neovascularization. This result corroborates the gene regulatory and tissue-level evidence of increased inflammation and angiogenesis during revascularization, spanning multiple pathways and numerous genes. The entire transcriptome is altered during repeated neovascularization, and this alteration rendered dexamethasone only minimally effective in suppressing vasodilation and ineffective in preventing new angiogenic sprouting. This suggests that a tissue primed for revascularization may require stronger suppression of inflammation and angiogenesis; however, a higher dosage of corticosteroids may increase the risk for glaucoma and cataract development in the eye. Alternatively, other nonsteroidal approaches should be considered for suppressing the aggressive repeat neovascularization response.

In the present study, using longitudinal in vivo imaging serially in the same corneas, it was observed that resolution of the initial neovascular response—in this model triggered by the removal of the initial pathologic stimulus—led to the restoration of corneal transparency. Although this transparency was clinically evident paralleling the ghost vessels observed in

![Figure 8](image-url)
In summary, repeat corneal neovascularization is a rapid process characterized by a stronger inflammatory and vasodilation response mediated by activation of numerous pro-inflammatory signaling pathways and genes, as well as inhibition of anti-inflammatory signaling. Rapid tissue reperfusion originates from incompletely-regressed vessels and immune-mediated processes that may prime the corneal tissue for a more aggressive neovascular phenotype after repeated insult, injury, or removal of antiangiogenic treatment. This highlights the necessity of achieving as complete vessel regression as possible, confirmation of complete regression, and development of strategies targeting an enhanced and more aggressive neovascular phenotype after repeated insult, injury, or removal of antiangiogenic treatment. This highlights the necessity of achieving as complete vessel regression as possible, confirmation of complete regression, and development of strategies targeting an enhanced and more aggressive neovascular phenotype after repeated insult, injury, or removal of antiangiogenic treatment.

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References

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