Anthony Mukwaya

Neil Lagali

Regulation of ocular inflammation and angiogenesis using corneal models

– new insights based on recent research

ANTHONY MUKWAYA PHD AND NEIL LAGALI PHD, ASSOCIATE PROFESSOR
DEPARTMENT OF OPHTHALMOLOGY, INSTITUTE FOR CLINICAL AND EXPERIMENTAL MEDICINE,
LINKÖPING UINVERSITY, 581 85 LINKÖPING, SWEDEN. E-MAIL: NEIL.LAGALI@LIU.SE

What is angiogenesis and why is it important in the eye?

Angiogenesis is the biological process whereby new blood vessels grow from pre-existing ones in order to expand the vascular bed and supply blood and oxygen to tissues. Angiogenesis can occur in a healthy state as part of normal development of an organism, but it also occurs in disease. In disease, angiogenesis it is referred to as pathological angiogenesis. Pathological angiogenesis can have devastating consequences in the eye and is a leading cause of blindness. In the retina and choroid, blood vessel networks exist but are normally stable. Pathological angiogenesis in these tissues, however, can lead to proliferative diabetic retinopathy (PDR) or the wet form of age-related macular degeneration (AMD). In the cornea, pathological angiogenesis is often referred to as corneal neovascularization and can lead to edema, scarring, blindness, breakdown of immune privilege and poor prognosis for transplantation.

How is ocular angiogenesis treated?

Pathological angiogenesis can be treated with corticosteroids, which effectively suppress inflammation and subsequent angiogenesis; however, their prolonged use as an immunosuppressant is associated with adverse side effects such as corneal ulcers, cataract and glaucoma. An alternative treatment strategy is to specifically target a key molecule required for blood vessel growth, namely vascular endothelial growth factor (VEGF). Anti-VEGF agents have been used widely in recent years, for example given as intravitreal injections for treating AMD, proliferative diabetic retinopathy (PDR) and diabetic macular

edema. Anti-VEGF has also been given by subconjunctival injection and topically in cases of corneal neovascularization. Particularly in the back of the eye, anti-VEGF treatments have become the best available therapy for maintaining vision in patients who otherwise would be blind.

What are the limitations of current anti-angiogenic treatments?

Despite the broad use of anti-angiogenic treatments in the eye, a number of challenges remain. Anti-VEGF agents have only a temporary effect, so multiple repeated injections are required to keep angiogenesis under control to maintain vision. In a subset of patients called non-responders, anti-VEGF treatment confers minimal benefit. Even more concerning is that in some patients the pathological angiogenesis continues despite anti-VEGF treatment, a phenomenon referred to as anti-VEGF resistance. Although, VEGF is undoubtedly a key molecule regulating angiogenesis, it represents just one of many redundant signaling molecules and pathways, and blocking VEGF may activate these alternative angiogenic signals. Moreover, many ocular conditions involving angiogenesis occur in the presence of inflammation, which can promote and sustain the growth of new blood vessels, and anti-VEGF may be ineffective in suppressing inflammation. A further consideration is that an abrupt cessation of anti-VEGF therapy, even temporarily, may carry a risk of reactivating capillaries leading to a subsequent aggressive angiogenic growth, as has been observed in tumors upon cessation of anti-VEGF treatment.

Investigating alternative approaches for treating ocular angiogenesis

The limitations of corticosteroids and anti-VEGF treatment highlights the need for alternative treatment approaches. In order to develop alternative strategies, a deeper understanding of ocular angiogenesis and inflammation is essential. In a recent series of research studies conducted at Linköping University in Sweden, the cornea has been used as a model to investigate ocular angiogenesis and inflammation, and its regulation at the gene expression level (Figure 1).

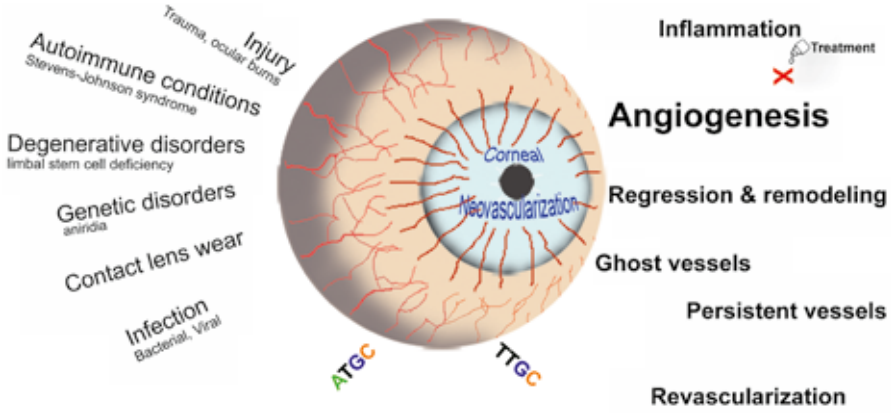


Figure 1. A number of pathologies listed on the left are potential causes of corneal neovascularization, which is the growth of new blood vessels into the cornea from the limbus. Inflammation plays an important role in the development of new blood vessels, resulting in pathological angiogenesis if not treated. The phenomena listed on the right hand side have been investigated in greater detail in a cornea model, and are described in the text below. Below the eye, the genetic regulation of these phenomena is depicted, representing upregulation (red) and downregulation (green) of various genes. Figure credit: A. Mukwaya.

Angiogenesis and capillary remodeling in the cornea

Normally devoid of blood vessels, the cornea is surrounded by an arcade of limbal vessels demarcating the boundary between the cornea and the scleral tissue containing blood vessels. In a healthy cornea, a tightly regulated balance between pro- and anti-angiogenic molecules keeps the cornea free of blood vessels. However, factors such as injury, genetic disorders and/or infection can tip the balance in favour of the proangiogenic molecules leading to inflammation. If this inflammation is not controlled it can result in angiogenesis (Figure 2A). Over time, the newly formed blood vessels partially regress and remodel to build a more stable and mature vasculature. During this remodeling and regression process, which may occur naturally or in response to treatment, distinct structures form in the tissue, referred to as 'ghost vessels' and 'persistent vessels'. Ghost vessels and persistent vessels may in turn facilitate revascularization of the cornea (Figure 2B).

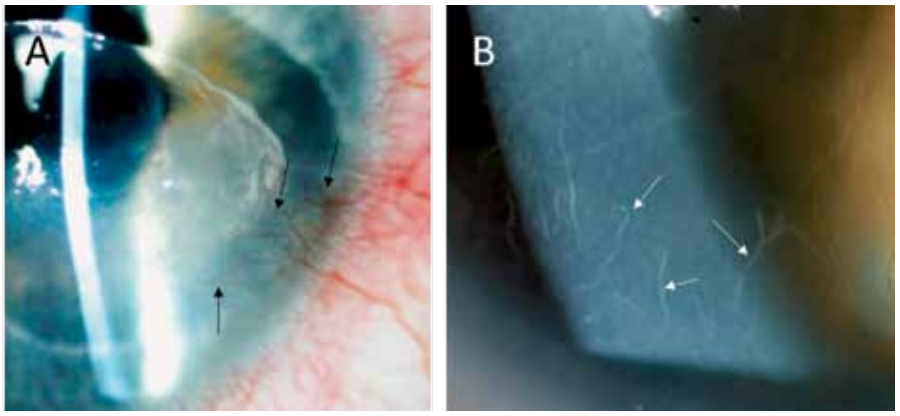


Figure 2. Slit lamp biomicroscopy images of (A) corneal neovascularization, with arrows indicating newly sprouted blood vessels invading the corneal periphery and growing in a direction towards the central cornea. These vessels leak fluid and this disrupts corneal transparency and vision. (B) Ghost vessels in the cornea (arrows) after regression of neovascularization. The impact of these structures over the long term is not fully known. Figure credit: B.Peebo, N.Lagali.

Santen

A Clear Vision For Life®

Forkortet produktresumé for Taflotan (tafluprost 15 mikrogram/ml), øjendråber, opløsning. Terapeutiske indikationer: Reduktion af forhøjet intraokulært tryk ved åbenvinklet glaukom og okulær hypertension. Som monoterapi hos patienter, som ville have gavn af øjendråber uden konserveringsmiddel; hos patienter, som ikke responderer tilstrækkeligt på første behandlingsvalg; hos patienter, som er intolerante eller kontraindicerede over for første behandlingsvalg. Som supplerende behandling til betablokkere. Taflotan er indiceret til brug hos voksne ≥ 18 år. Dosering og administration: Den anbefalede dosis er én dråbe i det/de syge øjne en gang dagligt om aftenen. Dosis bør ikke overskride én dråbe daglig, da en hyppigere administration kan mindske den sænkende virkning på det intraokulære tryk. Hvis der anvendes mere end et topikalt oftalmisk lægemiddel, skal hvert lægemiddel administreres med mindst 5 minutters mellemrum. Patienten skal informeres om korrekt håndtering af flasken. Kontraindikationer: Overfølsomhed over for de aktive stoffer eller over for et eller flere af hjælpestofferne. Særlige advarsler og forsigtigheds-regler: Før behandlingen påbegyndes, skal patienterne informeres om risikoen for vækst af øjenvipperne, mørkfarvning af huden på øjenlåget og forøget pigmentering af iris. Nogle af disse ændringer kan være permanente og føre til forskelle i udseendet på øjnene, når kun det ene øje behandles. Det anbefales at udvise forsigtighed ved brug af tafluprost hos afakiske patienter, pseudofakiske patienter med bristet bagerste linsekapsel eller forkammerlinser eller hos patienter med kendte risikofaktorer for cystoidt makulært ødem eller iritis/uveitis. Der er ingen erfaring hos patienter med alvorlig astma. Disse patienter skal derfor behandles med forsigtighed. Hvis synet bliver forbigående sløret efter inddrypning, skal patienten vente med at køre bil eller betjene maskiner, til synet er klart igen. Graviditet og amning: Tafluprost kan have skadelige farmakologiske virkninger på graviditeten og/eller fostret/det nyfødte barn. Dyrestudier har påvist reproduktionstoksicitet. Derfor bør Taflotan ikke anvendes under graviditet, medmindre det er klart nødvendigt. Taflotan må ikke anvendes af kvinder i den fødedygtige alder, medmindre der er truffet tilstrækkelige forholdsregler mht. prævention. Et studie med rotter har vist udskillelse af tafluprost og/eller dets metabolitter i brystmælk efter topikalt administration. Derfor må tafluprost ikke anvendes under amning. Interaktioner: Der forventes ikke interaktioner hos mennesker, da systemisk koncentration af tafluprost er ekstremt lav efter okulær dosering. Derfor er der ikke udført specifikke interaktionsstudier med andre lægemidler med tafluprost. I kliniske studier blev tafluprost anvendt samtidigt med timolol uden tegn på interaktion. Bivirkninger: Almindelige: hovedpine, øjenkløe, øjenirritation, øjensmerte, konjunktival/okulær hyperæmi, ændringer i øjenvipper (forøget længde, tykkelse og antal af vipper), tørre øjne, fornemmelse af fremmedlegeme i øjnene, misfarvning af øjenvipper, erytem på øjenlåget, overfladisk punktformig keratitis, fotofobi, forøget tåredannelse, sløret syn, reduceret visuel skarphed og øget irispigmentering. Ikke almindelige: pigmentering på øjenlåget, øjenlågsødem, astenopi, konjunktivalt ødem, øjenudflåd, blefaritis, celler i det forreste kammer, ubehag i øjnene, flare i det forreste kammer, konjunktival pigmentering, konjunktivale follikler, allergisk konjunktivitis og unormal følelse i øjet, hypertrikose på øjenlåget. Meget sjældne: Corneaforkalkning i forbindelse med brug af øjendråber, som indeholder fosfat, hos nogle patienter med signifikant beskadiget cornea. Hyppighed ikke kendt: iritis/uveitis, øjenlågets indkærvning fordybet, makulaødem/cystoidt makulaødem; eksacerbation af astma, dyspnø. Overdosering: Det er usandsynligt, at der vil forekomme overdosering efter okulær administration. Hvis der forekommer overdosering, bør behandlingen være symptomatisk. Pakningsstørrelser: Æsker med 1 eller 3 flasker à 3 ml. For dagsaktuel pris se: www.medicinpriser.dk. Udlevering: B. Tilskudsstatus: Generelt tilskud. Indehaver af markedsføringstilladelsen: Santen Oy, Niittyhaankatu 20, 33720 Tampere, Finland. Markedsførings-tilladelsesnummer: 60174. Denne produktinformation er forkortet i forhold til det af Lægemiddelstyrelsen godkendte produktresumé 22. marts 2018. Produktresumeet kan vederlagsfrit rekvireres fra indehaveren af markedsføringstilladelsen eller findes på Lægemiddelstyrelsens hjemmeside: http://www.produktresume.dk.
LÆS PRODUKTRESUMÉET FØR ORDINATION, ISÆR MED HENSYN TIL DOSERING, BIVIRKNINGER, ADVARSLER OG KONTRAINDIKATIONER.

Referencer:
1. Taflotan® SmPC.
2. Konstas AGP et al. Adv Ther 2017;34(1):221–35.
3. Taflotan® SmPC endose.

PP-TAFLMD-DK-0008 / MARTS 2019

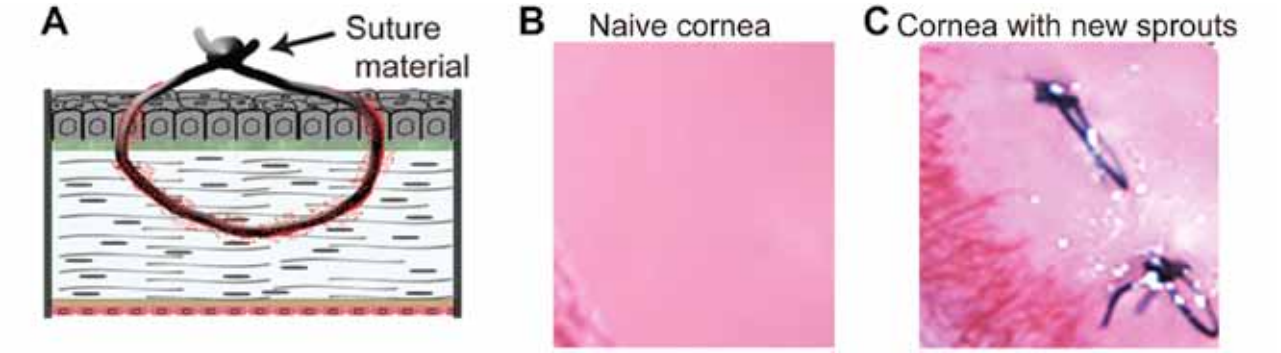


Figure 3. The suture model of inflammatory corneal angiogenesis. (A) A schematic depicting a suture traversing the corneal stroma with the knot left exposed above the corneal surface to trigger a sustained inflammatory response. (B) A naïve rat cornea without blood vessels. (C) New vessels sprouting from the limbus towards the suture sites on the temporal side of the right eye of a rat, four days following suture placement. Figure credit: A. Mukwaya.

Unanswered questions regarding ocular angiogenesis

In a series of studies recently performed at Linköping University in Sweden, regulation of inflammation and angiogenesis in the cornea was investigated at the whole genome level. The aim was to address the following questions of relevance for ocular pathologies:

1. Which genes speed up the remodeling and maturation of new capillaries? This is important because mature vessels do not leak fluid but are typically resistant to treatment while immature ones tend to leak fluid and cause tissue damage, but may be more sensitive to treatment.

2. How is inflammatory angiogenesis in the cornea regulated with time? This is important to know because inflammation and angiogenesis are dynamic events and this may have implications for the type and timing of treatment.

3. What is the role of ghost vessels versus persistent vessels in revascularisation of the cornea? Ghost vessels are observed clinically, but little is known about the impact of their presence in the long term.

4. What are the key targets for corticosteroids? To understand the basis for the effectiveness of steroids, their key targets are investigated. This may facilitate new approaches focusing on key molecules or pathways, to avoid the side effects of broad-acting corticosteroids.

A cornea model of inflammation and angiogenesis

To address the above research questions, a model of induced inflammation leading to corneal angiogenesis was used. In this model, surgical sutures are placed intra-stromally in the cornea of the rat. The suture knot is not buried into the stroma, which, in the absence of treatment, serves to act as a wound and a foreign body stimulating inflammation. Normally a healthy cornea has no vessels, however following suture stimulation, new vessels sprout from the limbus towards central cornea (Figure 3).

This model has several advantages. The response is robust and reproducible from cornea to cornea. The model is also controllable since the extent and timing of new angiogenic vessel sprouting can be defined by the location of sutures. This model also has the key advantage that it is reversible, for example, capillary regression can be induced by removing the sutures. The model recapitulates an early inflammatory response leading to angiogenesis, which mimics pathologic processes in ocular disease. In addition, because the cornea is externally visible and accessible, the model also allows for direct imaging techniques to be used in live animals, for example *in vivo* confocal microscopy (IVCM), and slit lamp photography as in Figure 3 B and C. Finally, the model reflects clinically relevant inflammation and angiogenesis occurring after suture placement in the cornea, and can be used to study the suppression of these processes using commonly used treatments, such as corticosteroids.

Capillary remodeling in the cornea model

To address the first research question, two sutures were placed into the rat cornea and vessel sprouting was allowed to proceed for four days. At this point, sprouting vessels typically extend from the limbus a distance of halfway to the sutures. On the fourth day, (referred to as the 0h time point) animals were divided into two groups. In a group called 'suture IN', both sutures are left in place, while in a group called 'suture OUT', both sutures are removed at the 0h time point. In both groups, animals were examined at 24h, 72h, and 120h time points by *in vivo* examination methods (Figure 4).

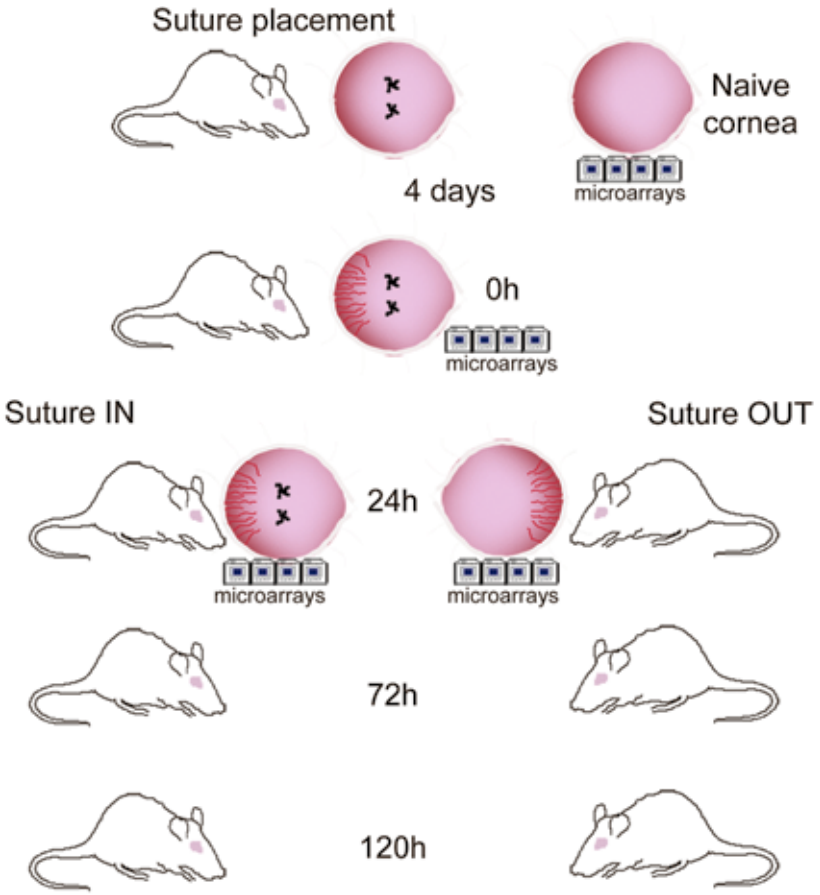
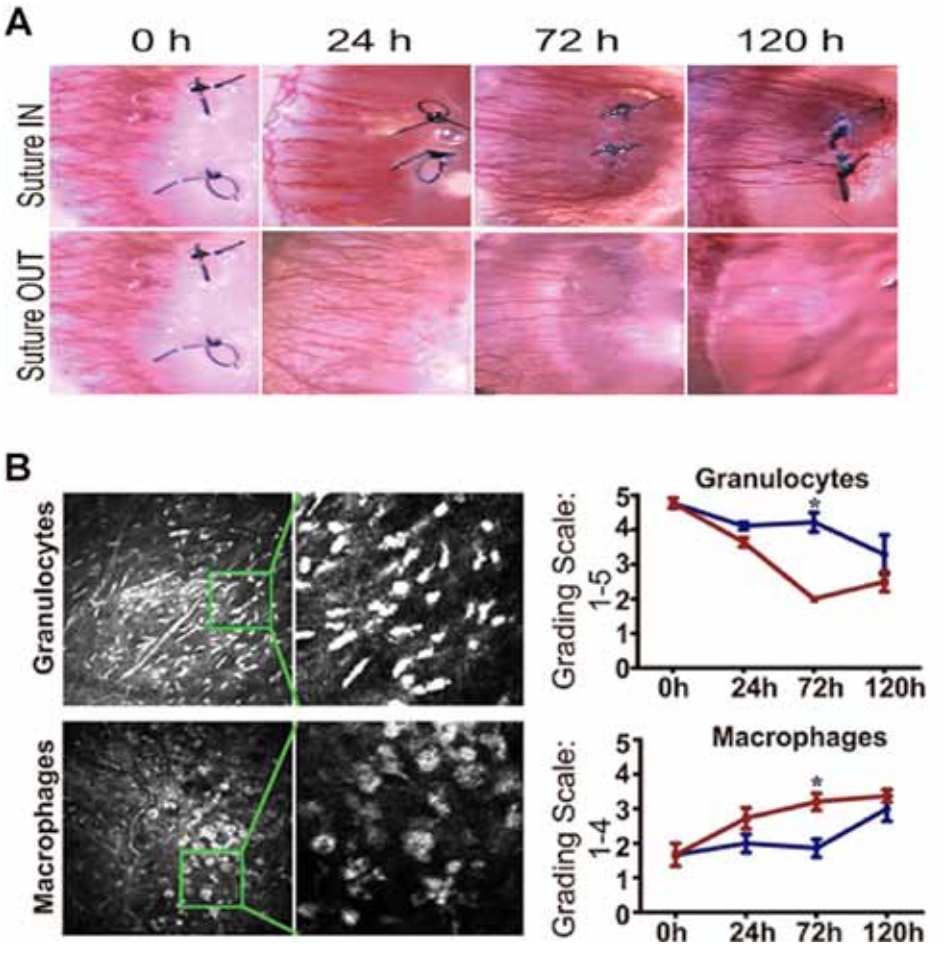


Figure 4. The experimental design followed to investigate genes involved in regression, remodeling and maturation of new capillaries. Regression was triggered by removing the corneal sutures after a 4 day initial sprouting phase ('Suture OUT' group). The 'Suture IN' group retained sutures as a positive control group. Gene microarray chips were used as indicated (4 animals per group) to compare whole-genome expression in regression and remodeling versus continued angiogenic sprouting. Figure credit: A. Mukwaya.

As expected, vessel density decreased with time after suture removal compared to the sutures being retained (Figure 5A). By in vivo confocal microscopy, it was observed that suture removal resulted in a decrease in the number of inflammatory granulocytes and an increase in the number of anti-inflammatory macrophages in the cornea (Figure 5B).

Figure 5. Phenotypic characterization of capillary remodeling and regression in the rat cornea. (A) Over time, new angiogenic capillaries in the cornea regress after suture removal. With the sutures still in place however, capillaries remain active. (B) Within the corneal tissue, inflammatory granulocyte density decreases while anti-inflammatory macrophage density increases. These macrophages are of the M2 phenotype, characterized by marker expression (data not shown). The red lines in the graphs indicate the Suture OUT group, while the blue line indicates Suture IN. Figure credit: A. Mukwaya.



Gene microarray analysis of the entire genome in the corneal tissue indicated that many genes were significantly upregulated or downregulated after placing the sutures, and after suture removal. With sutures still in place, inflammation-related genes were strongly upregulated. Twenty-four hours after suture removal, these genes were still upregulated, but to a lesser degree, while a host of other genes were downregulated. Interestingly, many genes had much higher expression than VEGF-A during both active sprouting and after suture removal.

Detailed analysis of microarray data revealed the most suppressed genes after removing the sutures were CXCL5 (a gene controlling neutrophil recruitment), CCL2 (a gene controlling monocyte recruitment) and SERPIN-B2 (a monocyte-derived blood coagulation factor, which is also known to regulate monocyte proliferation and differentiation). These three genes are known pro-inflammatory mediators. The most upregulated genes with suture removal were also identified, and these were SLIT-2, RASA-2 and GSK-3B, whose roles in the cornea are not well studied (Table 1).

Table 1. Genes most suppressed (negative fold change) and upgregulated (positive fold change) 24h after the suture stimulus is removed, compared to the suture being left in place.

Gene Symbol	Fold change with suture removal, relative to suture remaining in place
CXCL5	-41.7
CCL2	-15.6
SERPIN-B2	-15.2
SLIT-2	2.56
RASA-2	2.55
GSK-3B	2.23

To summarize, active sprouting of capillaries is associated with an influx of inflammatory neutrophil-granulocytes and expression of many pro-inflammatory factors, to a much greater level than VEGF-A. Removing the inflammatory stimulus, the cornea naturally restores its avascularity by first strongly suppressing neutrophil and monocyte recruitment through suppression of the chemokines CXCL5 and CCL2. Anti-inflammatory macrophages are recruited to remodel the newly formed vasculature and a host of other genes are expressed to dampen inflammation and angiogenesis, although the exact function of many of these genes is unknown (Figure 6).

Clinically, the importance of these findings is that inflammation is an important mediating factor in ocular neovascular disease (at the front and back of the eye) and should be addressed alongside the pathological angiogenesis. In this regard, additional targeting of specific inflammatory cytokines could be a more effective strategy to address inflammation and angiogenesis than the use of anti-VEGF alone. This hypothesis requires further studies.

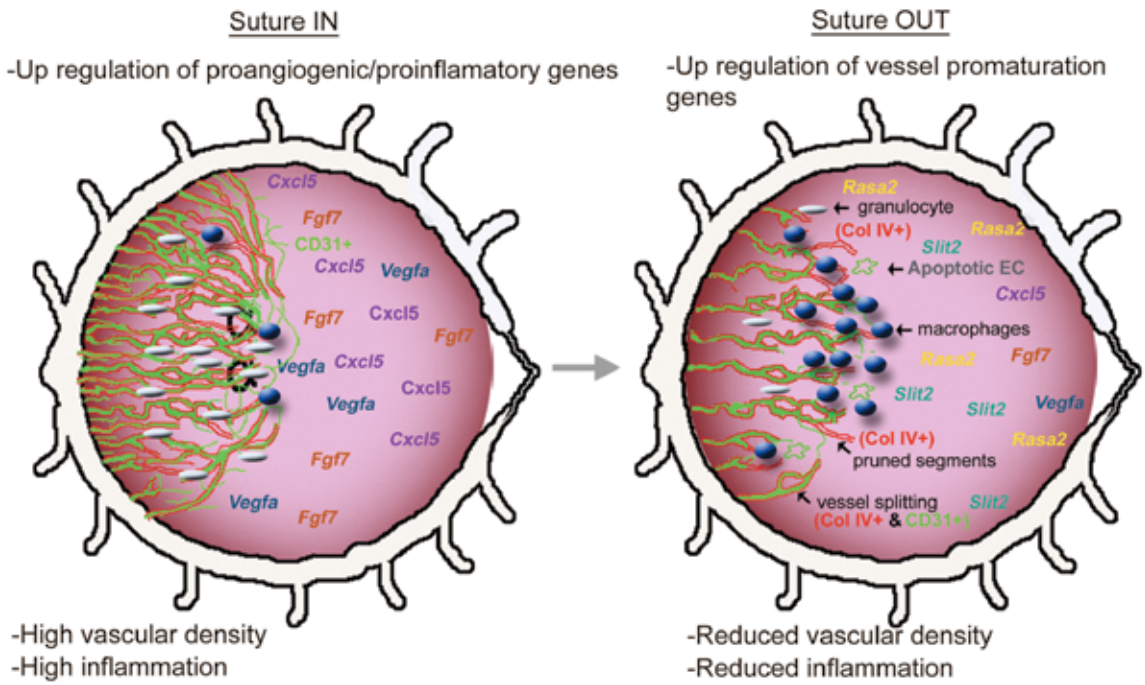


Figure 6. Summary of the angiogenic and remodeling responses in the cornea. With the sutures left IN (cornea on the left side), inflammation and angiogenesis are active and this corresponds to the expression of pro-angiogenic and pro-inflammatory factors along with granulocyte invasion (elongated white cells), while the number of anti-inflammatory macrophages (blue cells) is low. After suture removal (cornea on the right side), granulocytes are reduced, anti-inflammatory macrophages increase in number, factors related to remodeling are expressed and inflammatory factors are suppressed, leading to a regressing, remodeling vascular phenotype. Figure credit: A. Mukwaya.

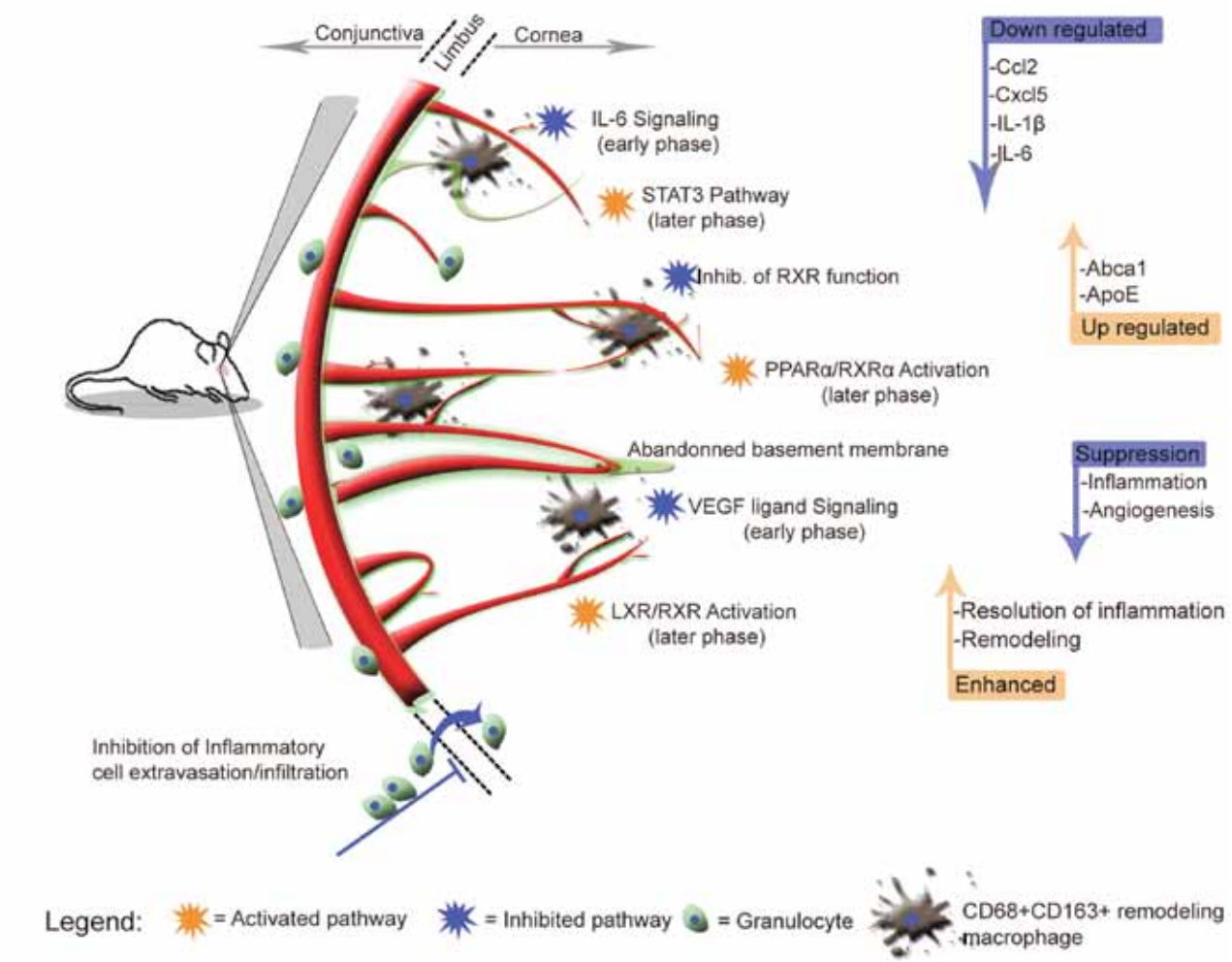


Figure 7. Summary of resolution of inflammation and capillary regression in the cornea. In an early phase of regression, pro-inflammatory pathways such as IL-6, LPS/IL-1 inhibition of RXR, and VEGF ligand signaling are inhibited, leading to a reduced expression of CCL2, IL-1β and IL-6. This dampens inflammation and reduces the granulocyte population in the tissue. In a later phase, anti-inflammatory pathways such as LXR/RXR activation, STAT3, and PPARα are activated to further suppress inflammation and remodel the vasculature. Remodeling occurs with recruitment of anti-inflammatory macrophages and is enhanced by cholesterol efflux from macrophages through the LXR downstream target genes ABCA-1 and APO-E. Figure credit: A. Mukwaya.

Time dependence of inflammation and angiogenesis

The second research question above was addressed using the experimental design presented in Figure 4, but with additional analysis of corneal tissue at the later time points of 72h and 120h with whole genome microarrays (4 corneas per group and time point). Analysis of the microarray data revealed a time-dependent gene expression during different phases of inflammation and angiogenesis. Where sutures remained in place, active sprouting continued and many genes were upregulated with the upregulation becoming stronger with time (in terms of both number of significantly upregulated genes and the degree of upregulation). Where sutures were removed, upregulation was not observed but instead many genes were downregulated, in particular inflammatory genes, and this downregulation became stronger with time (both the number of genes and degree of downregulation).

Analysis of relevant molecular pathways revealed that during active capillary sprouting (sutures in place), inflammation-related pathways were activated (such as IL-8, ILK, endothelin-1, IGF-1, CXCR4, integrin and actin cytoskeleton signaling pathways) whereas in the group with sutures removed, these pathways were not active, and instead several pathways were actively inhibited in an early phase 24h after suture removal (such as ILK, endothelin-1, ERK5, JAK/STAT, VEGF and IL-6 signaling). Of particular interest, in a later phase at 72h after initial inflammatory pathway modulation, the LXR/RXR (liver X-receptor/retinoid-X receptor) pathway was activated during remodeling (and inhibited during sprouting). The inhibitor of this pathway (LPS/IL-1 inhibition of RXR function) was likewise inhibited during remodeling, conferring a dual effect reinforcing the activation of LXR/RXR. In addition, pathways such as STAT3 and PPARα were activated during remodeling (i.e., upon suture removal).

Given the prominence of the LXR/RXR pathway during the resolution of inflammation, further analysis by Western blot, qRTPCR, and immunostaining of corneas revealed that LXR receptors α and β were expressed in the cornea and localized to anti-inflammatory M2 macrophages. Moreover, LXR downstream genes ABCA-1 and APO-E were strongly activated during capillary remodeling but not during sprouting, and their

protein products were localized to M2 macrophages. These proteins are responsible for efficient cholesterol transport from macrophages, which is essential to maintain their cellular function and prevent intracellular cytotoxicity.

To summarize, remodeling and regression of new angiogenic capillaries was characterized by an early-phase suppression of pro-inflammatory pathways such as IL-6 signaling, LPS/IL-1 inhibition of RXR, and VEGF signaling. In a later phase, anti-inflammatory pathways such as LXR/RXR, PPARα, and STAT3 were activated, while inflammatory genes such as CCL2, IL-1β and IL-6 were suppressed. LXR target genes ABCA-1 and APO-E were upregulated, and this upregulation is believed to enhance removal of cholesterol buildup within macrophages leading to effective remodeling of capillaries (Figure 7). These findings highlight the time-dependence of the natural resolution of inflammation and angiogenesis. First, an early dampening of inflammation occurs, that is later followed by activation of specific anti-inflammatory pathways to orchestrate vascular remodeling by mobilizing anti-inflammatory macrophages.

Clinically, this suggests that inhibition of not only VEGF signaling but other inflammatory pathways may be necessary to suppress potent inflammatory mediators such as CCL2 and IL-1β. The initial suppression of inflammation alone, however, appears inadequate to initiate effective capillary regression. Other, anti-inflammatory pathways such as LXR/RXR may require activation in a later phase. Also, recruitment of anti-inflammatory macrophages may be required for effective capillary remodeling. A complex, time-dependent balance and interplay of various pathways thus characterizes effective capillary regression. While this occurs during the natural restoration of corneal transparency in our model, for pathological angiogenesis observed clinically, current treatments do not act at this level of complexity. Typically, only a single factor or pathway (such as the VEGF pathway) is blocked at regular intervals.

Ghost vessels and persistent vessels

To address the third research question, non-perfused ghost vessels in the cornea were induced. To achieve this, the suture model was again used, but with sprouting allowed to proceed for seven days to allow for a limited maturation of vessels and remodeling of sprouts into functional vascular loops. On the 7th day, sutures were removed to induce regression over a 30-day period. On the 30th day, almost complete regression of vessels occurred, leaving a transparent cornea where only a few very thin non-regressed capillaries (called 'persistent vessels') could be detected by *in vivo* confocal microscopy examination and careful slit lamp examination. These persistent vessels were so thin that they permitted only single, serial erythrocyte flow along their length. In addition to these persistent vessels, a second type of structure termed 'ghost vessels' was also apparent after regression. In contrast to persistent vessels however, the ghost vessels were not perfused and did not have a cellular component, but were composed of only empty basement membrane sleeves of primarily type IV collagen.

At this point, the cornea was then re-sutured to simulate a repeated inflammatory angiogenic stimulus, such as repeat injury or high-risk corneal transplantation. Twentyfour h after re-suturing, the persistent vessels became reactivated and by 72h became hyper-dilated leading to a severe corneal vascular phenotype. The ghost vessels, however, did not re-perfuse, and remained without a cellular component. Only the persistent, perfused vessels were capable of reactivation. Over time, new sprouting occurred from these reactivated persistent vessels, without ghost vessel involvement.

Severity increases after repeated inflammatory stimulus

To investigate the stronger phenotypic angiogenic response after repeated stimulus, whole genome expression was analyzed using microarrays. More pathways were activated and to a greater degree during revascularization (the second stimulation) compared to the initial angiogenic phase (the initial stimulation). Many of these activated pathways were associated with inflammation. In particular, the LXR/RXR pathway (anti-inflammatory) was not active during the initial angiogeneic phase, but was inhibited during revascularization. Concurrently, its inhibitor (LPS/IL-1β inhibition of RXR function) was activated in the initial angiogenic phase, and to a greater degree during revascularization, reinforcing LXR/RXR inhibition. Other pathways such as nitric oxide and leukocyte extravasation were activated during revascularization. At the gene level, inflammatory and pro-angiogenic genes such as VEGF-A, CXCL2, IL-6, and IL-1β were more strongly expressed after the second stimulus compared to the first.

To summarize, corneal ghost vessels are non-perfused structures without cells that do not re-perfuse upon repeat injury. Instead, persistent non-regressed vessels can reactivate and give rise to new vessel sprouting in a faster and more aggressive manner than in the initial injury. This accelerated angiogenesis is characterized by inhibition of the anti-inflammatory LXR/RXR pathway, activation of nitric oxide-related vasodilation and enhanced expression of factors such as VEGF-A and IL-1β.

The clinical significance of these results is that in the eye, treatment-induced vascular regression is almost never total, with some persistent vessels remaining in the tissue. These persistent vessels appear to be 'primed' to respond faster and more aggressively with subsequent stimulation (or upon the removal of anti-angiogenic treatment). Once completely regressed however, vessels lose their supporting endothelial cells, forming empty conduits that do not regain the ability to reactivate. A decline in the tissue levels of pro-angiogenic factors (such as VEGF) can stimulate some vessels to mature, normalize, and persist in the tissue. These persistent vessels then serve as the source for future re-perfusion and angiogenesis. Repeated anti-VEGF treatment may simply be partially regressing vessels while leaving a few persistent ones behind, that later reactivate due to the underlying disease.

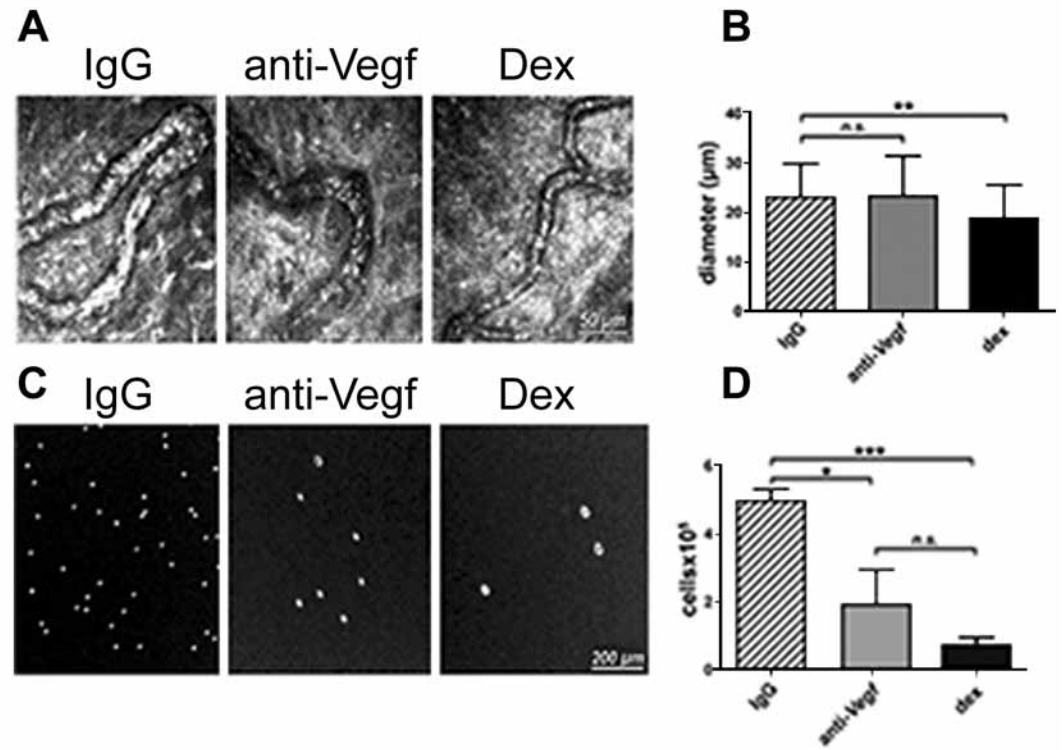


Figure 8. Differential 48 hours following corneal suture placement, the dilation of pre-existing limbal vessels was compared by measuring limbal vessel diameter by in vivo confocal microscopy (A, B). Inflammatory cell infiltration into the aqueous humor was also measured by cell counting in the aqueous fluid (C, D). Dexamethasone effectively suppressed both limbal vessel dilation and inflammatory cell infiltration into the aqueous humor. n.s. = non-significant, * P < 0.05, ** P < 0.01, *** P < 0.001. Figure credit: A. Lennikov.

Such treatment is not as efficient as the natural regression in our model, highlighting a possible benefit of ensuring a more complete regression by additionally targeting the inflammatory component, for instance by activating the anti-inflammatory LXR/RXR pathway or directly targeting factors such as IL-1β.

Why are corticosteroids so effective in suppressing inflammation?

To address the fourth research question, the corneal suture model was used with treatment given immediately after suture placement. Dexamethasone treatment was compared with anti-VEGF treatment, with IgG as a control. Treatment was given in the form of eye drops applied 4x/daily for two days to study the effect of suture placement on early-phase inflammation that eventually leads to angiogenesis. From characterization of the response by *in vivo* confocal microscopy, it was observed that dexamethasone treatment inhibited vasodilation of limbal vessels, whereas anti-VEGF did not (Figure 8 A-B). This early vasodilation is a precursor to angiogenic sprouting. In addition, Dexamethasone treatment more effectively suppressed inflammatory cell invasion into the aqueous humor (Figure 8 C-D).

Gene expression analysis of steroid versus VEGF activity

From whole genome microarray data in the different treatment groups, pathway analysis revealed dexamethasone treatment resulted in a fewer number of genes activated in most inflammatory pathways relative to anti-VEGF treatment (Figure 9). Anti-VEGF treatment did not effectively suppress inflammatory factors, as the response was similar to the control IgG treatment.

Whole genome analysis of the top 10 genes suppressed by dexamethasone and not by anti-VEGF treatment included several pro-inflammatory genes such as CCL2 and CXCL5, and a complement inhibitor CFI. Of note, dexamethasone suppressed expression of CCL2 by over 38-fold and CXCL5 by over 7-fold relative to anti-VEGF. This result highlights the broad-acting nature of corticosteroids that effectively suppress inflammation through numerous pathways and factors such as CCL2 and CXCL5 associated with neutrophil and monocyte recruitment. Clinically, corticosteroids efficiently suppress inflammation, a key step in the process of new vessel sprouting. This anti-inflammatory activity is broad, encompassing numerous pathways and factors, but certain chemokines mediating neutrophil and monocyte recruitment to the site of injury are overly suppressed by steroids, highlighting their importance. For a less broadly acting, more targeted suppression of inflammation leading to angiogenesis, one should consider specific inhibition of these chemokines.

Lessons learned from investigations of ocular inflammation and angiogenesis

Collectively, the above studies led to several insights, that are summarized here.

1. In an inflammatory environment, the expression of pro-inflammatory genes (eg. CXCL5, CCL2) and recruitment of inflammatory cells may be of equal importance as angiogenic factors such as VEGF for initiating and sustaining pathologic angiogenesis. Many other factors besides VEGF are activated and/or suppressed during active sprouting

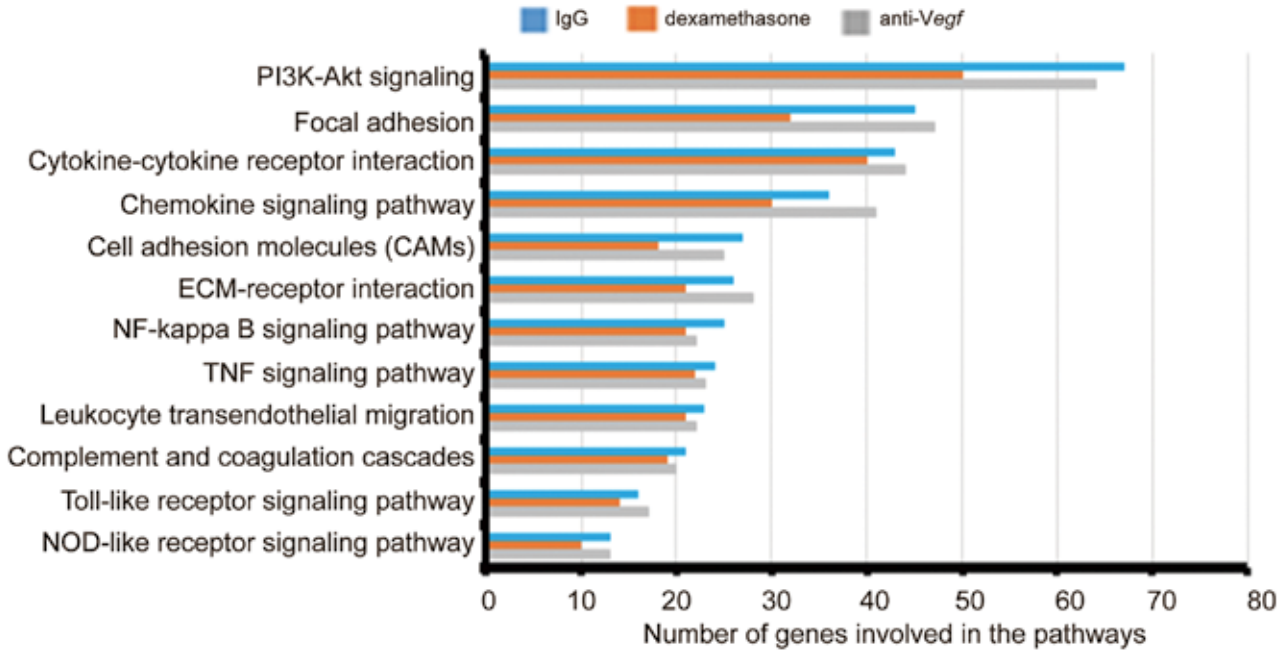


Figure 9. Activated inflammatory pathways 48h after suture placement and treatment by either steroid, anti-VEGF or IgG (control). The number of activated genes in the given pathways is indicated for the various treatment groups. In all inflammatory pathways, dexamethasone achieved the greatest suppression of genes.

and during remodeling, and these deserve closer investigation.

2. The natural regression of angiogenic capillaries is a complex process involving an early suppression of numerous inflammatory pathways and a subsequent activation of anti-inflammatory LXR/RXR, PPARα/RXRα and STAT3 signaling. This leads to subdued inflammation in the cornea and capillary remodeling and regression, a process associated with enhanced anti-inflammatory macrophage activity and suppression of inflammatory factors such as CCL2, CXCL5, IL-1*β* and IL-6. The timing and type of anti-angiogenic therapy are important considerations in order to achieve effective resolution of inflammation and angiogenic capillary regression.

3. Non-perfused ghost vessels without cellular components are dormant remnants of once active vessels that do not re-perfuse during repeated injury in the corneal neovascularization model. Instead, natural or treatment-induced vascular regression is not 100% effective and is associated with ghost vessels as well as persistent capillaries. The persistent capillaries, formed when angiogenic factors decline, are mature, stable and treatment-resistant. Although, they may be severely constricted and have very weak blood flow, they have the potential to rapidly dilate and form the basis for renewed, aggressive inflammation and angiogenesis during repeat injury or when the effect of anti-angiogenic treatment subsides. Pro-inflammatory pathways such reactive oxygen species and nitric oxide signaling may mediate this revascularisation response.

4. Corticosteroids such as dexamethasone suppress many inflammatory pathways and genes, which anti-VEGF treatment does not. This leads to an early suppression of vascular permeability and inflammatory cell recruitment via chemokines such as CXCL5 and CCL2 – an effect not achieved with anti-VEGF treatment. To avoid the broad effects of corticosteroids while achieving effective suppression of angiogenesis may require targeted inflammatory pathway and/or chemokine blockade in addition to targeting VEGF. This approach should be investigated further.

In summary, the complexity, redundancy, and time dependence of mechanisms regulating inflammation and angiogenesis may partially explain the observed limitations of current therapies for ocular angiogenesis. Numerous regulatory factors and pathways remain unexploited as potential targets for treating ocular inflammation and angiogenesis. Future therapies could benefit by augmentation with specific inflammatory molecule or pathway agonists or inhibitors and possibly alternative anti-angiogenic agents to more completely eradicate new pathologic vessels and maintain a regressed, more stable vasculature. ■

References

1. Mukwaya A, Peebo BB, Xeroudaki M, Ali Z, Lennikov A, Jensen L, Lagali N. Factors regulating capillary remodeling in a reversible model of inflammatory corneal angiogenesis. Sci Rep. 2016 Aug 26; 6:32137.
2. Mukwaya A, Lindvall JM, Xeroudaki M, Peebo BB, Ali Z, Lennikov A, Jensen LD, Lagali N. A microarray whole-genome gene expression dataset in a rat model of inflammatory corneal angiogenesis. Sci Data. 2016 Nov 22;3:160103.
3. Mukwaya A, Lennikov A, Xeroudaki M, Mirabelli P, Lachota M, Jensen L, Peebo BB, Lagali N. Time-dependent LXR/RXR pathway modulation characterizes capillary remodeling in inflammatory corneal neovascularization. Angiogenesis. 2018 May;21(2):395-413.
4. Mukwaya A, Lennikov A, Mirabelli P, Thangavelu M, Peebo BB, Jensen LD, and Lagali N. Excessive inflammation and angiogenesis characterizes vascular rebound in the murine cornea. Manuscript (2018).
5. Mirabelli P, Mukwaya A, Lennikov A, Xeroudaki M, Peebo B, Schaupper M, Lagali N. Genome-wide expression differences in anti-Vegf and dexamethasone treatment of inflammatory angiogenesis in the rat cornea. Sci Rep. 2017 Aug 15; 7(1):7616.
6. Mukwaya A, Mirabelli P, Lennikov A, Xeroudaki M, Schaupper M, Peebo B, Lagali N. Genome-wide expression datasets of anti-VEGF and dexamethasone treatment of angiogenesis in the rat cornea. Sci Data. 2017 Aug 15;4:170111

All publications are open access. Publications 2 and 6 provide open access to full microarray data sets for re-use by other researchers.