REGULATION OF INFLAMMATION AND ANGIOGENESIS IN THE CORNEA

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Regulation of inflammation and angiogenesis in the cornea

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"The most beautiful thing we can experience is the mysterious. It is the source of all true art and science".

- Albert Einstein

ABSTRACT

Inflammation and angiogenesis, the growth of new blood vessels from pre-existing ones, are involved in tumor growth, ocular diseases and wound healing. In ocular angiogenesis, new pathological vessels grow into a specific eye tissue, leak fluid, and disrupt vision. The development of safe and effective therapies for ocular angiogenesis is of great importance for preventing blindness, given that current treatments have limited efficacy or are associated with undesirable side effects. The search for alternative treatment targets requires a deeper understanding of inflammation and how it can lead to angiogenesis in the eye in pathologic situations. This thesis provides new insights into the regulation of inflammation and angiogenesis, particularly at the gene expression and phenotypic levels, in different situations characterized by angiogenesis of the cornea, often called corneal neovascularization. For instance, specific genes and pathways are either endogenously activated or suppressed during active inflammation, wound healing, and during resolution of inflammation and angiogenesis, serving as potential targets to modulate the inflammatory and angiogenic response. In addition, as part of the healing response to restore corneal transparency, inflammation and angiogenesis subside with time in the cornea. In this context, LXR/RXR signaling was found to be activated in a time-dependent manner, to potentially regulate resolution of inflammation and angiogenesis. During regression of new angiogenic capillaries, ghost vessels and empty basement membrane sleeves are formed, which can persist in the cornea for a long time. Here, ghost vessels were found to facilitate subsequent revascularization of the cornea, while empty basement membrane sleeves did not revascularize. The revascularization response observed here was characterised by vasodilation, increased inflammatory cell infiltration and by sprouting at the front of the reperfused vessels. Importantly, reactive oxygen species and nitrous oxide signaling among other pro-inflammatory pathways were activated, and at the same time anti-inflammatory LXR/RXR signaling was inhibited. The interplay between activation and inhibition of these pathways highlights potential mechanisms that regulate corneal revascularization. When treating corneal neovascularization clinically, corticosteroids are in widespread use due to their effectiveness. To minimize the many undesirable side effects associated with corticosteroid use, however, identifying new and more selective agents is of great importance. Here, it was observed that corticosteroids not only suppressed pro-inflammatory chemokines and cytokines, but also activated the classical complement pathway. Classical complement may

represent a candidate for further selective therapeutic manipulation to investigate its effect on treatment of corneal neovascularization.

In summary, this thesis identifies genes, pathways, and phenotypic responses involved in sprouting and remodeling of corneal capillaries, highlights novel pathways and factors that may regulate inflammation and angiogenesis in the cornea, and provides insights into regulation of capillary regression and re-activation. Further investigation of these regulatory mechanisms may offer alternative and effective targets for the treatment of corneal inflammation and angiogenesis.

SAMMANFATTNING

Kärlnybildning, vilket innebär tillväxt av nya blodkärl, och inflammation är involverade i sårsläkning, tumörtillväxt och vid en rad olika ögonsjukdomar. Vid kärlnybildning i ögat är de nybildade kärlen ofta omogna, vilket leder till att de läcker vätska, och skapar svullnad och blödning, som skadar ögats vävnader och ger synnedsättning. Utvecklingen av säker och effektiv behandling av kärlnybildning i ögat har stor betydelse för att bättre kunna förhindra blindhet, inte minst då dagens behandlingar ofta är förenade med otillräcklig effekt och oönskade bieffekter. Sökandet efter nya behandlingsmetoder kräver en djupare förståelse av den inflammatoriska processen, och på vilket sätt den leder till kärlnybildning i ögat vid olika sjukliga tillstånd. Denna avhandling ger ny insikt i hur inflammation och kärlnybildning regleras, speciellt avseende genuttryck och fenotypnivåer, vid olika tillstånd med kärlnybildning i hornhinnan, också kallad korneal kärlnybildning. Olika gener och signaleringsvägar visade sig vara antingen endogent aktiverade eller hämmade vid pågående inflammation och sårsläkning, respektive vid tillbakagång av inflammation och kärlnybildning, och skulle kunna utgöra alternativa behandlingsmål för att reglera tillväxt av blodkärlen. Som ett led vid sårsläkning i hornhinnan, för att återskapa vävnadens klarhet, återgår långsamt inflammation och kärlnybildning. I samband med denna reaktion kunde det visas att LXR/RXR signalering var tidsberoende aktiverat för att reglera tillbakagång av inflammation och kärlnybildning. Vid återbildning av de nybildade blodkärlen formades så kallade spökkärl och tomma basalmembranssträngar, vilka kan kvarstå under lång tid i hornhinnan. Det visade sig att spökkärlen underlättar revaskularisering i hornhinnan vid en ny skada medan de tomma basalmembranen lämnades opåverkade. Revaskulariseringen karakteriserades i nämnd ordning av; 1. vidgning av befintliga blodkärl (spökkärl), 2. infiltration av inflammatoriska celler och 3. nybildning av blodkärl, så kallad "sprouting", i toppen på de revaskulariserade spökkärlen. Parallellt noterades en aktivering av reaktiva syremolekyler och kväveoxidsignalering tillsammans med andra pro-inflammatoriska signaleringsvägar samtidigt som LXR/RXR aktivitet var hämmad. Samspelet mellan aktivering och hämning av dessa signaleringsvägar belyser viktiga mekanismer som reglerar kärlnybildning i hornhinnan. Vid behandling av korneal kärlnybildning i kliniken används idag kortikosteroider, ofta med god effekt, men också med hotande biverkningar såsom högt ögontryck (glaukom) och katarakt. För att minska risken för oönskade sidoeffekter är det av stor vikt att finna nya selektiva

behandlingsmetoder. I denna avhandling visar det sig att kortikosteroider inte bara hämmar proinflammatoriska cytokiner utan också aktiverar klassiska komplementfaktorer. Det klassiska komplementet kan således utgöra en kandidat för framtida selektiv manipulering för att identifiera nya behandlingsprinciper av korneal kärlnybildning.

Sammanfattningsvis identifieras och belyses i avhandlingen gener, signaleringsvägar och fenotypnivåer som är involverade vid tillväxt och utmognad av inflammatorisk kärlnybildning i hornhinnan. Fynden ger en ny kännedom om reglermekanismer för nybildning och tillbakagång av sjukliga blodkärl, kunskap som i framtiden kan ge svar på hur inflammation och blodkärlstillväxt kan regleras och hämmas för att spara syn hos patienter med inflammatoriska och kärlnybildande tillstånd i ögats hornhinna.

LIST OF PUBLICATIONS INCLUDED THE THESIS

- I. **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.
- II. **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
- III. **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.
- IV. **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).
- V. 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.
denotes equal contribution
- VI. **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

Associated data citations

- I. **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. *NCBI Gene Expression Omnibus* GSE87330 (2017).
- II. **Mukwaya A**, Peebo B, Xeroudaki M, Ali Z, Lennikov A, Jensen L, Lagali N. A microarray whole-genome gene expression dataset in a rat model of inflammatory corneal angiogenesis. *NCBI Gene Expression Omnibus* GSE81418 (2016).

RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

- I. Lennikov A, Mirabelli P, **Mukwaya A**, Schaupper M, Thangavelu M, Lachota M, Ali Z, Jensen L, Lagali N. Selective IKK2 inhibitor IMD0354 disrupts NF- κ B signaling to suppress corneal inflammation and angiogenesis. *Angiogenesis*. 2018 May; 21(2):267-285.
- II. Harada F, Morikawa T, Lennikov A, **Mukwaya A**, Schaupper M, Uehara O, Takai R, Yoshida K, Sato J, Horie Y, Sakaguchi H, Wu CZ, Abiko Y, Lagali N, Kitaichi N. Protective Effects of Oral Astaxanthin Nanopowder against Ultraviolet-Induced Photokeratitis in Mice. *Oxid Med Cell Longev*. 2017; 2017:1956104.
- III. Ali Z, **Mukwaya A**, Biesemeier A, Ntzouni N, Ramsköld D, Giatrellis S, Mammadzada P, Cao R, Lennikov A, Rossi A, Marass M, Stone O, Deng Q, Peebo BB, Peso L, Kvanta A, Belting H, Affolter M, Sandberg R, Schraermeyer U, Andre H, Steffensen JF, Stainier DY, Lagali N, Cao Y, Kele J, and Jensen LD. Productive and non-productive intussusception as a novel mechanism of occult choroidal neovascularization in wet AMD. Manuscript (2018).

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ABBREVIATIONS

ARVO	The Association for research in vision and ophthalmology
BSA	Bovin Serum Albumin
CCL	Chemokine C-C motif ligand
CD	Cluster of differentiation
cDNA	Complementary DNA
CNV	Choroidal neovascularisation
cRNA	Complementary RNA
CXCL	Chemokine C-X-C motif ligand
DAPI	4', 6-diamidino-2-phenylindole
DME	Diabetic macular edema
DR	Diabetic retinopathy
EC	Endothelial cells
ECM	Extracellular matrix
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HUVECs	Human umbilical vein endothelial cells
IHC	Immunohistochemistry
IL	Interleukin
IVCM	In vivo confocal microscopy
IVT	<i>In Vitro</i> Transcription
LXR	Liver X receptors
LXRE	Liver X receptor response element
PBS	Phosphate buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PPAR	Peroxisome proliferator-activated receptor
qRTPCR	Quantitative real time polymerase chain reaction
RIN	RNA integrity number
RNA	Ribonucleic Acid
ROP	Retinopathy of prematurity
RVO	Retinal vein occlusion
RXR	Retinoid X receptors
ss-cDNA	Single-Stranded cDNA
ST	Sense Target
TAC	Transcription analysis console
TdT	Terminal deoxynucleotidyl transferase
UDG	Uracil-DNA glycosylase
VEGF	Vascular endothelial growth factor
WT	Whole Transcript

Genes and protein names in this thesis have been formatted according to the bioscience writer guidelines available at:
<http://www.biosciencewriters.com/Guidelines-for-Formatting-Gene-and-Protein-Names.aspx>.

1. INTRODUCTION

1.1 Blindness

Blindness can be defined as the inability to see either clearly or completely. Being blind reduces the quality of life ¹, increases the risk of death ² and can be an economic burden for the affected ³. The World Health Organization (WHO) estimates that 253 million people live with vision impairment worldwide, of these 36 million are blind, and 217 million have moderate to severe vision impairment ⁴. Globally, corneal blindness is the fourth largest cause of blindness and is one of the major causes of visual deficiency after cataract, glaucoma and age-related macular degeneration (AMD) ⁵. Corneal blindness can be caused by trauma and ulceration ⁶, by childhood corneal blindness ^{5,7} or by infections such as trachoma ⁸. Several of these causes are associated with inflammation and angiogenesis in the cornea, which in turn can cause persistent inflammation and scarring, irreversibly affecting the transparency of this tissue, thus reducing vision. Angiogenesis is defined as the growth of new blood vessels from pre-existing ones, and angiogenesis of the cornea is often called corneal neovascularization. As of today, there are no safe and effective treatments specifically targeting corneal neovascularization, making it a major clinical challenge in ophthalmology. As a contribution towards addressing this challenge, work in this thesis focused on gaining a better understanding of inflammation and angiogenesis in the cornea, by identifying genes and pathways that regulate these processes using an inflammatory corneal model of angiogenesis.

1.2. Anatomy of the cornea

The cornea is the outermost part of the eye that serves to protect the eye from mechanical injury. Transparency of the cornea allows for the transmission of light for proper vision, while its curved refractive nature is responsible for focusing light onto the retina for proper vision ⁹, with 2/3 of the light refracted in the cornea. The cornea consists of five distinct anatomic layers; epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium. The epithelium is the outermost layer and measures approximately 50 microns in thickness in humans. Anteriorly, the epithelium is kept moist by the tear film. The epithelium is stratified into several cell layers. The basal layer of epithelial cells is the most posterior layer and is attached to the

underlying basal lamina (or basement membrane) by hemidesmosomes and adhesion complexes. Epithelial cells are attached to each other via tight junctions, a characteristic important for the physical and chemical barrier function of the epithelium⁹. The corneal epithelium is one of the mostly densely innervated structures of the body, a property important for survival of the epithelial cells, protection of the epithelium from external stimuli (through the blink reflex) and for the wound healing function of epithelial cells. A self-renewing ability of the epithelium facilitates continuous repair in case of epithelial damage. The tear film oxygenates the epithelium so that it does not require a direct blood supply, and the tears contain factors important for epithelial wound healing. Beneath the epithelium is 8-14 microns thick Bowman's layer, which is composed of a thin acellular layer composed of randomly-oriented Type I and V collagen fibrils and proteoglycans (Fig.1).

Figure 1. Schematic representation of an eyeball, and the cross section of the cornea. **A** is an eyeball identifying the cornea among other parts. **B** is a cross sectional representation of the cornea showing the different layers that comprise the cornea. The cornea is bathed anteriorly by a tear film, which is depicted by the blue circular arrows, and is bathed posteriorly by the aqueous humour, depicted by the grey circular arrows in B above. The schematic of the cornea in B is not to scale.

Posterior to the Bowman's layer is the stroma, which constitutes 80-90% of the entire corneal thickness. The stroma consists mainly of collagen fibers (Type I and V). These collagen fibrils run parallel to each other to form layers (or lamellae), which in turn are stacked with perpendicular orientation of the collagen fibrils between adjacent lamellae. The collagen fibril diameter and periodicity are important for minimizing light scatter and this configuration is believed to be the origin of corneal transparency¹⁰. Posterior to the stroma is the Descemet's membrane, which consists of Type IV and Type VIII collagen. The Descemet's membrane is continuously deposited throughout life by the endothelium, and thus gets thicker with age⁹.

Posteriorly, the cornea is covered by the endothelium, a single layer of cells which maintains a fluid and ion balance between the stroma and the anterior chamber, by actively pumping fluid, keeping the stroma properly hydrated to maintain optimal transparency. The cells of the endothelium are metabolically active, and the aqueous humor provides the required nutrients to the endothelium and to the cornea in general, mainly by passive diffusion ¹¹, to prevent formation of edematous haze ¹², which would otherwise affect transparency of this tissue. Corneal endothelium should not be confused with vascular endothelium, which are two different cell types, and although they share the same name (endothelium), they have very different functions.

1.2.1. Avascularity of the cornea

The term “angiogenic privilege” refers to the ability of the cornea to maintain avascularity by preventing neovascularization from the surrounding tissues. Avascularity of the cornea has been attributed to factors such as the expression of soluble VEGF receptor-1 (sVEGFR-1 or sflt1), which binds and blocks the activity of vascular endothelial cell growth factor (VEGF)-A to prevent angiogenesis ¹³. Mutations in genes such as paired box protein Pax-6 (Pax6), a gene important for the development of the eye, results in aniridia and spontaneous corneal neovascularization ^{14,15} due to the lack of sVEGFR-1 ¹⁶. In addition, the presence of anti-angiogenic molecules within the cornea are thought to contribute to the avascularity of this tissue ¹⁷. For example, thrombospondin-1 (TSP-1) and thrombospondin-2 (TSP-2) members of the thrombospondin family are expressed in the healthy native cornea ¹⁸, and were shown to have anti-angiogenic properties ^{19,20}. Other examples of anti-angiogenic mediators normally present in the cornea include endostatins ²¹, angiostatins, and pigment epithelial-derived factor (PEDF) ²². Apart from factors at the molecular level, anatomically, the dense collagen fibers, and the barrier function of limbal cells are also thought to contribute to the avascular nature of this tissue ²³. However, when the cornea is stimulated leading to inflammation, pro-angiogenic factors are activated and outweigh these anti-angiogenic factors, leading to neovascularization of the cornea.

1.3. Angiogenesis in health and in disease

The formation of new blood vessels from pre-existing ones (angiogenesis) is a tightly regulated process mediated by factors such as vascular endothelial growth factor (VEGF), transforming growth factor (TGF), or by family members of the platelet derived growth factor (PDGF) among others. Angiogenesis differs from vasculogenesis, the latter referring to the *de novo* formation of blood vessels during development. Angiogenesis occurs in both health and in disease. Blood vessels of the central nervous system expand via angiogenesis ^{24,25}. In the developing retina, hypoxia in retinal astrocytes leads to the expression of VEGF ²⁶ resulting in angiogenesis ²⁷ to meet the oxygen demands of the tissue. In disease, such as in tumor angiogenesis, hypoxia is the main driving force for this pathology ^{28,29}. Hypoxia results from excessive tumor growth such that tumor tissue extends beyond the reach of the existing vasculature, leading to inadequate perfusion ³⁰. Activation of transcriptional factors (mainly HIF1 α) by hypoxia leads to the expression of target genes that include VEGF-A ³¹ to promote angiogenesis. In ocular pathologies such as in proliferative diabetic retinopathy, hypoxia resulting from capillary occlusion drives angiogenesis by promoting expression of angiogenic factors via HIF1 α activation ^{32,33}. In corneal neovascularization (described in detail below), for instance after injury, an early inflammatory response leads to the expression of pro-angiogenic factors including VEGF-A, that promote neovascularization ^{34,35}.

Overall, angiogenesis involves a sequence of events. The pre-existing vessels from which the new vessels emerge are in a state of quiescence stabilized by mural cells (or pericytes) and by basement membrane ³⁶. In a pre-angiogenic phase (for example during inflammation), these supporting structures are degraded by matrix-degrading proteases ³⁷. The activated vascular endothelial cell then leads the way as a tip cell of the newly forming vessel. The tip cell extends filopodia along the angiogenic molecule concentration gradient(s) ^{38,39}. The tip cell is followed by a stalk cell, which is highly proliferative, and establishes tight junctions to stabilize the newly forming vessel ^{40,41}. The interplay between stalk and tip cell phenotype is key for efficiency and directionality of the angiogenic response ⁴². This phenotype is characterized by distinctive expression profiles for example of VEGFR2 and Delta-like ligand 4 (DLL4) by the endothelial cells. Signaling of the tip cell through VEGFA/VEGFR2 enhances the expression of DLL4, the ligand for Notch, and instructs the neighboring endothelial cell

via Dll4/Notch1 to become the stalk cell. The stalk cell responds by downregulating VEGFR2 and Dll4⁴³, limiting the tip cell phenotype^{44,45}. Angiogenesis involving sprouting of new vessels constitutes sprouting angiogenesis, the main form of angiogenesis modelled in this thesis. Besides sprouting angiogenesis, other forms of angiogenesis have been described and they include intussusceptive angiogenesis⁴⁶ and looping angiogenesis⁴⁷. Intussusceptive angiogenesis is thought to occur in mature capillaries, characterised by the formation of intraluminal pillars within the vessel, mediated by shear stress⁴⁸, to divide the vessel into two separate functional vessels. In corneal angiogenesis, intussusceptive angiogenesis has been observed during capillary remodeling⁴⁹, and is thought to be a means of expanding the number of circulatory conduits within the neovascularised tissue, without new sprouting.

1.4. The crosstalk between inflammation and angiogenesis

In a broad sense, inflammation is the body's response to infection or external stimulation, and is characterized by swelling, redness, heat and pain. In addition to defending the body against pathogens, inflammation can affect the surrounding tissue to promote angiogenesis as part of a wound healing response. During inflammation, leukocytes enter the affected site by a process of leukocyte rolling, activation, adhesion and extravasation from pre-existing vessels, followed by migration to the site of injury⁵⁰. These cells express early response inflammatory mediators such as IL-1 α , IL-1 β and TNF- α ⁵¹ to further promote inflammation. TNF- α acts by upregulating VCAM-1 and ICAM-1 in endothelial cells, mediated by endothelial cell Ang-2 to enhance further leukocyte extravasation. Ang-2 serves to amplify the effects of TNF- α , given that TNF- α is of suboptimal concentrations at this early stage of the inflammatory response⁵². This interplay between Ang-2 and TNF- α is an example of the crosstalk between inflammation and angiogenesis. In addition, the pro-inflammatory mediators IL-1 β and IL-1 α enhance endothelial cell proliferation by stimulating increased expression of VEGF⁵³. From a clinical standpoint, ocular diseases such as pterygium⁵⁴, diabetic retinopathy^{55,55} and choroidal neovascularization⁵⁶ all involve a component of inflammation and angiogenesis. With the currently available evidence^{57,58} it is tempting to speculate that inflammation and angiogenesis are mutually interdependent processes.

1.5. Inflammatory corneal angiogenesis

The angiogenic privilege of the cornea can be impaired by inflammation resulting from a range of stimuli such as infection, injury, and extended use of contact lenses leading to hypoxia ^{59,60,61}. Following corneal injury, necrosis of corneal cells can lead to expression of cytokines and chemokines ⁶² to promote inflammation. For example, IL-1 α is expressed in the cornea following injury ⁶³, promoting expression of pro-inflammatory mediators from corneal epithelial cells ⁶⁴. Cytokines such as IL-1 β ⁶⁵, and Ccl2 ⁶⁶ are highly expressed in the injured cornea to promote inflammation by regulating recruitment of inflammatory cells ⁶⁷. Damage to the epithelium also results in the expression of many pro-angiogenic factors such as VEGF and TGF β ^{68,69}. The vasculature of the limbal arcade responds to VEGF levels by dilating, presumably to facilitate inflammatory cell extravasation ^{34,70,71}, characterised by increased expression of adhesion molecules like ICAM-1 ⁷² and VCAM-1 ⁷³. In addition, inflammation can lead to expression of proteases that degrade the extracellular matrix ^{74,75} to promote corneal neovascularization. The infiltrating inflammatory cells can lead to edema in the stroma which occurs prior to, and may facilitate the neovascularization response. Neutrophils and monocytes secrete factors that lead to vasodilation and to recruitment of more inflammatory cells ⁷⁶. The recruited inflammatory cells express pro-inflammatory genes via activation of Nuclear Factor- κ B (Nf κ B) pathway, to promote inflammation ^{77,78}.

Once angiogenic sprouting has initiated, the newly formed sprouts are immature and leaky, further promoting edema in the stroma ⁷⁹. Persistent inflammation, edema, and scarring affect the transparency of the cornea, hence reducing overall visual acuity.

Besides the formation of blood vessels from pre-existing ones (called hemangiogenesis), inflammation in the cornea can lead to the formation of lymphatic vessels from pre-existing lymphatic vessels (called lymphangiogenesis)^{80,81}. Lymphangiogenesis occurs in the cornea, but is delayed relative to hemangiogenesis ⁸². The lymph vessels serve to drain the tissue of excess fluid ^{83,84}. However, lymphatic vessels in the cornea can have adverse effects such promoting graft rejection by enhancing trafficking of antigens from the cornea to the regional lymph nodes via the transport of antigen presenting cells ⁸⁴.

In an inflammatory response, the innate and adaptive arms of the immune system play different functions. The adaptive immune response develops slowly and is necessary for an efficient immune response. The innate immune response on the other hand is activated immediately by stimuli such as infection, to defend the host ⁸⁵. Innate immunity of the cornea involves cells such as epithelial cells which secrete TNF- α , IL-1, IL-6 and IL-8, and fibroblasts which secrete IL-1, IL-6, IL-8, TNF- α , and α -defensin, to fight infections ⁸⁶. Toll like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) on the surface of invading pathogens, to elicit an immune response ⁸⁷. TLRs link the innate immune response to the adaptive immune response by initiating inflammatory cell recruitment. In inflammatory corneal angiogenesis, neutrophils migrate to the injured site within a few hours after stimulation ³⁴. In addition, inflammatory cells such as dendritic cells, in turn present antigens to naïve T-cells to initiate the adaptive immune response ^{88,86}, to completely clear the invading pathogen. Complement is part of the innate immunity and serves to rapidly eliminate the invading pathogen by opsonization, and by activation of inflammation ⁸⁹.

1.6. Capillary remodeling and regression

Following corneal neovascularization, the fate of the newly formed vessels can be determined by either the continued presence or absence of an angiogenic stimulus over time. For example, removing the angiogenic stimulus (such as a foreign body) results in remodeling and regression of the newly formed vessels ⁹⁰. Remodeled vessels can become covered by pericytes, which makes them potentially resistant to anti-VEGF therapy, given that pericytes stabilize the capillaries, whereas anti-VEGF treatments efficiently target immature non-stabilized and leaky sprouts ^{91,92,93}. Therefore, keeping capillaries in an immature state can be a means to avert resistance to anti-VEGF therapy. To achieve this, a better understanding of the process of capillary remodeling is required to identify potential factors regulating maturation. From studies in developmental angiogenesis ^{94,95,96}, and in adults ⁹⁷, different modes of capillary remodeling have been described, and these include endothelial cell apoptosis observed during hyaloid vessel regression ⁹⁸, and during pupillary membrane vessel regression ^{99,100,101}. Endothelial cell migration as another mode of remodeling is observed in remodeling of zebrafish midbrain ¹⁰².

Perfusion of vessels is also key for their survival, and this is so because shear stress promotes endothelial cell survival through the Akt pathway and Krüppel-like factor 2 (KLF2) activation leading to the upregulation of nitric oxide(NO)-synthase and superoxide dismutase to mediate vasodilation ¹⁰³. In addition, inhibition of VEGF signaling induces capillary regression characterized by cessation of flow and by endothelial cell apoptosis ^{104,105}. Another signaling axis implicated in capillary remodeling is Wnt signaling, which regulates endothelial cell migration, proliferation, and survival ¹⁰⁶. Angiopoietin and Tie signaling is also another axis involved in capillary remodeling and regression ¹⁰⁷. Overall, extensive capillary remodeling in the cornea over time can lead to the formation of persistent vascular networks, and VEGF-B, a member of the VEGF family of ligands, was shown to be important for the survival of a remodeled capillary bed, by promoting the activity of pro-survival genes neuropilin-1(NP-1) and VEGFR-1¹⁰⁸.

1.7. VEGF family ligands

The VEGF family of ligands are structurally related members including; VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF). Some of these are discussed below.

VEGF-A is an important growth factor involved in vasculogenesis and angiogenesis ¹⁰⁹, and consists of different isoforms resulting from a single gene by process of alternative splicing ^{110,111}. In humans, VEGF-A has isoforms having 121, 145, 165, 189 and 206 amino acids ^{112,113,114,115}, which differ in their affinity for heparin sulfate determined by their exon composition i.e. exons 6 and 7 are important for heparin-binding ^{116,117}, and isoforms containing these exons are less diffusible. In murine species, VEGFA-164 is a homologue for the human VEGFA-165 isoform, and is the most abundant isoform ¹¹⁸. VEGF-A is most studied for its role in angiogenesis ^{119,120}, and VEGFA-165 is the most potent isoform for angiogenesis ¹²¹. VEGF-A signals via transmembrane tyrosine kinase receptors VEGFR-1(FLK-1) and VEGFR-2 (KDR). VEGFR-2 is expressed mainly by endothelial cells, and signaling through this receptor by VEGF can regulate processes such as cell proliferation and migration to promote angiogenesis ^{122,123}. VEGFR-1 is shown to heterodimerize with VEGFR-2 to regulate endothelial cell homeostasis ¹²⁴. In addition, VEGFR-1 binds VEGF-A with greater

affinity compared to VEGFR-2, however, the kinase activity downstream of VEGFR-1 is weaker ¹²⁵, and VEGFA-VEGFR1 is shown to negatively regulate angiogenesis during embryogenesis ¹²⁶. VEGFA-VEGFR2 signaling is important for angiogenesis, and is the most studied pathway ^{127,122,125,128}.

In an inflammatory milieu, VEGF acts as a pro-inflammatory cytokine by promoting expression of cell adhesion molecules, other cytokines, and acts as a monocyte chemoattractant ^{129,130,131}. Monocytes express VEGFR-1 and respond to VEGF by enhanced cell migration ¹³², to further promote inflammation. A subpopulation of circulating neutrophils express VEGFR-1, and respond to VEGFA during inflammation ¹³³. The central role of VEGF-A in angiogenesis has led to the development of anti-VEGF treatments (discussed below) for tumor and for ocular angiogenesis; however, efficacy of anti-VEGF for example in treating corneal angiogenesis is variable, an effect partially explained by this thesis.

VEGF-B is expressed in many tissues, and by a range of cells including endothelial cells ^{134,135}. VEGF-B consists of two isoforms VEGFB-167 and VEGFB-186, both of which can heterodimerize with VEGF-A when co-expressed. VEGF-B₁₆₇ is speculated to determine the bioavailability of heterodimers to potentially control bioavailability of VEGF-A ^{125,136,137}. VEGF-B is not as well described as VEGF-A with regard to angiogenesis. From recent studies, the loss of VEGF-B in corneal angiogenesis does not affect neovascularization¹³⁸. Deletion of VEGF-B during developmental angiogenesis is not lethal in mice, but rather causes cardiovascular abnormalities ^{138,139}. However, in the developing zebrafish embryo, *Vegfb*a knockdown is lethal, demonstrating the importance of VEGF-B in developmental angiogenesis in this model ¹⁴⁰. Notably, developmental angiogenesis in zebrafish embryos is not hypoxia-dependent as is the case in mice and other mammals ¹⁴¹.

VEGF-C can signal via both VEGFR-2 and VEGFR-3, to regulate lymphangiogenesis mainly via VEGFR-3 ¹⁴². VEGF-C is important during embryogenesis, as shown by lethality of mice containing a global deletion of this gene ¹⁴³. In adults, constitutive expression of VEGF-C regulates lymphangiogenesis via VEGFR-3 ¹⁴⁴. In addition, VEGF-C is known to stimulate lymphangiogenesis and hemanagiogenesis under inflammatory conditions ^{145,142}, and its expression can be induced by pro-inflammatory

mediators such as TNF- α ¹⁴⁶. Lymphangiogenesis can also occur during inflammation in the cornea, but this was not the focus of this work.

1.8. Treatment of corneal neovascularization

Currently, treatments for corneal neovascularization include corticosteroids (often simply referred to as 'steroids') and non-steroidal anti-inflammatory drugs, laser photocoagulation ¹⁴⁷, fine needle diathermy ¹⁴⁸, and photodynamic therapy ^{149,150}. These treatments are of variable efficacy, and some treatments such as steroids have undesirable side effects ^{151,152}. Given that VEGF is an important angiogenic molecule for corneal neovascularization ³⁵, one could argue that targeting this molecule as a therapeutic intervention against corneal neovascularization would be ideal. Along these lines, the currently available anti-VEGF agents initially intended for treating tumor angiogenesis are now finding their way into ophthalmology for the treatment of ocular pathologies, but are used off-label in the cornea. For instance, bevacizumab (Avastin®) is a recombinant full-length humanized monoclonal antibody against VEGF-A approved by the FDA for treating cancers ¹⁵³. Bevacizumab is used off-label for treating ocular pathologies such as age-related-macular degeneration, proliferative diabetic retinopathy, and retinopathy of prematurity ^{154,155}, and for corneal neovascularization ^{156,157,158}.

On a positive note, some anti-VEGF treatments have been approved for treatment of ocular pathologies in the posterior eye, for instance Ranibizumab (Lucentis®) which comprises of the Fab fragment of the same mouse monoclonal antibody as bevacizumab ¹⁵⁹. This modification is meant to improve penetration efficiency of the antibody, given that the full-length antibody was found to poorly penetrate the retina ¹⁶⁰, though this is debatable ^{161,162}. Ranibizumab is approved for wet-AMD ¹⁶³, with efficacy similar to that of bevacizumab ^{164,165}. However, ranibizumab is much more expensive ¹⁶⁶, hence creating a potential economic burden. Another approved anti-VEGF treatment is Pegaptanib (Macugen®), which is an aptamer that specifically binds VEGF₁₆₅. Pegaptanib is approved for treating the wet form of AMD ¹⁶⁷, but has only limited efficacy. Aflibercept (EYLEA®), another anti-VEGF treatment is a recombinant fusion protein of human VEGFR-1 and 2 extracellular domains fused to the Fc portion of human IgG1. Aflibercept acts as a VEGF trap sequestering VEGF-A, B and PlGF ^{168,169} with high affinity, and reported to be of a longer intravitreal half-life ¹⁷⁰. Aflibercept

is approved by the FDA for treating neovascular AMD ¹⁷¹, diabetic macular edema (DME) ¹⁷², retinal vein occlusion (RVO) ¹⁷³ and myopic choroidal neovascularisation (CNV) ¹⁷⁴, and with promising results ¹⁷⁵.

In line with the gene therapy approach, Aganirsen (GS-101®) is a DNA antisense oligonucleotide targeting insulin receptor substrate-1 (IRS-1). Aganirsen has shown promise in inhibiting keratitis-induced corneal neovascularization ^{176,177}.

Of the currently available anti-VEGF treatments, none is yet approved for treating corneal neovascularization, and when used off-label, such agents are of limited efficacy as demonstrated experimentally and in clinical settings ^{71,178,179}. In addition, these treatments do not directly target inflammation. Gene-therapy based treatments are typically transient and are associated with off-target effects. These shortfalls highlight the need for alternative treatments.

1.9. Modeling angiogenesis

To identify alternative targets for improved treatment of corneal neovascularization requires the use of models that mimic the pathophysiology of the disease as closely as possible. The process of angiogenesis is complex in that it involves many different cell types and signaling molecules. However, many models have been developed over the years to study angiogenesis in contexts such as tumor angiogenesis, adipose tissue angiogenesis and ocular angiogenesis ¹⁸⁰. These include models such as the zebrafish model of angiogenesis which has been used to study tumor angiogenesis ¹⁸¹, retinal angiogenesis ¹⁸¹, choroidal neovascularisation ¹⁸² and regenerative angiogenesis ¹⁸³. The rat ischemic hind limb model is important for addressing questions concerning impaired angiogenesis such as in wound healing, stroke and in myocardial infarction ^{184,185}. The oxygen induced retinopathy (OIR) model in mouse pups is another model that has been used extensively to investigate retinopathy of prematurity (ROP), yielding a better understanding of this pathology ¹⁸⁶. Another model system of angiogenesis is corneal angiogenesis. The cornea is naturally avascular, which means that all vessels that grow into the cornea are pathologic and a result of the stimulus. In other non-cornea models, pre-existing vessels are confounding factors, which make it difficult to differentiate between the angiogenic and pre-existing vessels, particularly *in vivo*. In addition, transparency of the cornea means the newly formed

vessels can easily be observed; since they are perfused with blood, the vessels appear as reddish/pinkish structures under a light microscope. The cornea is an externally accessible tissue, a property that allows for live *in vivo* imaging, for instance to document angiogenesis in a time-dependent manner ³⁴. Furthermore, compounds under investigation can easily be implanted in the cornea using an intra-stromal micropocket ¹⁸⁷, and medications can easily be given locally as eye drops or by sub-conjunctival injection ¹⁸⁸.

Use of the cornea as a tissue to study angiogenesis has been reported over the years, in models such as the mouse micropocket assay. The micropocket assay entails making a pouch intra-stromally in the cornea where the factor(s) under investigation are implanted in the form of a polymer pellet that slowly releases the substance ^{189,190}. This model has enabled investigation of angiogenic properties of molecules such as bFGF ^{191,192}, VEGF, PDGF ¹⁹⁰ and Fgf7 ¹⁹³. The model elucidates the angiogenic potential of single substances in isolation; however, if the angiogenic response in a physiological setting is to be studied, the multifactorial nature of angiogenesis and the role of inflammation are not adequately represented by this model.

More physiologic models of angiogenesis are inflammatory models, which are intended to mimic pathological scenarios of corneal neovascularization. For instance, alkaline cauterization/burn of the cornea using a mixture of silver nitrate or by NaOH induces inflammatory corneal angiogenesis ^{194,195,196}. The alkaline cauterization/burn model is effective, however, an investigator in this setting has limited control of the stimulus. In this thesis, being able to control the angiogenic stimulus was a key factor in deciding the model of choice. In particular, a model was sought where angiogenesis could be induced reproducibly and reversibly in the cornea, to study not only the neovascularization response but also vascular regression, remodeling, and re-vascularization by repeated stimulus. This brings us to the suture model of inflammatory corneal angiogenesis, which involves placing surgical sutures into the cornea intra-stromally (Fig. 2). The suture breaks the epithelial barrier and introduces a foreign body into the stroma to induce an inflammatory response, which in turn leads to angiogenesis.

Figure 2. Schematic illustration of the suture model of inflammatory corneal angiogenesis, showing the suture traversing the corneal stroma before it exits to the surface where it is knotted to fasten it in place.

The neovascularization response in the suture model of inflammatory corneal angiogenesis can be controlled, depending on parameters such as the number of sutures, pattern and depth of suture placement, suture size and material, and the distance of the sutures to the limbus. The closer the sutures are to the limbus (where the pre-existing vessels are present), the faster the neovascularization response. The greater the number of sutures spread out across the cornea, the larger the neovascularized area. In a clinical setting, surgical sutures are placed into the cornea following corneal transplantation, and these sutures can trigger neovascularization, which is typically handled by postoperative prophylactic use of steroids. In this sense, the suture model is a clinically relevant model for corneal neovascularization, mimicking other pathologic situations leading to inflammation and angiogenesis in the cornea. In this thesis, the suture model of angiogenesis was used and is elaborated in detail in the later chapters.

Despite the benefits of the cornea as a model of angiogenesis, compared to other assays, the cornea model of angiogenesis can be considered relatively expensive since a single cornea can allow for a few laboratory downstream analyses, and is hence not a practical choice for larger screening purposes. For screening purposes, there are *in vitro* models typically using Human umbilical vein endothelial cells (HUVECs), but these do not replicate physiological parameters such as blood flow, and do not take other cell types (such as inflammatory cells) into account. In addition, assays such as the rat aortic ring, allow for quantitative angiogenesis, and can be transfected to study gene function^{197,198}. Nevertheless, this *in vitro* assay does not also accurately mimic the physiological state of angiogenesis. The cornea itself has also been questioned as a suitable model for angiogenesis because of its angiogenic and

immune privilege, meaning that it may not properly represent other tissues in the body, which already have a pre-existing vascular bed. Therefore, the different models and assays of angiogenesis should be seen as complementary to each other, with the research question under investigation guiding the choice of model to use.

2. RESEARCH QUESTIONS AND FINDINGS IN THIS THESIS

Corneal neovascularization is a clinical challenge, mainly due to the lack of safe and effective treatments. Currently, anti-VEGF therapies are used off-label, and are of limited efficacy ⁷¹. Steroids and nonsteroidal anti-inflammatory drugs (NSAIDs) are potent in blocking inflammation, but they are associated with undesirable side effects like corneal thinning and ulceration, cataract, glaucoma, corneal melting, and increased risk of infection ^{151,152}. Alternative treatments such as gene therapy are under investigation ¹⁹⁹, however, these have drawbacks such as the transient nature ²⁰⁰ and immune reactivity ²⁰¹ of siRNAs, and their association with off-target effects ²⁰². Addressing these shortfalls requires a better understanding of the process of corneal neovascularization, which this thesis aims to address. The main research questions addressed in this thesis are:

1. Which genes are responsible for the rapid remodeling and maturation of newly formed angiogenic capillaries in the cornea, making them less susceptible to treatment?
2. How is inflammatory angiogenesis in the cornea regulated with time?
3. What are the characteristics and the role of ghost vessels in the cornea?
4. What are the important targets for corticosteroids, as a treatment for inflammatory corneal angiogenesis?

These lines of investigation are graphically illustrated in Fig. 3 below.

Figure 3. An overview of the lines of investigation in this thesis. Neovascularization of the cornea can be caused by stimuli such as injury, infections, contact lens wear for prolonged periods, or by genetic disorders such as aniridia. These stimuli cause inflammation of the cornea, which in turn can facilitate angiogenesis with sprouts originating from the limbus towards the center of the cornea (red lines from the red circle). Remodeling and regression of the hemangiogenic vessels leads to the formation of ghost

vessels and empty basement membrane sleeves, which can persist in the cornea. The red arrows indicate the processes investigated within this thesis.

Following the research questions outlined above, this thesis addresses the following specific research objectives:

1. To identify the mediators of capillary remodeling and regression
2. To investigate possible time-dependent expression patterns of inflammatory angiogenesis
3. To better elucidate the role of corneal ghost vessels and basement membrane sleeves in a repeat event of corneal neovascularization
4. To identify key targets of corticosteroids in treating corneal neovascularization

To address these objectives, the suture model of inflammatory corneal angiogenesis in the rat was used. The rat was preferred over the mouse because the rat eye is larger (diameter of about 6.41mm in rats and 3.32mm in mice ²⁰³, but both are much smaller compared to 28mm in humans ²⁰⁴). The rat eye therefore offers more surface area for *in vivo* examination and more tissue for laboratory downstream analysis from a single eye, a parameter important to minimize variation. One disadvantage of rat models is the difficulty in obtaining transgenic strains; for the purposes of this thesis gene knockdown was not required, but would be of interest in future work.

Importantly, the suture model of inflammatory corneal angiogenesis also offers control of the angiogenic stimulus, a property that was instrumental for studying regression and remodeling of capillaries, which was triggered by a timed removal of the angiogenic stimulus. In addition, the suture model is robust, highly reproducible and adaptable. Ease of reproducibly suturing the rat cornea and locating the suture site for analysis and re-suturing were also important considerations in the choice of species. Briefly, the model involved placing two sutures intrastromally into the cornea (at 1.5mm from the limbus). The sutures induced an inflammatory response characterized by vasodilation of limbal vessels, and inflammatory cell extravasation and infiltration into the cornea, starting a few hours after suture placement. The migrating inflammatory cells express cytokines, chemokines and growth factors, building a concentration gradient towards the site of injury. This environment then leads to the sprouting of new capillaries from the limbal vessels in a direction towards the sutures. By day four after

suture placement, the newly formed sprouts extend from the limbus to about half of the distance to the sutures (Fig.4).

Figure 4. The suture model of inflammatory corneal angiogenesis used in this thesis. **A.** The appearance of a naïve cornea. The vessels observed are the iris vessels behind the cornea, and should not be confused with corneal vessels. **B.** The site for suture placement is determined using a Vernier caliper, corresponding to a distance of 1.5mm from the limbus and marked at the eight and nine o'clock positions of the right eye. **C.** Two intra-stromal sutures are then placed at the demarcated sites using a wedge-shaped suture needle using 10-0 nylon sutures. After traversing the stroma, two knots are made on the surface of the epithelium to fasten the sutures in place. **D.** The sutured animals are then examined at various times after suture placement, starting from the first few hours. By day four after suture placement, capillary sprouts extend from the limbus to about halfway the distance to the sutures. This response is predictable and reproducible. Painkillers and antibiotics are used to manage pain and prevent infection, soon after suture placement and upon suture removal.

This model was used throughout this thesis with slight adjustments to meet the needs of each defined objective. The adjustments are detailed in the respective articles. Following neovascularization of the cornea, the sutured eye was examined using *in vivo* confocal microscopy (IVCM) and by a specialized slit lamp camera adapted for rodent eyes. *In vivo* analysis was instrumental for monitoring, and for the time-dependent noninvasive analysis of the neovascularization response. Quantifiable indicators of inflammation and angiogenesis such as infiltrating inflammatory cell density, and the diameter of capillaries were determined from IVCM image sequences, and analysed. Vascular density was determined from slit lamp images, and analysed. Following euthanasia of the animals, *ex vivo* tissue analysis was limited to only the vascularized area of the cornea. The neovascularized area of the cornea was preserved in 'RNA-later' medium for gene expression analysis. Total RNA was extracted from the samples, and the quality verified prior to sample preparation for

whole transcriptome analysis, performed mainly using GeneChip Rat 2.0 ST microarrays. As a verification of the microarray data, gene expression analysis was performed for specific genes of interest by qRT-PCR, mainly using custom primers. Tissue for Western blot analysis was frozen at -80°C until processing. Tissue for immunohistochemical analysis was frozen in optimal cutting temperature (OCT) media and temporarily stored at -20°C until needed. Immunofluorescence was used to localize the expression of target genes in corneal cross-sections, and images were captured with a laser-scanning confocal fluorescence microscope. Statistical significance of quantifiable parameters was defined by a two-tailed p-value < 0.05. The Shapiro-Wilk normality test was used to verify the normality of the data. Analysis of variance (ANOVA) was used when performing multiple comparisons and the Student-t test was used when comparing two independent groups. For the microarray data, differentially expressed genes were defined by the fold change, and by either p-value or false discovery rate (FDR), or both. Details of the methods and statistical considerations can be found in Papers I - VI.

The regional ethics committee for animal experiments in Linköping approved all animal experimental procedures under the ethical permit numbers (585 and 7-13) given in the respective papers. The conducted experiments were also in line with the Association for Research in Vision and Ophthalmology (ARVO) guidelines for the use of animals in vision research.

2.1. Results

The findings herein describe the phenotypic characteristics associated with inflammatory corneal angiogenesis, and the associated dysregulated genes and pathways. In general, the results address each specific research question, one at a time.

2.1.1. Upregulation of pro-maturation and suppression of pro-inflammatory genes drives capillary remodeling and regression in inflammatory corneal angiogenesis (PAPERS I & II)

To better understand the process of capillary remodeling thought to promote capillary resistance to anti-angiogenic treatments, the suture model of inflammatory corneal angiogenesis was used. Two intra-stromal sutures placed at 1.5mm from the limbus in rat corneas induced sprouting of capillaries from the limbus towards the sutures. The sprouts extended halfway the distance from the limbus to the sutures by day four after suture placement. This time point of aggressive, active sprouting was re-named as the zero hour (0h) time point, at which sutures were removed to induce capillary regression (Suture OUT). In another group, sutures were kept in place at 0h (Suture IN) to provide a continuous angiogenic stimulation, to serve as a positive control for the suture OUT group. For both groups, animals were subsequently examined at 24h, 72h, and 120h (1, 3, and 5 days after the time point of sprouts extending halfway to the sutures). Microarray analysis (GeneChip Rat 2.0 ST arrays) was performed in the naïve, non-sutured corneas as a negative control, at the 0h time point and at the 24h time point in both suture IN and OUT groups. A total of four-microarray chips were used per group, with no pooling of samples (Fig.5).

Figure 5. The experimental design used to characterize capillary remodeling and regression. Two sutures were placed into the cornea and sprouting was allowed to proceed for four days. On the fourth day (0h) animals were divided into two groups. In the suture IN group, both sutures were left in place from 0h and onwards, while in the suture OUT group, both sutures were removed at the 0h time point. In both groups animals were examined at 24h, 72h and 120h time points by *in vivo* examination methods. In addition, animals were sampled at each time point to harvest tissue for laboratory analysis. Microarray analysis was performed in the naïve, 0h, and at 24h in both the suture IN and suture OUT groups. The gene expression data was compared between 0h, 24h suture IN and 24h suture OUT relative to the naïve cornea. In addition to the microarrays at 0h, whole-genome microarray analysis at 72h and 120h were the subject of a subsequent study focusing on time-dependent responses (Paper III).

Following removal of the angiogenic stimulus, an overall reduction in vascular density characterized the suture OUT group. *In vivo* confocal microscopy examination revealed a transition of the inflammatory cell response from granulocytes (appearing as hyper-reflective rounded and spindle-like cells ³⁴) to macrophage (large polymorphic with dark nucleus ²⁰⁵) in the suture OUT group. The accumulating macrophages were of the M2 anti-angiogenic or remodeling phenotype (CD 204+). In addition, vessel splits (intussusception) increased with time in both suture IN and suture OUT groups. By immunohistochemistry analysis, removing the angiogenic stimuli delayed the deposition of basement membrane (Collagen IV) onto the newly formed capillaries. In

general, the phenotypic characteristics described above were distinctly different between the suture IN and suture OUT groups at 72h following suture removal. Based on this observation, it was reasoned that the genes regulating the observed phenotypic responses would be expressed starting at an earlier time, i.e. at the 24h time point. In addition, preliminary gene expression analysis by qRT-PCR showed suppression of *Vegfa* expression by 24h after suture removal. With this in mind, whole transcriptome microarray analysis was performed at the 24h time point in both groups. The resultant microarray data from the 24h time points in both suture IN and suture OUT groups were normalized to that of the naïve cornea, to determine the differentially expressed genes for downstream analysis.

The analysis revealed an upregulation of pro-inflammatory mediators C-X-C Motif Chemokine Ligand 5 (*Cxcl5*), C-C motif chemokine ligand 2 (*Ccl2*), Serpin Family B Member 2 (*Serpinb2*), S100 calcium-binding protein A8 (*S100a8*) among others, in sutured corneas actively undergoing angiogenic sprouting. Removal of the sutures led to suppression of these genes in the suture OUT group, compared to the suture IN group. For instance, *Cxcl5* was the most suppressed gene with a 41.7 fold change difference between suture IN and suture OUT at 24h. Genes such as *Reg3g*, *Krt16*, *Ccl2* and *Serpinb2*, had a fold change difference of 24.8, 19.28, 15.6, and 15.18 respectively, between suture IN and suture OUT. These genes are mostly associated with inflammation. Among other genes, *Vegfa* was suppressed with suture removal, but to a more modest level with a fold change difference of 2.3. On the other hand, genes such as RAS p21 protein activator 2 (*Rasa2*), slit guidance ligand 2 (*Slit2*), CYLD lysine 63 deubiquitinase (*Cyld*) and glycogen synthase kinase 3 beta (*Gsk3b*) were upregulated in the suture OUT group, and were down regulated in the suture IN group. Comparing suture IN and suture OUT, the fold change difference between suture IN and suture OUT was 2.82, 2.56, 2.55, and 2.23 for *Cyld*, *Slit2*, *Rasa2* and *Gsk3b* respectively. Remodeling and inhibition of angiogenesis generally had a much weaker effect than inflammation and active angiogenesis, at the gene expression level.

Taken together, results indicated that capillary remodeling stabilized some of the newly formed capillaries, suppressed granulocyte infiltration, promoted the accumulation of M2 macrophages and decreased the overall vascular density. This response was potentially mediated by the suppression of pro-inflammatory mediators such as *Cxcl5*, *Ccl2*, and *Serpinb2*, and at the same time an upregulation of putative capillary pro-

remodeling genes such as Slit2, Rasa2, Gsk3b and Cyld (Fig.6). A detailed description of these results can be found in Papers I & II.

Figure 6. Graphical summary of capillary remodeling and regression in inflammatory corneal angiogenesis. Suture OUT represents a cornea where capillary remodeling was induced by suture removal, while suture IN represents a cornea where sutures were left in place for continued angiogenic stimulation, to serve as a control for the suture OUT group. The color coding of the genes is only for easy identification. (See Papers I & II).

2.1.2. LXR/RXR activation suppresses corneal inflammation time dependently (PAPER III)

Capillary remodeling described in Papers I & II above is associated with the time-dependent resolution of inflammation. This resolving of inflammation may promote the establishment of functional and persistent capillary networks in the cornea. Regulation of the resolution of inflammation has not been investigated in detail previously, but could reveal mechanisms important for the establishment of persistent corneal vessels, as potential therapeutic targets. To investigate these mechanisms, the suture model of inflammatory corneal angiogenesis was used as described above. In brief, after four days of sprouting following suture placement, the angiogenic stimulus (sutures) were removed to induce capillary remodeling and regression, with this experimental group labelled as the 'remodeling arm'. In the parallel group termed the 'sprouting arm', both sutures were left in place after the four days of sprouting to provide constant angiogenic stimulation. As in Paper I, the sprouting arm (suture IN) served as a positive control for the remodeling arm (suture OUT). Animals in both arms were examined at 24h, 72h

and at 120h. Whole transcriptome analysis was performed using microarrays (GeneChip RatGene 2.0 ST arrays) at 0h, 24h, 72h and at 120h time points. The 0h time point was used to normalize the microarray data from the other time points (Fig. 7).

Figure 7. The experimental design used to investigate the time-dependent resolution of inflammation in the cornea. Two sutures were placed into the cornea and sprouting was allowed to proceed for four days. On the fourth day, (0h) animals were divided in to two groups. In the one group, both sutures were left in place from the 0h, through to the 120h time point (referred to as the sprouting arm). In another group, both sutures were removed at the 0h time point, and animals examined at 24h, 72h and 120h time points (referred to as the remodeling arm). In addition to the *in vivo* examination, animals in both arms were sampled at each time point to harvest tissue for laboratory analysis by microarray. The number of microarray chips used for analysis is indicated beside each schematic representation of the rat, with no pooling of samples. The above experimental design was similar to that used in Papers I & II, but with additional microarrays performed at 72 and 120h, and the data from these time points were normalised to data previously generated at the 0h time in the first study (Paper I).

From analysis of the corneal response *in vivo*, removal of the angiogenic stimulus led to regression of capillaries characterized by a time-dependent vasoconstriction. Resolution of inflammation was indicated by the progressive absence of granulocytes, and appearance of macrophages in the cornea, maintaining the trend observed in the early stages of capillary remodeling as shown Paper I. Hierarchical cluster analysis of the microarray data indicated sample partitioning with time and with treatment.

Focusing on the remodeling arm, removal of the angiogenic stimulus led to an immediate inhibition of pathways such as VEGF ligand signaling, Jak/stat and ERK5

signaling, Endothelin-1 signaling and IL6 signaling, at 24h – pathways that are strongly associated with promoting inflammation and angiogenesis.

At 72h, pathway inhibition continued, with interferon signaling being the most inhibited pathway with inhibition z-score of -2.5, while Wnt β -catenin was the least inhibited with inhibition z-score of -1.5. The pathways LXR/RXR activation, STAT3 and Dendritic cell maturation were on the other hand activated to a comparable extent. Of note, LXR/RXR activation in the remodeling arm was contrary to the inhibition status of the same pathway observed in the sprouting arm at 72h, highlighting a differential activation/suppression pattern between the two arms.

At 120h in the remodeling arm, LPS/IL-1 inhibition of RXR function, and Wnt/beta-catenin were persistently inhibited, maintaining an inhibition profile observed at both 24h and 72h time points during remodeling. In addition, pathways STAT3 and LXR/RXR were still active at 120h, with an even higher activation z-score. For example, LXR/RXR activation increased from 1 to 1.8 z-score between 72h and 120h, indicating a time-dependent activation. In addition, PPAR signaling was also activated at 120h.

Given the differential activation and time dependence of LXR described above, this pathway was analyzed in detail in the remodeling arm. By immunohistochemistry analysis, LXR α and LXR β proteins were expressed by CD45+, CD68+ and by CD163+ cells (M2 remodeling macrophages), and in addition, their target genes ApoE and Abca1 were upregulated, and also expressed by CD68+ and CD163+ cells.

Taken together, the results indicated that resolution of inflammation in the cornea involves an early-phase inhibition of pro-inflammatory and pro-angiogenic pathways like VEGF ligand signaling, and in a later-phase, activation of anti-inflammatory pathways such as LXR/RXR and PPAR signaling. The activated anti-inflammatory pathways upregulate the expression of their target genes in this case such as ApoE and Abca1 in remodeling macrophages. This presumably promotes cholesterol transport out of these cells, leading to improved macrophage function in remodeling capillaries and suppressing the expression of pro-inflammatory genes such as Ccl2, IL-1 β and IL-6 (Fig.8). A detailed description of these results can be found in Paper III.

Figure 8. Graphical summary of the time-dependent resolution of inflammatory corneal angiogenesis, highlighting the cells, pathways and genes involved along with their activation/expression status. An early-phase inhibition of pro-inflammatory and pro-angiogenic pathways such as VEGF ligand signaling, IL-6 signaling, and inhibition of RXR function, and a later-phase activation of anti-inflammatory pathways such as LXR/RXR, STAT3 pathways and PPAR signaling, was noted. Pro-inflammatory mediators such as Ccl2, Cxcl5, and IL-1β, IL-6 were suppressed, and target genes for LXR activation ApoE and Abca1 were upregulated. (See Paper III).

2.1.3. Ghost vessels facilitate rapid corneal revascularization (PAPER IV)

The regression of capillaries along with resolution of inflammation described in Papers I - III, results in the formation of ghost vessels and empty basement membrane sleeves in the cornea. Ghost vessels are thought to be the remnants of previously perfused angiogenic capillaries in the cornea, and have been observed clinically in patients; however, little is known beyond their existence in the cornea. Empty basement membrane sleeves are structures observed in tissues after vessel/capillary regression, where previous functional and perfused vessels have lost their vascular endothelium and flow, leaving only the bare basement membrane tubes (sleeves) present 90,206,207,208. To investigate the characteristics and role of ghost vessels and empty

basement membrane sleeves during a corneal revascularization response, the suture model of inflammatory corneal angiogenesis was again used. Here, the model was used as described above, but with slight modifications. Briefly, corneal neovascularization was induced using two sutures. Sprouting of the newly formed capillaries was allowed for seven days (instead of four days as in Papers I – III). This modification was made to enable sprouting over a longer distance (at seven days the new sprouts reach the sutures), and allows time for the new capillaries to partially stabilize, to better mimic the clinical situation of corneal neovascularization. On the seventh day, sutures were removed to induce capillary regression over a thirty-day period. On the thirty-seventh day, the same cornea was re-sutured with two sutures (0h time point), and the animals were subsequently examined at 24h, 72h and 96h (Fig.9). Whole transcriptome analysis was performed using microarrays (GeneChip RatGene 2.0 ST arrays) at 96h after re-suture.

Figure 9. The experimental design used to investigate the role of ghost vessels and empty basement membrane sleeves in revascularization of the cornea. **1.** Two sutures are placed into the cornea at 1.5mm from the limbus and sprouting occurs for seven days. **2.** On the seventh day, sutures are removed to induce capillary regression over a thirty-day period. **3.** After 30 days of regression, the same cornea is re-sutured (0h) and the animals are examined at 24h, 72h and 96h. **4.** At 96h, animals are euthanized and corneal tissue is collected for laboratory analysis.

Regression of capillaries over a thirty-day period resulted in the formation of both ghost vessels and empty basement membrane sleeves. Here, we differentiate these two phenotypic structures by *in vivo* observation by IVCN, *ex vivo* observation by immunohistochemistry and by TEM analysis of the tissue. The main difference is that ghost vessels, although severely constricted, are still functional, possess vascular endothelium and have minimal blood/plasma flow (often permitting only single erythrocyte flow within the lumen), while basement membrane sleeves do not possess vascular endothelium or flow. TEM analysis of the ghost vessels revealed, however,

that the endothelium of ghost vessels was abnormal, characterized by partial endothelial cell detachment from the basement membrane, microvilli-like projections into the capillary lumen, partial collapse of the capillary lumen, and with single erythrocytes flowing within the collapsing capillary wall.

Upon re-suture of the cornea with this regressed capillary bed, the ghost vessels became rapidly reperfused with blood. TEM analysis of the reperfusing vessels showed a greatly expanded vascular lumen with tightly packed erythrocytes, and with a normalized, functional vascular endothelial cell phenotype lining the re-perfused vessel wall. Endothelial cells were characterized by numerous cytoplasmic mitochondria, and were bordered by pericytes and by basement membrane on the external side of the capillary wall.

In comparison to the primary neovascularization response at 96h, corneal revascularization was of a much stronger magnitude, as illustrated by extensive dilation of capillaries and increased numbers of inflammatory cells infiltrating the cornea. Sprouting of new capillaries occurred at the front of the re-perfused capillaries, and were prominent by 96h after re-suture. The empty basement membrane sleeves were not reused during the revascularization process. This was illustrated by an overall reduction in vascular density of the revascularized cornea compared to the vascular density of the same cornea on day 7 (prior to the induced regression). Furthermore, a side-by-side comparison of slit lamp images of the same cornea at day 7, and at 96 of revascularization showed that many of the initial capillaries that regressed were not re-perfused.

At the whole transcriptome level of analysis, an upregulation of pro-inflammatory mediators such as *Ccl2*, *Ccl6*, *IL-1 β* , and *IL-6* was associated with revascularization. In comparison to the initial angiogenic phase, these genes were on average 20-fold upregulated during revascularization. Pathway enrichment analysis revealed activation of pro-inflammatory pathways such as production of nitric oxide and reactive oxygen species and ILK signaling, and at the same time, inhibition of anti-inflammatory pathways such as LXR/RXR signaling. Genes *IL-6*, *IL-1 β* and *Vegfa* are examples of upstream regulators for the pathways enriched in the revascularization phase.

Taken together, the results indicate that a revascularization response is robust and more severe than the initial angiogenesis, in terms of phenotype and gene expression.

This may be attributed to persistent ghost vessels in the cornea, which are functional and maintain at least a single cell flow, and are composed of a degenerate vascular endothelium. The vascular endothelium of ghost vessels is characterized by loosely attached endothelial cells with multiple microvilli-like projections into the capillary lumen. Upon re-suture, the ghost vessel endothelium rapidly normalizes while the capillary lumen dilates significantly and refills with a strong flow of blood. Sprouting of new capillaries occurs at the front of the re-perfused capillaries and not through re-activation of basement membrane sleeves or repopulation of such sleeves with vascular endothelial cells, as has been suggested in other models²⁰⁹. Activation of pro-inflammatory pathways such as production of nitric oxide and reactive oxygen species signaling, and at the same time inhibition of anti-inflammatory pathway such as LXR/RXR signaling, may explain the observed hyper dilation. (Fig.10). A detailed description of these results can be found in Paper IV.

Figure 10. Summary of events characterizing the regression and the revascularization response of the cornea to a repeated stimulus. Revascularization of the cornea was associated with the activation of pro-inflammatory pathways such as leukocyte extravasation, LPS/IL-1 mediated inhibition of RXR function, and production of nitric oxide and reactive oxygen species, indicated by orange color arrows and stars. At the same time, the anti-inflammatory pathway LXR/RXR activation was inhibited, indicated by the blue colored lines above. Activated upstream regulators for the observed pathway enrichment include IL-1 β , Vegfa and IL-6. Pro-inflammatory mediators such as Ccl2, Ccl3, Cxcl2 and Serpinb2 (referred to in the above as 'downstream effectors') were upregulated. Sprouting occurred at the front of the re-perfused vessels. (See Paper IV).

2.1.4. Corticosteroid treatment suppresses pro-inflammatory genes and activates complement component factors (PAPERS V & VI)

Corticosteroids are effective in resolving ocular inflammation, but with their use comes associated side effects. Understanding the differential mode of action of steroids in comparison to more specific anti-neovascularization treatments could help identify alternative treatment targets for treating corneal neovascularization, that avoid the broad activity of steroids but may mimic their suppressive effects. To address this, the suture model of inflammatory corneal angiogenesis was used as described above but with some modifications. Here, following suture placement, eye drops (IgG, anti-Vegf and dexamethasone) were given to the sutured cornea 4 times per day for two days. On the second day (48h), rats were examined *in vivo* before tissue was harvested for analysis using GeneChip Rat 230 2.0 microarrays. Different from the study designs described above, here the early inflammatory response in a pre-sprouting phase (48h) was investigated, to better understand the mode of action of steroids in an early phase not complicated by active sprouting (Fig.11).

Figure 11. The experimental design used to investigate the differential effect of steroids and anti-Vegf in treating corneal neovascularization. Two sutures were placed into the cornea at 1.5mm from the limbus. The sutured rats were divided into three groups corresponding to topical treatment with IgG (A), anti-Vegf (B) or dexamethasone (C). Animals were treated four times per day for two days. After the second day (48h), animals were examined *in vivo* and corneal tissue was harvested for laboratory analysis.

From *in vivo* analysis, capillary dilation as an early indicator of inflammation preceding sprouting angiogenesis in this model was suppressed by dexamethasone treatment relative to the IgG treated group. On the contrary, anti-Vegf treatment did not suppress early limbal vasodilation relative to the IgG-treated group. In comparison to the anti-

Vegf treated group, dexamethasone treatment suppressed vasodilation by a 5-fold magnitude. Early inflammatory cell infiltration into the cornea at 5h post-suture, as another inflammatory indicator, was not suppressed by any of the treatments.

At the whole transcriptome level of analysis, dexamethasone treatment suppressed expression of pro-inflammatory genes such as Ccl2 and Cxcl5 by 40 and 8-fold, respectively, relative to anti-Vegf treatment. Contrary to the suppression of pro-inflammatory mediators, dexamethasone treatment promoted the upregulation of complement C3 (C3), complement C1s (C1s), C2, and other classical complement pathway factors relative to anti-Vegf treatment. In addition, genes such as complement factor I (Cfi), a complement inhibitor, was suppressed by dexamethasone treatment, relative to anti-Vegf and IgG treatments.

Taken together, the results indicated that steroid treatment in the cornea suppresses the expression of potent pro-inflammatory genes such as Cxcl5 and Ccl2 to subdue inflammation in an early phase. In addition, steroid treatment promotes activation of the classical complement system by activating classical complement factors and inhibiting the expression of Cfi, thereby enhancing synthesis of C3 and C5, by C3 and C5 convertase respectively, leading to enhanced inflammatory cell infiltration (Fig.12). A detailed description of these results can be found in Papers V & VI.

Figure 12. Graphical summary (adapted from del Zoppo ²¹⁰) of the proposed mode of action of steroids in activating the classical complement pathway. The represented genes were differentially regulated by dexamethasone relative to the anti-Vegf treatment in the cornea model. Upregulated genes are shown

in red color, while down regulated genes are shown in green color. The difference in fold change i.e. (fold change dexamethasone minus fold change anti-Vegf) is shown in parentheses, along with the corresponding p-value. The differentially expressed genes activate the classical complement pathway, leading to C3 upregulation, which in turn promotes inflammatory cell infiltration into the cornea. (See Papers V & VI).

2.2. Discussion and future aspects

The lack of effective treatments against corneal neovascularization presents a major clinical challenge. Currently, corticosteroids are used to treat corneal angiogenesis but the side effects associated with steroid use ²¹¹ need to be addressed if these are to be safely used for this purpose. Anti-VEGF treatments on the other hand are used off-label in the cornea, and are of limited efficacy ^{71,178,212,179,213,214}. The drawbacks of both steroids and anti-VEGF warrants the need for safer and more effective treatments. The search for alternative treatment approaches and targets ^{78,215,216,217,218}, and ways to circumvent resistance to treatment is underway. One of the hypothesized means of resistance to anti-VEGF treatment is by the rapid remodeling and maturation of capillaries, making them less susceptible to treatment. Pro-remodeling/maturation genes such as *Cyld*, *Slit2*, *Rasa2* and *Gsk3b* identified here, as being upregulated during the remodeling response are potential mediators of capillary remodeling. In line with this, *Slit2*/roundabout (*Robo*) signaling axis has been shown to regulate vascular remodeling in other systems ²¹⁹. *Slit2* was shown to impair VEGF-induced tumor angiogenesis ²²⁰. In corneal angiogenesis, *Slit2* was shown to interact with *Robo 1* and *Robo 4* to impair neovascularization ²²¹. *Slit2* in other conditions is shown to have anti-inflammatory properties ²²². The differential upregulation of *Slit2* in remodeling capillaries observed here corroborates these reports. Therefore, *Slit2* and the other pro-remodeling/maturation genes identified here warrant further investigation as targets to inhibit capillary remodeling, to keep the newly formed capillaries immature and hence more susceptible to treatment.

The remodeling capillaries here were associated with the accumulation of M2 macrophages (*CD204+* and *CD163+*), which likely mediate anti-inflammatory properties, given that M2 macrophages have previously been reported to have anti-inflammatory properties ^{223,224,225}. However, macrophages are key inflammatory mediators and are of variable plasticity ^{226,227,228}. Some studies have shown M2 macrophages promoting angiogenesis ^{229,230,231}. In addition, proper cholesterol transport from macrophages is important for their functionality. Impairment of this process can lead to pathological vascular proliferation due to the accumulation of cholesterol within the cell resulting in an abnormal macrophage phenotype ²³². Therefore, the role of the M2 macrophage population might be tissue- and disease-specific.

Initial capillary remodeling is associated with attenuation of inflammation over time in the cornea ²³³. The longitudinal whole transcriptome analysis of the time dependent resolution of inflammation in the cornea revealed inhibition of pro-angiogenic pathways such as VEGF signaling ²³⁴, and at the same time, activation of anti-inflammatory pathways such as LXR/RXR signaling ²³⁵. This balance between pro and anti-inflammatory pathways is intriguing, as it provides clues to the regulation of inflammation in this model system. Not surprisingly, LXRs are known to mediate anti-inflammatory properties ^{236,237,238,239,240}. LXRs mediate their anti-inflammatory properties by transrepression of NF-κB-signaling ^{241,240,242}, a pathway known to be involved in corneal neovascularization ^{77,78,243}. It is probable that LXRs suppresses inflammation in the cornea through transrepression of NF-κB-signaling, as reported in other conditions ²⁴⁴. In addition, the expression of LXR target genes *Abca1* ²⁴⁵ and *ApoE* ²⁴⁶ by corneal macrophages, is in line with the reported activity of LXRs in this cell type ^{235,247,248}. If LXR agonists are to be adopted for use in the cornea, their associated side effects such as hypertriglyceridemia and hepatic steatosis ²⁴⁹ will need to be circumvented. However, on a positive note, reports are emerging with means to reduce these effects. For instance, site-specific antibody delivery of LXR agonist is shown to localize treatment, hence reducing the undesired side effects ²⁵⁰. For the corneal indications, it is inevitable to think of LXR formulations in the form of eye drops, to localize their effects and minimize undesired side effects. This route of treatment administration is continuously being improved to further reduce undesirable side effects ²⁵¹. However, the efficacy of LXR agonists in suppressing inflammation in the cornea remains to be evaluated.

Even where capillary regression is apparent and treatment of corneal neovascularization is successful, extensive remodeling and regression of capillaries and the time-dependent resolution of inflammation results in the formation of ghost vessels and empty basement membrane sleeves ^{233,252,253}. These features are distinguished here as separate structures with differing characteristics. The ultrastructure of corneal ghost vessels revealed that they contain an endothelium, however, the endothelium was rather in a degenerative state. The ghost vessels were characterized by endothelial cell detachment from the basement membrane, by collapse of the capillary lumen, and multiple projections of microvilli-like processes into the capillary lumen from the endothelial cells. These ghost vessels often had single erythrocyte flow. These characteristics are potential indicators of

regressing vessels, as reported previously²⁵⁴. The characteristics of ghost vessels observed here were of particular interest, given that ghost vessels were previously thought to be non-functional^{255,256,257}, to have no vascular endothelium^{258,259,260}, and to be non-perfused^{261,262}. The definition of ghost vessels could be tissue and disease specific, or may be confused with empty basement membrane sleeves in other studies that do not use the cornea as a model. In other models, such as in tumor models²⁰⁹, it is difficult or impossible to image such structures in vivo, to observe their perfusion status, and to examine the same structures longitudinally to determine their fate during revascularization. The revascularization response was robust and quicker compared to the initial angiogenic response, and this is probably due to some sort of immunological priming and memory of the cornea.

Extensive vasodilation during revascularization is likely to be a result of the activation of nitric oxide (NO) and reactive oxygen species (ROS) signaling. In line with this, previous reports have implicated ROS in vasodilation, vascular permeability^{263,264}, and in the activity of matrix metalloproteinases²⁶⁵. Re-perfusion of capillaries may have induced shear stress activation of endothelial cell²⁶⁶ to promote their rapid normalization, reactivation and survival. In addition, the numerous mitochondria observed in the endothelial cells might be responsible for the activation of ROS, since mitochondria in endothelial cells are known to mediate ROS signaling²⁶⁷. Besides ROS, LXR/RXR signaling was inhibited during revascularization, which illustrates the potential importance of inflammation in the response, given that LXR/RXR activation was recently shown to have anti-inflammatory properties in the cornea (Paper III). Revascularization of the cornea by re-perfusion of the ghost vessels, and not the empty basement membrane sleeves contradicts previous reports that showed that empty basement membrane sleeves can serve as scaffolds for the reforming vessels²⁰⁹. However, attempts to degrade empty basement membrane sleeves to slow down revascularization were futile²⁶⁸. From our current understanding, it is possible that the empty basement membrane sleeves were not principally reused during revascularization in that study, but rather it was the ghost vessels that facilitated this process. The limiting factor of testing this hypothesis in other models will be the inaccessibility of the experimental site for a longitudinal in vivo examination making it impossible to follow capillaries before and after revascularization. Such drawbacks of other models leave the cornea, and the suture

model of inflammatory corneal angiogenesis as very useful tools for further investigation to answer questions such as: Are the re-perfused capillaries susceptible to antiangiogenic therapies? Do anti-angiogenic treatments influence the manner of capillary regression, and future revascularization? Addressing these questions could for instance provide information with clinical relevance in cases where there is a high risk of revascularization of the cornea due to incomplete eradication of the angiogenic or inflammatory stimulus. In another context, the model mimics the situation of reactivation of tumors after the initial regression of angiogenic capillaries due to acquired treatment resistance or cessation of anti-angiogenic therapy. Persistent ghost vessels may therefore represent an important treatment target in the cancer setting ²⁰⁹.

To maintain the efficacy of corticosteroids while minimizing their side effects ^{269,270} is another treatment strategy for inflammatory corneal neovascularization, which is under investigation ⁷¹. It is hypothesized that by identifying the most efficient targets for steroids, these can then be selectively targeted for effective results with less or no side effects. Here, it was found that in addition to suppressing the expression of pro-inflammatory genes such as Cxcl5 and Ccl2, steroid use seemed to activate complement, specifically upregulating complement C2, C3 and C1s, to potentially suppress angiogenesis. In support of this idea, activation of complement specifically C5a-C5aR has been shown to inhibit angiogenesis in a model of retinopathy of prematurity (ROP) ²⁷¹, and some studies in tumor angiogenesis have also shown inhibitory effects of complement system in angiogenesis ²⁷². However, the complement system should be approached with caution, because it has the capacity to both defend against invading pathogens, and at the same time to inflict tissue damage ^{273,274}. In addition, complement activation has been observed in the pathogenesis of retinal diseases such as in AMD ^{275,276,277}. The findings here could also imply that classical complement pathway seems to be important in cornea while the alternative pathway seems to be more important in the retina. More generally, targeting inflammation, for instance by inhibiting factors such as Cxcl5 and/or Ccl2 would be an interesting approach to investigate as a potential supplement to anti-VEGF therapy.

2.3. Conclusions

The search for safer and more effective treatments against corneal neovascularization is an ongoing process, and the work presented here generates new knowledge regarding the regulation of inflammation and angiogenesis, highlighting the role of previously unidentified pathways and genes in this model system. Therefore, we concluded that;

1. Keeping the newly formed capillaries in an immature state, hence susceptible to treatment may be further investigated by focusing on pro-remodeling genes such as Slit2, Rasa2 and Cyld, and at the same time by suppressing pro-inflammatory mediators such as Cxcl5 and Ccl2, among others, to suppress inflammatory corneal angiogenesis.
2. LXR/RXR signaling and PPAR α /RXR α pathways are activated with time in the cornea to subdue inflammation. The anti-inflammatory property of LXR/RXR is at least partially mediated by tissue macrophages, leading to the suppression of pro-inflammatory mediators such as Ccl2, Cxcl5, Il1 β , and Il6.
3. Ghost vessels and not empty basement membrane sleeves facilitate corneal revascularization. The revascularization response is a rapid process characterized by vasodilation, inflammatory cell infiltration and by sprouting at the front of the re-perfused capillaries. The response is also more severe compared to the initial angiogenesis. Activation of pro-inflammatory pathways such ROS and nitrous oxide signaling, and at the same time inhibition of anti-inflammatory pathways such as LXR/RXR signaling, are potential regulatory mechanisms for this response.
4. Activation of the classical complement pathway, and at the same time suppression of pro-inflammatory genes such as Cxcl5 and Ccl2, is a potential mode of action of corticosteroids to suppress inflammatory corneal angiogenesis, effects that are not achieved with anti-VEGF treatment.

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5. APPENDIX: Publications and a manuscript used in the thesis

Papers

The papers associated with this thesis have been removed for copyright reasons. For more details about these see:

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-147979>