Emerging roles for the CD36 scavenger receptor in neovascular ocular disease

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Here I raise my Ebenezer:

« stone of help » - for upto

this point, the Lord has helped

me. 1 Samuel 7:12

To my dearest husband,

family, and friends who have

been my stone of help

throughout this journey.

ABSTRACT

Ocular neovascularization (NV) associated with corneal NV, ischemic retinopathies and age-related macular degeneration is a leading cause of severe vision loss. While numerous contributing factors have been identified, the potential role of the CD36 scavenger receptor has been largely overlooked notwithstanding its crucial involvement in normal retinal function. Accordingly, the central aim of this work was to elucidate the contribution and regulation of CD36 during ocular NV using the cornea as a model.

Initial work investigating the role of CD36 in maintaining corneal avascularity, an important feature of the normal cornea, revealed that genetic ablation of CD36 elicits age-related corneal NV. Subsequent studies using a pathophysiologically relevant model of inflammatory corneal NV showed constitutive expression of CD36 in the normal cornea with marked induction in the neovascularized cornea. Importantly, activation of CD36 suppressed and induced regression of corneal NV, effects that proceeded via concerted inhibition of VEGFA, JNK-1, and cJun.

Because hypoxia is a fundamental stimulus for angiogenesis, it was pertinent to explore the role and regulation of CD36 during hypoxia. We demonstrate that CD36 expression was significantly elevated in hypoxia-exposed corneal and retinal tissue and in hypoxic retinal pigment epithelial cells. Essential contributions of hypoxia-inducible factor (HIF)-1 and reactive oxygen species were also established. Functional consequences were depicted by augmentations in CD36 phagocytic and anti-angiogenic activities.

Collectively, data disclose CD36 as an important modulator of corneal avascularity and inflammatory corneal NV; this imparts several interesting avenues for future research on the involvement of CD36 in neovascular diseases of the eye. Novel data further identify CD36 as a hypoxia and HIF-1 regulated gene thus creating a framework for future elucidation of the regulatory aspects of this receptor.

RÉSUMÉ

La néovascularisation oculaire (NV), associée à une NV cornéenne, une rétinopathie ischémique et une dégénérescence maculaire liée à l'âge, cause une perte de vision sévère. Bien que plusieurs facteurs ont été identifiés dans ce processus, le rôle potentiel du récepteur éboueur CD36 n'a pas été très étudié malgré son implication crucial dans la fonction rétinienne normale. Alors le but majeur de ce travail a été d'élucider la contribution et la régulation du CD36 durant la NV oculaire en utilisant le modèle de la cornée.

Le travail initial visait à étudier le rôle de CD36 dans le maintient de l'avascularité cornéenne, phénomène important de la cornée normale, a révélé que l'ablation génétique de CD36 amène un développement de la NV cornéenne liée à l'âge. Les études suivantes utilisant un modèle pathophysiologique pertinent de NV cornéenne inflammatoire ont montré une expression constitutive de CD36 dans la cornée normale avec une induction marquée dans la néovascularisation cornéenne, des effets qui se produisent via l'inhibition concertée des voies de signalisations de VEGF et de JNK-1/cJun.

Comme l'hypoxie est un stimulus fondamental pour l'angiogénèse, il était pertinent d'explorer le rôle et la régulation de CD36 durant l'hypoxie. Nous avons démontré que l'expression de CD36 est significativement élevée dans la cornée et le tissu rétinien exposé à l'hypoxie ainsi que dans les cellules épithéliales pigmentaires de la rétine exposées à l'hypoxie. Les contributions essentielles du facteur induit par l'hypoxie (HIF)-1 et des produits dérivés de l'oxygène ont aussi

été établies. Les conséquences fonctionnelles ont été démontrées par l'augmentation de la phagocytose de CD36 et l'activité anti-angiogénique.

Dans l'ensemble, les données révèlent que le CD36 est un modulateur puissant de l'avascularité cornéenne et de la NV cornéenne inflammatoire; ouvrant ainsi plusieurs avenues intéressantes pour de futures recherches sur l'implication de CD36 dans les maladies oculaires néovasculaires. Ces nouvelles données identifient que le CD36 est modulé par l'hypoxie et par le gène HIF-1 créant une ébauche qui permettra d'élucider les aspects régulateurs de ce récepteur.

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Over the years that it took to complete my Ph.D., many people have passed through the laboratories of the 2nd floor, specifically the doors of Dr. Pierre Hardy's Lab, and made a contribution to my work by providing pertinent advice, discussion, friendship, or support. Without their contributions this thesis would have been a lot thinner.

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CHAPTER 2

ABBREVIATIONS

ActD	actinomycin D
ALDH3	aldehyde dehydrogenase
AMD	age related macular degeneration
Ang	Angiopoietin
bFGF	basic fibroblast growth factor
CHX	cycloheximide
$CoCl_2$	cobalt chloride
DHA	docosahexaenoic acid
DR	diabetic retinopathy
ELISA	enzyme-linked immunosorbent assay
EC	endothelial cell
ECM	extracellular matrix
ETC	electron transport chain
FAT	fatty acid translocase
FGF	fibroblast growthfactor
GHRP	growth hormone releasing peptide
GSH	glutathione
HIF	hypoxia-inducible factor
H_2O_2	hydrogen peroxide
HRE	hypoxia response element
IsoP	Isoprostane
JNK	c-Jun N-terminal kinase

LCFA	long chain fatty acid
mAb	monoclonal antibody
MAPK	mitogen activated kinase
MDA	malondialdehyde
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
NADPH	nicotinamide adenine dinucleotide phosphate
NOS	nitric oxide synthase
NOX	NADPH oxidase
NV	neovascularization
oxLDL	oxidized low density lipoprotein
oxPL	oxidized phospholipid
$O_2^{\bullet-}$	superoxide anion
$OH^{\bullet-}$	hydroxyl radical
PC	phosphatidylcholine
PEDF	pigment epithelial derived factor
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PHD	prolyl-hydroxylase domain-containing protein
PI3K	phosphoinositide-3-kinase
POS	photoreceptor outer segments
PS	phosphatidylserine
PUFA	polyunsaturated fatty acids

qRT-PCR	quantitative reverse transcription polymerase chain reaction
RPE	retinal pigment epithelium
ROP	retinopathy of prematurity
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
sFlt-1	soluble VEGFR-1
SOD	superoxide dismutase
SR	scavenger receptor
TIMP	tissue inhibitor of metalloproteinase
TSP	thrombospondin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VPF	vascular permeability factor
XO	xanthine oxidase

PREFACE: AUTHOR CONTRIBUTIONS

This thesis is written in manuscript form as permitted by the McGill University Faculty of Graduate Studies and Research. It comprises three original research articles, as listed below, with the contributions of each author.

Mwaikambo BR, Sennlaub F, Ong H, Chemtob S, Hardy P (2008). Genetic ablation of CD36 induces age-related corneal neovascularization. *Cornea* 27(9):1037-41.

1) *In vivo* manipulations were conducted by the candidate under the guidance of Florian Sennlaub. Remaining experiments and manuscript preparation were completed by the candidate. Huy Ong generously provided the CD36 knock-out mice. Sylvain Chemtob and Pierre Hardy assisted with data interpretation and revised the manuscript.

2) **Mwaikambo BR,** Sennlaub F, Ong H, Chemtob S, Hardy P (2006). Activation of CD36 inhibits and induces regression of inflammatory corneal neovascularization. *Invest Ophthalmol Vis Sci.* 47(10):4356-64.

In this study, the candidate conducted all the experiments and prepared the manuscript. Florian Sennlaub, Sylvain Chemtob, and Pierre Hardy assisted with experimental design, data interpretation, and manuscript editing. Huy Ong assisted with animal studies.

3) **Mwaikambo BR**, Yang C, Chemtob S, Hardy P (2008). Hypoxia upregulates CD36 expression and function via hypoxia inducible factor-1 and ROS dependent mechanisms. Submitted for revision *Free Radic Biol Med.*

All experiments in this study were performed by the candidate. Chun Yang, Sylvain Chemtob and Pierre Hardy provided editorial assistance of the manuscript prepared by the candidate.

OTHER PUBLICATIONS

Mwaikambo BR, Yang C, Ong H, Chemtob S, Hardy P (2008). Emerging roles for the CD36 scavenger receptor as a therapeutic target for corneal neovascularization. *Endocrine Metabolic Immune Diseases-Drug Targets* Review In Press

Mwaikambo BR, Yang C, Zhu T, Gagnon C, Lafleur J, Seshadri S, Lachapelle P, Lavoie JC, Chemtob S, Hardy P (2007). Lymphocytic microparticles inhibit angiogenesis by stimulating oxidative stress and negatively regulating VEGF-induced pathways. *Am J Physiol Regul Integr Comp Physiol. 294(2):R467-76*

Yang C, Lafleur J, **Mwaikambo BR**, Zhu T, Gagnon C, Chemtob S, Hardy P (2008). Lysophosphatidic acid (LPA1) receptor mediates oxygen-induced retinal ganglion cell degeneration. *Invest Opthalmol Vis Sci.* In Press

Hardy P, Beauchamp M, Sennlaub F, Jr Gobeil F, **Mwaikambo B**, Lachapelle P, Chemtob S (2005) Inflammatory lipid mediators in ischemic retinopathy. *Pharmacol Rep* 57 Suppl:169-190.

Hardy P, Beauchamp M, Sennlaub F, Gobeil F, Jr., Tremblay L, **Mwaikambo B**, Lachapelle P, Chemtob S (2005) New insights into the retinal circulation: inflammatory lipid mediators in ischemic retinopathy. *Prostaglandins Leukot Essent Fatty Acids* 72:301-325.

Sennlaub F, Valamanesh F, Vazquez-Tello A, El-Asrar AM, Checchin D, Brault S, Gobeil F, Beauchamp MH, **Mwaikambo B**, Courtois Y, Geboes K, Varma DR, Lachapelle P, Ong H, Behar-Cohen F, Chemtob S (2003) Cyclooxygenase-2 in human and experimental ischemic proliferative retinopathy. *Circulation* 108:198-204.

CHAPTER 1: INTRODUCTION

1.1 Rationale

The vast majority of diseases that cause devastating visual impairment do so as a result of abnormal neovascularization (NV) often in response to chronic hypoxia or inflammatory stimuli. Neovascular ocular diseases such as corneal NV, retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and neovascular age-related macular degeneration (AMD) are major health concerns that when left untreated can potentially cause blindness in infants, working age adults, and the elderly (Lee et al., 1998; Bradley et al., 2007). Oxidative stress, which refers to cellular or molecular damage caused by reactive oxygen species (ROS), is also a key stimulus for the genesis of these ailments (Beatty et al., 2000; Imamura et al., 2006). While conventional treatments for ocular NV such as steroids, laser photocoagulation and photodynamic therapy are somewhat effective in arresting the progression of aberrant neovessels or ablating established ones, they lack specificity and often culminate in tissue destruction (van Wijngaarden et al., 2005; Campochiaro, 2007). As such, there is a tremendous need for treatments that selectively target molecular mediators of these disorders.

Among a host of novel molecular therapeutics, anti-angiogenic therapies offer great promise in preventing the onset or progression of ocular NV. Two licensed agents, pegaptanib and ranibizumab, are currently available for the treatment of AMD (via intraocular injection) by specifically targeting vascular endothelial growth factor (VEGF) which plays a central role in stimulating hypoxia or ischemia induced neovessel formation (Bradley et al., 2007).

However, despite these advances, present treatments can be improved and there exists a niche for research and development of new pharmacological agents that can be administered less invasively, are relatively inexpensive, and have complementary modes of action allowing for combination therapies (Afzal et al., 2007). To this end, elucidating the involvement of the CD36 anti-angiogenic receptor may enhance our understanding of how to better target neovascular or degenerative diseases of the eye.

CD36, an integral membrane glycoprotein comprising the scavenger receptor family features prominently in angiogenesis, cardiovascular biology, lipid homeostasis, parasitology, Alzheimer's disease, diabetes, platelet biology, and even food choice; knowledge that has been largely facilitated by the creation of the CD36 knock-out mouse (Febbraio et al., 1999). The multifunctional attributes of CD36 are accredited to the many distinct ligands with which it interacts, the numerous downstream effectors upon which it acts, and the large panel of CD36 expressing cells and tissues [for reviews refer to (Febbraio et al., 2001; Simantov and Silverstein, 2003; Febbraio and Silverstein, 2007)]. In the eye, distribution of CD36 has not been examined in an exhaustive fashion. However, expression of CD36 in the retina, particularly the retinal pigment epithelium (RPE), is well established and is in line with its scavenging functions, namely the phagocytosis of photoreceptor outer segments (POSs) (Ryeom et al., 1996a; Finnemann and Silverstein, 2001; Sun et al., 2006). We also previously reported that inhibition of the immediate-early pro-inflammatory gene product, cyclooxygenase-2, suppresses pre-retinal neovascularization via novel induction of the anti-angiogenic CD36/thrombospondin-1 pathway (Sennlaub et al., 2003). On the

other hand, CD36 expression and function in the cornea has only been loosely reported in studies screening the anti-angiogenic potential of various molecules in models of corneal angiogenesis using CD36 null mice (Jimenez et al., 2000; Simantov et al., 2001). It was also recently postulated that CD36 deficiency triggers age- and gender-related spontaneous corneal lesions in mice (Barcia et al., 2007), however complete documentation of these results is yet to be published in a peer reviewed journal. In the end, despite the impressive literature on CD36, knowledge of the functional and regulatory aspects of this receptor in the context of ocular health and disease remains obscure, albeit pertinent, given the importance of CD36 in retinal function and in the regulation of angiogenesis.

1.2 Objectives of research

On the basis of the aforementioned considerations, we hypothesized that in the setting of ocular NV, specifically of the cornea, CD36 is important for the maintenance of corneal avascularity and in suppression of inflammatory corneal NV, in part by negatively regulating VEGF-induced pathways. Given that hypoxia is a critical stimulus of ocular NV and that CD36 is a well-established anti-angiogenic receptor, we further speculated that hypoxia modulates CD36 expression and function following hypoxic injury.

In order to address our hypotheses, the following experimental aims were established:

1) Determine the contribution of CD36 in the maintenance of postnatal corneal avascularity by histologically examining corneas from CD36 wild-type and knock-out mice at different ages.

2) Using a mouse model of inflammation-induced corneal NV, determine the expression profile of CD36 in normal and neovascularized corneas and the effects of pharmacologically modulating its expression on the outcome of corneal NV. The roles of VEGF, the stress-activated protein kinase JNK, and the cJun transcription factor were also assessed.
3) Determine the effect of hypoxia on CD36 expression and function *in vivo* (in hypoxia exposed corneal and retinal tissue) and *in vitro* in RPE cells. Delineate the roles of HIF-1, ROS, and other potential mechanisms in the hypoxic regulation of CD36.

1.3 Thesis organization

In order to familiarize the reader with the subject matter at hand, an extensive literature survey of topics covered in this dissertation is presented in Chapter 2. Specifically, an introduction to the structural anatomy and function of the cornea and retina is provided, with detailed coverage of the current knowledge in the field of corneal NV. Also included is an overview of hypoxia, ROS and oxidative stress as they pertain to corneal and retinal biology and pathology. Finally, the reader is introduced to the protagonist, the CD36 scavenger receptor, including pertinent information regarding its function, regulation, and contribution to angiogenesis and ocular physiology.

While Chapter 3 introduces the reader to the role of CD36 in the maintenance of corneal avascularity, Chapter 4 investigates the therapeutic potential of modulating CD36 expression in targeting inflammatory corneal NV (Mwaikambo et al., 2006). Novel regulation of CD36 by hypoxia and HIF-1 is

described in Chapter 5 specifically using the cornea, retina, and RPE cells as experimental models. In Chapter 6, the research achievements of this work are discussed and topics for future work are presented.

CHAPTER 2: LITERATURE SURVEY

2.1 The Eye

To see well, we must maintain a clear visual axis and normally functioning cellular phototransduction (Friedlander, 2007). Light entering the eye passes through the smooth, wet surface of the cornea (the major refractive surface), which, along with corneal transparency, enables light to proceed through the lens, the vitreous (gel in the posterior chamber of the eye), and onto the retina for photoreceptor activation and subsequent conversion of light into electrical signals to be transmitted to the brain (Figure 1) (Friedlander, 2007). To maintain a visual system that is conducive for light entry, a highly organized tissue structure is necessary; any disruption in normal cell-cell interactions can lead to biological dysfunction and ensuing visual distortion or impairment (Friedlander, 2007). This section will describe the structural anatomy and function of the anterior and posterior segments of the eye, with a major focus on the cornea and retina.

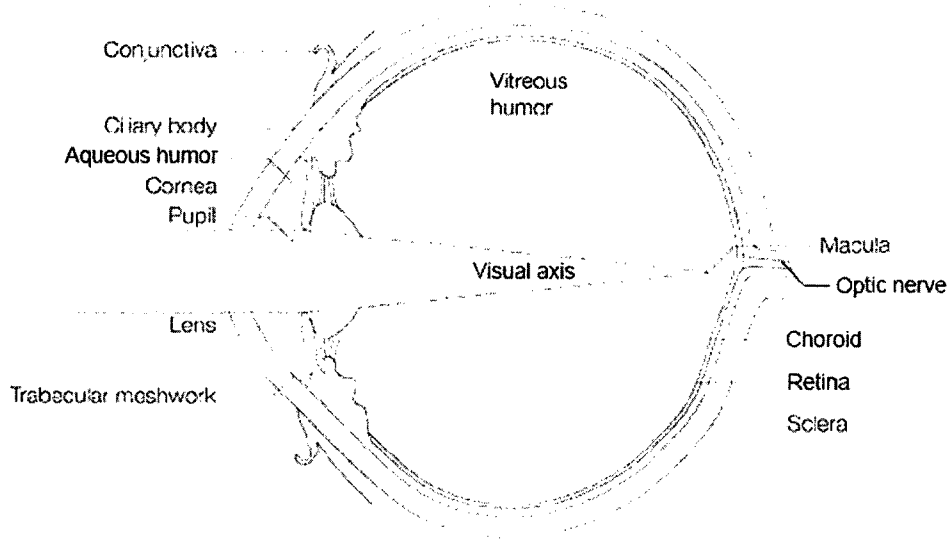

Figure 1. Anatomy of the visual axis of the eye (adapted from Streilin, 2003).

2.1.1 The ocular surface: cornea, conjunctiva, and limbus

The term "ocular surface" was coined in 1977, by Thoft and Friend to describe the continuous epithelial sheet that covers the anterior surface of the eye (Thoft and Friend, 1977). This concept prompted numerous discoveries including the remarkable identification of corneal epithelial stem cells at the limbus (Schermer et al., 1986), a transitional zone between the cornea and conjunctiva. The ocular surface is composed of three distinct epithelia: corneal, limbal and conjunctival. Although they are all stratified, squamous, non-keratinizing epithelia, their characteristics and functions differ considerably, as will be articulated in the subsequent sections (Kinoshita et al., 1982; Azar, 2006).

2.1.1.1 Anatomy and function of the cornea

While there are those who consider the cornea simply as a "dustcover" for the retina, (Friedlander, 2007) it is in fact one of the simplest and, at the same time, most fascinating tissues. The cornea is a transparent tissue located at the front of the eye. It provides two-thirds of the eye's optical power (refracting and focusing incident light onto the retina) and serves a protective role by acting as an external barrier to infectious agents (Friedlander, 2007). A cross-section of the cornea reveals five distinct layers (Figure 2), which organized from the anterior to posterior are:

1. **Corneal epithelium**. The cornea is covered by a tough, transparent stratified epithelium that functions to protect the front of the eye from physical and chemical agents. The epithelium is composed of five to seven layers of cells, with the basal layer containing prominent nuclei and undergoing active mitosis. The outermost layers of these cells produce numerous microvilli which are in constant contact with the tear film, a key factor in maintaining a smooth outer surface and the optical characteristics of the epithelium. Moreover, the epithelium is continuously shed into the tear film, allowing for its regeneration and turnover almost every 7 days (Bazan and Ottino, 2002). The corneal epithelium is also an important source of anti-angiogenic molecules (Ambati et al., 2006; Cursiefen et al., 2006), the relevance of which will be discussed in Section 2.2.5.

2. **Corneal stroma**. The stroma constitutes 90 % of the total corneal thickness (500 μm) and is comprised of heterodimeric complexes of type I and type V collagen fibers that are arranged in bundles referred to as lamellae; this

marriage of collagen fibers ensures a uniformly small diameter maintained at close periodicity. The lamellae are aligned in parallel arrays within each layer and surrounded by specialized proteoglycans that confer unique water-retention properties. These specializations are imperative for corneal function because they impart clarity to the tissue, allowing free passage of light (Fini and Stramer, 2005; West-Mays and Dwivedi, 2006). Dispersed between the lamellae are the keratocytes, which are a population of flat, elongated cells whose function it is to secrete collagen and intercellular matrix and thus maintain corneal transparency (Müller et al., 1995). In response to corneal injury or edema, keratocytes are also activated to either undergo apoptosis or to transform into a fibroblast phenotype, which is necessary for promoting regeneration (Fini and Stramer, 2005).

3. **Bowman's membrane**. Classically described as a tough acellular layer that serves to protect the underlying stroma (Wilson and Hong, 2000).

4. **Corneal endothelium**. A monolayer of polygonal mitochondria rich cells that form a physical barrier between the corneal stroma and aqueous humour. The corneal endothelium functions to maintain corneal transparency by continuously pumping water out of the stroma (Tervo and Palkama, 1975). Unlike the tight barrier formed by the corneal epithelium, the endothelial barrier is permeable and leaky, hence permitting the slow, constant leak of aqueous humour into the stroma, which provides most of the cornea's nutritional requirements. Corneal endothelial cells also have a limited capacity to divide; therefore, loss of endothelial cells due to increasing age, trauma, disease, or previous corneal transplants causes the remaining cells to rapidly elongate in order to cover the damaged area.

5. **Descemet's membrane**. A thin acellular layer secreted by the corneal endothelium that functions to protect the stroma from infiltration of cells arriving at the cornea (Bazan and Ottino, 2002).

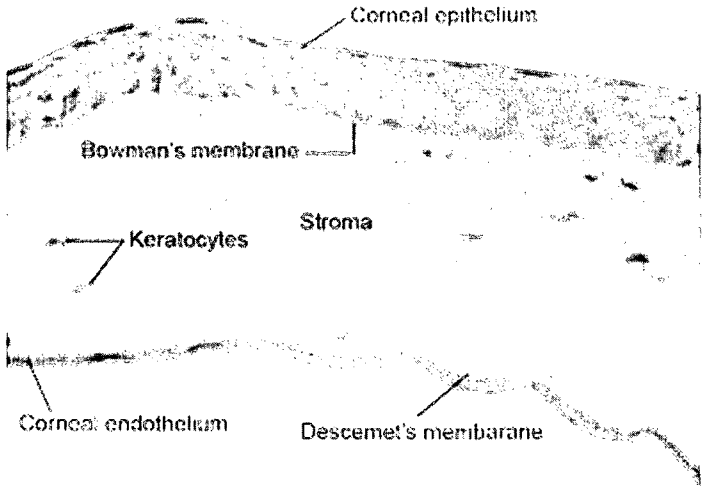

Figure 2. Anatomy of the cornea. Depicted is a histological section through the mouse cornea illustrating the five principal layers.

2.1.1.2 Anatomy and function of the conjunctiva

The conjunctiva is a thin transparent mucous epithelial barrier that lines the inside of the eyelids, and covers the anterior one-third of the eyeball. The cornea and the conjunctiva are physically apposed, being separated from each other by only a thin film of tear fluid. The conjunctiva is composed of two layers: an outer epithelium and its underlying stroma. The epithelium is covered with microvilli and consists of stratified epithelial cells whereas the stroma (containing structural and cellular elements, including goblet cells, nerves, lymphatics, and blood vessels) attaches to the underlying sclera. The conjunctiva has numerous functions including barrier protection of the ocular surface, production of tear film by way

of secreting electrolytes, fluid, and mucins (by goblet cells), and as a conduit for drug clearance into the systemic circulation or for drug transport to the deep tissues of the eye (Dartt, 2002).

2.1.1.3 Anatomy and function of the limbus

The epithelia of the cornea and of the conjunctiva are separated by a narrow transition zone known as the limbus. The limbal epithelium consists of a multilayer of cells dispersed in the stroma, rich in tiny blood vessels, with the presence of Langherans and melanocyte cells (which produce and secrete pigments). Although the limbus does not contain stem cells of the conjunctival epithelium, its basal cells do generate stem cells of the corneal epithelium (Revoltella et al., 2007). Interestingly, limbal epithelial cells, in their capacity as corneal epithelial cell progenitors, have been shown to possess anti-angiogenic properties (Ma et al., 1999; Azar, 2006), as will be explored in Section 2.1.2.3.

2.1.2 The Retina

The retina is the innermost layer of the eyeball. It is comprised of cells with vastly distinct functions, including six neuron types of the inner neurosensory retina: photoreceptor cells, horizontal cells, bipolar cells, amacrine cells, interplexiform cells, and ganglion cells. The retina is nourished by two vascular beds: the choriocapillaris in the posterior region and the retinal vasculature in the region beneath the vitreous. Separating these two vascular beds are Bruch's membrane and the retinal pigment epithelium (RPE).

The neural retina can be classified into distinct layers as depicted in Figure 3 (Kaufkan, 2002). However, for the purposes of our discussion, we will focus on the RPE and companion photoreceptors.

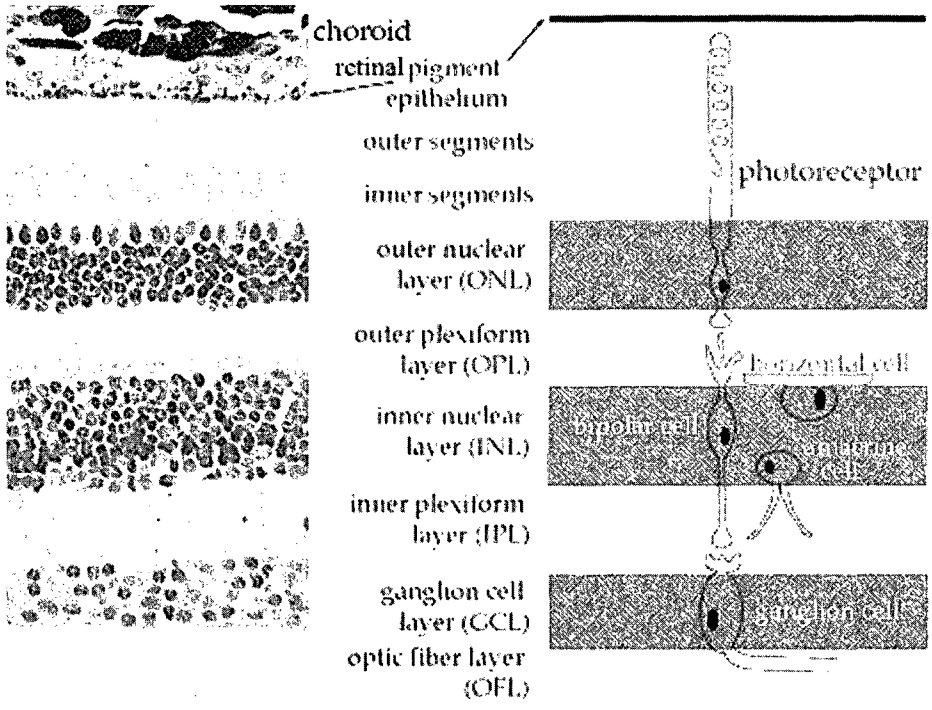

Figure 3. Anatomy of the human retina. Depicted are the principal layers of the neural retina (adapted from http://thalamus.wustl.edu/course/eyeret.html).

2.1.2.1 Photoreceptor cells

Photoreceptor cells are highly specialized and convert light into neural signals by a process called phototransduction. Their distal parts are adapted for capturing light whereas their proximal parts are adapted to transmit it. Two main types exist: rods and cones. The approximately 92 million rod cells are responsible for vision in dim light, whereas about 5 million cone cells are responsible for vision in bright illumination, in which colour cues are important.

Cones are highly concentrated in the central retina while rods are present throughout the retina except for the very central region known as the fovea. The visual pigment in rods responsible for capturing light is rhodopsin, which consists of a protein component, opsin and a chromophore, the vitamin A derivative, 11-*cis*-retinal. Phototransduction is triggered by the photic conversion of 11-cis-retinal to all-trans-retinal in rhodopsin.

Photoreceptors are reputed to have the highest oxygen consumption and metabolic rate of any tissue in the body (Chen et al., 2007). They are also constantly exposed to intense levels of light which damages their outer segments (OSs) to such an extent that they have to be rapidly replaced. Consequently, every morning, following stimulation by light and circadian rhythms, rods shed their OS tips which are then degraded by RPE-mediated phagocytosis. Cone cells are also constantly renewed in the evening, rather than the morning (Kaufkan, 2002).

2.1.2.2 The Retinal Pigment Epithelium

The RPE is a highly polarized and specialized monolayer of pigmented cells making up the outermost layer of the retina. Each eye contains an estimated 4 to 6 million RPE cells. As with other melanin producing cells, RPE cells are characterized by the presence of the melanosome, a lysosome-related organelle devoted to the biosynthesis and storage of melanin pigments (Marks and Seabra, 2001). The apical surface of the RPE faces the photoreceptor outer segment (POS), while its basolateral membrane faces Bruch's membrane, a collagenous layer separating the RPE from the choroid (Figure 4). As such, the photoreceptors,

choroidal vessels, and RPE are considered an interdependent functional unit and loss of any one component causes dysfunction of the others (Chen et al., 2007).

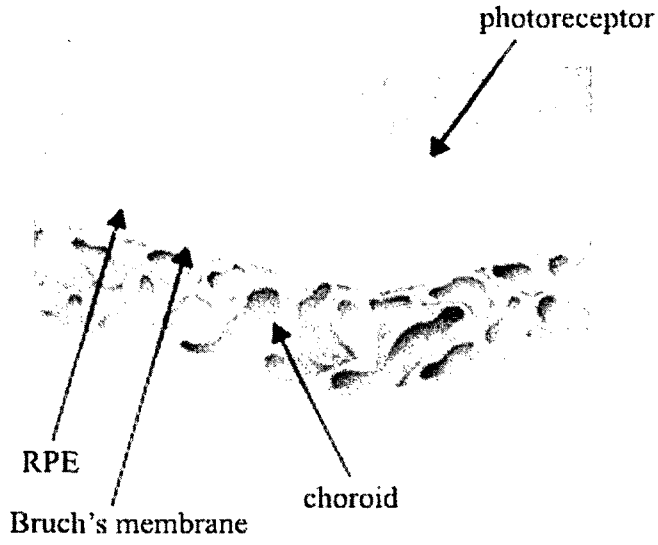

Figure 4. Cross section of the retina illlustrating the photoreceptor-RPE-choroid complex (adapted from www.amdcanada.com).

A selected list of critical RPE functions is presented below (Marmorstein et al., 1998; Kaufkan, 2002; Strauss, 2005):

- *Absorption of light energy.* Owing to its dark pigmentation, the RPE increases optical quality which aids in absorption of scattered light.

- *Transportation of nutrients and ions and removal of wastes.* Since the photoreceptors lack a direct blood supply, the RPE is responsible for regulation of traffic between the choroid and the photoreceptors. Thus the RPE cells manage the bi-directional transport of ions, water, and metabolic end-products from the subretinal space to the choroid; in the opposite direction, the RPE takes up

nutrients such as glucose, retinol, and fatty acids from the blood and delivers them to the photoreceptors.

- *Phagocytosis of shed POSs and transport and processing of vitamin A.* RPE cells are among the most efficient phagocytes in the body. In the mammalian retina, each RPE cell underlies about 30 photoreceptors which as mentioned, daily shed the aged, distal tips of their OSs. RPE cells promptly and efficiently recognize and engulf the shed POSs by receptor-mediated phagocytosis such that an individual post-mitotic RPE cell disposes of several thousand OSs once a day for decades (Marmorstein et al., 1998). In this process important molecules such as retinal, rhodopsin, and docosahexaenoic acid (DHA) are recycled back to the photoreceptors. These intricate steps engage RPE cell-surface receptors specifically Mer tyrosine kinase (MerTK) (D'Cruz et al., 2000), a mannose receptor (Boyle et al., 1991), the CD36 class B scavenger receptor (Ryeom et al., 1996b; Finnemann and Silverstein, 2001), and integrin adhesion receptors, $\alpha v\beta 3$/ $\alpha v\beta 5$ (Finnemann et al., 1997; Finnemann and Silverstein, 2001) that recognize, bind to, and/or internalize photoreceptor tips. More detail on RPE phagocytosis specifically in the context of CD36 will be provided in Section 2.5.5.

- *Formation of the outer blood–retina barrier.* As the part of the blood retinal barrier (provided in part by the tight junctions between RPE cells), the RPE functions in establishing immune privilege of the eye.

- *Secretion.* The RPE is known to produce and secrete a variety of factors that are essential for maintaining the structural integrity of the retina and

choriocapillaris while ensuring optimal circulation and supply of nutrients (e.g. VEGF, FGF, PEDF).

2.1.2.3 RPE, aging, and disease

RPE cells exhibit definite signs of aging and the reasons for this are at minimum three-fold: (1) RPE cells accumulate a lot of waste products due to their high metabolism, (2) RPE cells have to shoulder the oxidative burden of daily removing shed POS (see Section 2.4.7), and (3) the antioxidant capacity of the RPE declines with age (Cai et al., 2000; Strauss, 2005). When unable to sufficiently cope, aging or "injured" RPE cells accrue abnormal molecules such as lipofuscin or drusen (lipid or cellular debris-containing deposits). Such events not only interfere with normal cellular metabolism but result in a thickening of Bruch's membrane and subsequent RPE dysfunction and photoreceptor degeneration, processes that have been shown to result in retinal degeneration in experimental animal models and initiation and/or progression of age-related macular degeneration (AMD) in humans (Liang and Godley, 2003; Combadière et al., 2007; Justilien et al., 2007). Indeed, hallmark pathologic features of AMD, the leading cause of visual impairment in the elderly in the developed world, are lesions involving the RPE and Bruch's membrane, photoreceptor degeneration, and in extremely devastating cases, choroidal neovascularization (Chen et al., 2007).

2.2 Corneal Neovascularization

Neovascularization within the eye contributes to visual loss in several ocular diseases that include corneal neovascularization, neovascular AMD, and retinopathies of diabetes and prematurity (Figure 5). Together these diseases afflict persons in all stages of life and account for most instances of legal blindness (Lee et al., 1998; Chang et al., 2001).

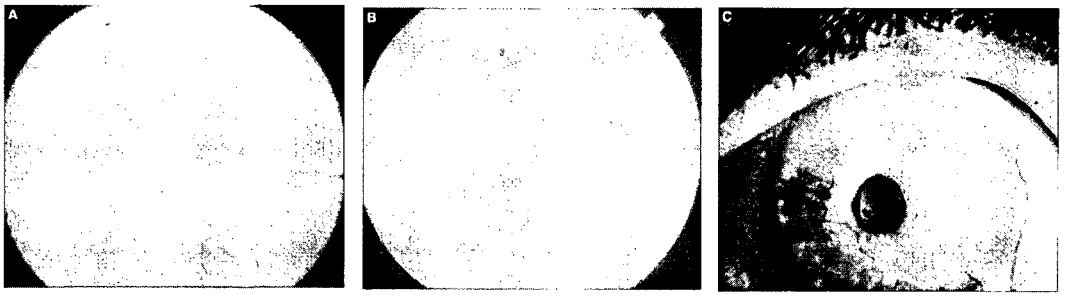

Figure 5. Ocular angiogenesis. (**A**) Retinal neovascularization in a patient with proliferative diabetic retinopathy. (**B**) Subretinal haemorrhage in a patient with choroidal neovascularization due to age-related macular degeneration. (**C**) corneal NV in a patient with infectious keratitis (from Kvanta, 2006).

While the posterior structures of the eye, such as the choroid are heavily vascularized, the normal cornea is one of the few sites in the human body that is devoid of vascular elements, i.e. it is *avascular* (Figure 6) (Cursiefen et al., 2006). The corneal blood supply arises from the ciliary arteries, which are branches of the ophthalmic artery that divide and terminate in the pericorneal plexus in the limbal area. As such, corneal NV is characterized by the sprouting of new blood vessels from the pericorneal plexus into the central cornea (Chang et al., 2001).

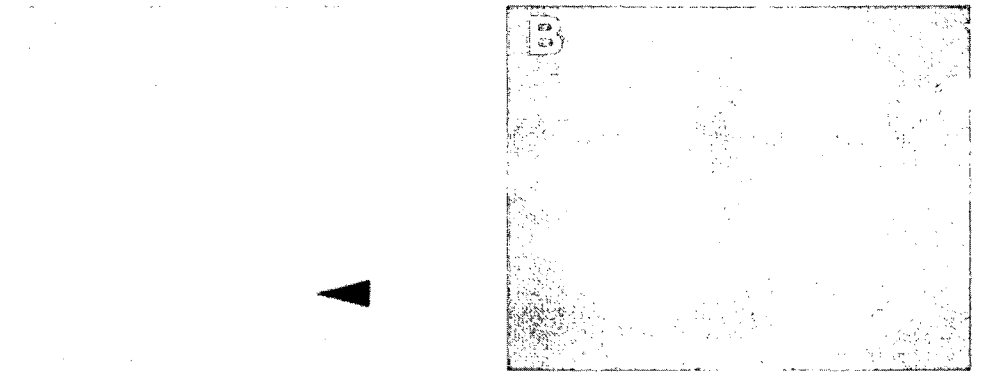

Figure 6. Sharp transition from vascularized limbus to normally avascular cornea. Normal murine (upper, A) and central (B) cornea is avascular and is sharply demarcated from limbus (lower, arrowhead (A); arrows (B)) (adapted from Cursiefen et al., 2006).

2.2.1 Neovascularization (NV)

Neovascularization (NV) is broadly defined as the formation of new vascular structures in areas that were previously avascular. This often occurs where inadequate blood supply and the resultant decline in tissue oxygenation (hypoxia) (see Section 2.3) causes compensatory development of blood vessels in response to the heightened tissue demands.

Neovascularization is believed to occur via two mechanisms: (1) *vasculogenesis*, the de novo recruitment of bone marrow–derived progenitor cells to yield new vascular structures, or (2) *angiogenesis*, the formation of new capillary vessels from pre-existing resident endothelial cells. The traditional perception that vasculogenesis is restricted to embryonic life is rapidly being challenged by studies documenting the identification of circulating endothelial progenitor cells (Asahara et al., 1997; Shi et al., 1998) and their participation in NV during ischemic episodes (Asahara et al., 1999; Tepper et al., 2005). In fact,

Ozerdem et al. recently revealed that during corneal NV, a significant proportion of neovascular pericytes originate from bone marrow-derived progenitor cells, rather than from pre-existing vessels, thus suggesting an extensive role for vasculogenesis in corneal NV (Ozerdem et al., 2005). Angiogenesis on the other hand occurs extensively during embryogenesis, while in the normal adult it is limited to physiological events such as wound healing (Ortega et al., 1998), ovulation, corpus luteum formation, and embryo implantation (Hyder and Stancel, 1999; Kvanta, 2006). Angiogenesis also plays an important pathogenic role in tumorigenesis and in the vision loss associated with corneal NV, ischemic retinal disorders, and the wet form of AMD (Friedlander, 2007).

2.2.2 Risk factors for corneal NV and Epidemiology

Corneal NV is associated with the major causes of corneal blindness worldwide (trachoma) and in developed nations (herpetic keratitis) (Whitcher et al., 2001). It is commonly observed in response to persistent hypoxia, alkali burns, graft rejections or various inflammatory, infectious, or degenerative disorders (Table 1) (Lee et al., 1998; Chang et al., 2001). The resultant NV, edema, corneal scarring, opacification, and lipid deposits may not only significantly alter visual acuity, but may also exacerbate the prognosis of subsequent penetrating keratoplasty (corneal transplantation) (Cursiefen et al., 1998; Chang et al., 2001). Indeed, graft failure accounted for more than 30% of vascularized corneal buttons diagnosed following corneal transplantation (Cursiefen et al., 1998).

Although no data currently exist documenting the exact incidence and prevalence rates of corneal NV, the incidence rate was estimated at 1.4 million

patients per year based on an extrapolation of the 4.14% prevalence rate at the Massachusetts Eye and Ear Infirmary in 1996 (Lee et al., 1998).

Table 1. Diseases associated with corneal neovascularization (adapted from Chang et al., 2001).

Inflammatory and immunologic diseases
Stevens-Johnson syndrome
Graft rejection
Cicatricial pemphigoid
Rosacea
Atopic conjunctivitis
Lyell's syndrome
Trauma and prior surgery
Contact lens wear
Alkali burns
Stem cell deficiency
Infections
Bacteria and other microorganisms, e.g., *Chlamydia, Staphylococcus, Streptococcus, Pseudomonas, Syphilis*
Viruses, e.g. herpes simples, herpes zoster
Protozoa, e.g. *Onchocerca volvulus, Leishmania Brasiliensis*
Fungi, e.g. Candida, Fusarium, Aspergillus
Degenerative disorders
e.g. pterygium, Terrien marginal degeneration, Aniridia

2.2.3 Types of corneal NV

While it has been reported that the primary histopathological localization of vascularized corneas occurs in the upper and mid-third regions of the stroma (Cursiefen et al., 1998; Chang et al., 2001), three clinical entities of corneal NV are generally discerned (Lee et al., 1998):

1) *superficial NV* is characterized by vessels that extend beneath the corneal epithelium, usually due to corneal trauma, mild chemical burns, inflammation, and infections;

2) *vascular pannus formation* is denoted by connective tissue proliferating in the superficial corneal periphery and is mainly associated with ocular surface disorders;

3) *deep stromal vascularization* occurs at any level of the stroma from beneath Bowman's layer to Descemet's membrane and it is associated with stromal keratitis, serious anterior segment injuries, tuberculosis, and syphilis.

2.2.4 Molecular basis of corneal NV

2.2.4.1 Balance between angiogenesis and anti-angiogenesis

Angiogenesis is a complex multistep process that involves the stimulation of angiogenic growth factor receptors on vascular endothelial cells (ECs), proteolytic breakdown of the basal membrane, EC proliferation and migration, extracellular matrix (ECM) remodelling, vessel maturation, recruitment of supporting cells (e.g. pericytes), and capillary tube formation [for increased detail see (Folkman, 1971; Carmeliet, 2003; Kvanta, 2006)] as shown in Figure 7.

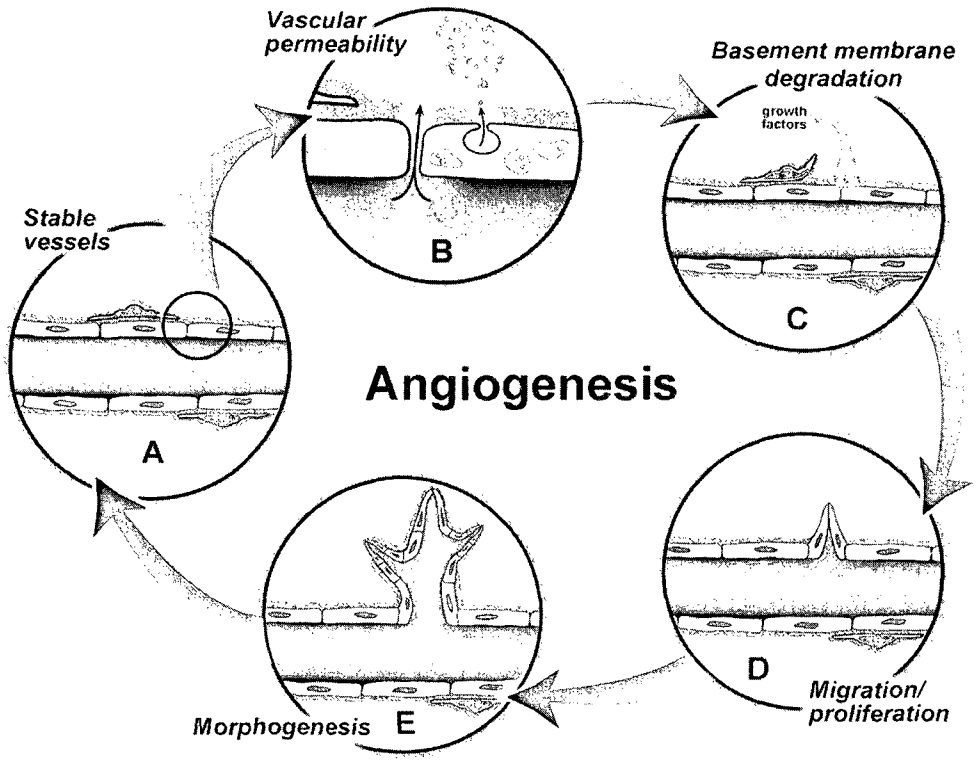

Figure 7. The angiogenic cascade. During the process of angiogenesis, stable vessels (a) undergo a vascular permeability increase, which allows extravasation of plasma proteins (b). Degradation of the ECM by MMPs relieves pericyte-EC contacts and liberates ECM-sequestered growth factors (c). ECs then proliferate and migrate to their final destination (d) and assemble as lumen-bearing cords (e). ECM, extracellularmatrix; MMPs, matrix-metalloproteases; EC, endothelial cell (Bryan and D'Amore, 2007).

A balance between naturally occurring stimulatory (*angiogenic factors*) and inhibitory (*anti-angiogenic factors*) molecules is responsible for the normally quiescent microvasculature. When this balance is upset, as occurs during corneal NV, the balance is shifted in favour of angiogenesis, an event termed the 'angiogenic switch' (Carmeliet and Jain, 2000; Kvanta, 2006). Corneal avascularity in itself requires low levels of angiogenic factors and high levels of anti-angiogenic factors under basal conditions (Chang et al., 2001).

2.2.4.2 Angiogenic factors involved in corneal NV

Numerous angiogenic factors such as vascular endothelial derived factor (VEGF), fibroblast growth factor (FGF), and the matrix metalloproteinases (MMPs) have been implicated in corneal NV (Table 2). Nonetheless, data supporting a causal role for VEGF are extensive including potent upregulation of VEGF in inflamed and vascularized corneas in both human and animal models (Amano et al., 1998; Philipp et al., 2000; Chang et al., 2001; Joussen et al., 2003; Cursiefen et al., 2004b; Singh et al., 2005).

VEGF: VEGF was originally identified as a vascular permeability factor (VPF) and has subsequently emerged as a major regulator of normal and pathological angiogenesis, promoting several steps during angiogenesis including ECM degradation, EC survival, proliferation, migration, and capillary tube formation (Ferrara et al., 2003). VEGF-A is the prototype member of a family of growth factors that includes placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E (Ferrara et al., 2003). Mainly produced by RPE cells, macrophages, T cells, astrocytes, and smooth muscle cells, VEGF induction has been demonstrated in hypoxic/ischemic and inflammatory conditions. Indeed, hypoxia is a key stimulator of VEGF (Shweiki et al., 1992) eliciting its expression through stabilization of hypoxia inducible factor (HIF)-1 which subsequently binds to the hypoxia response element (HRE) on the VEGF promoter (for more on HIF-1 refer to Section 2.3.1).

Alternative splicing of VEGF-A yields five main isoforms, of which the most abundant forms are $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, reflecting on the

number of amino acids after cleavage of the signal sequence. VEGF exerts its biological effects by binding to two surface receptor tyrosine kinases (RTKs), VEGF receptor-1 (VEGFR-1; also known as fms-like tyrosine kinase-1/Flt-1) and VEGFR-2 (also known as kinase domain region/Flk-1). Both VEGFR-1 and -2 are predominantly expressed on vascular ECs, however evidence to date attests that VEGFR-2 mediates the bulk of VEGF's angiogenic and vasopermeability activities (Ferrara et al., 2003). An alternatively spliced form of VEGFR-1, soluble Flt-1 (sFlt-1) binds to VEGF with high affinity and inhibits its mitogenic activity (Kendall and Thomas, 1993). Interestingly, Kendall and Thomas (1993) previously proposed that the *in vivo* inhibitory action of sFlt-1 on VEGF could act as a feedback mechanism to terminate angiogenesis, and prevent blood vessel growth into normally avascular tissues such as the cornea and cartilage. Indeed, these hypotheses were recently validated by Ambati and colleagues (Ambati et al., 2007) who reported on the requirement of sFlt-1 for the maintenance of corneal avascularity. This subject will be elaborated on in Section 2.2.5.

FGF: FGF-1 and basic fibroblast growth factor (bFGF or FGF-2) are two potent inducers of angiogenesis that comprise the prototypic members of the FGF family and exert their biological activity by binding to high affinity tyrosine kinase receptors, designated FGFR-1, -2, -3, and -4 (Presta et al., 2005). FGF-1 is expressed in the normal corneal epithelium. Interestingly, bFGF binds to Bowman's and Descemet's membranes in the normal cornea and vascular basement membranes in neovascularized corneas (Chang et al., 2001; Azar, 2006). Of notable mention, bFGF has been widely used in corneal angiogenesis assays.

MMPs: corneal NV is also regulated by MMPs, a 25 member family of zinc-containing proteolytic enzymes that are essential for ECM degradation and remodelling, as well as release of bioactive fragments during wound healing and pathologic processes. MMPs are secreted as pro-enzymes that are subsequently activated in the ECM by a variety of proteinases, including MMPs and serine proteases. Certain MMPs (types 1, 2, 7, 8, 9, and 13) are also known to degrade native collagens (types I, II, III, IV, VI, XVIII) and gelatins to generate proteolytic fragments such as endostatin (Lin et al., 2001).

Of the numerous MMPs described in the literature, MMPs 1, 2, 3, 7, 9, 13, and 14 have been identified in the normal cornea (Chang et al., 2001; Sivak and Fini, 2002). Although previous studies demonstrated up-regulation of MMP-2 in models of inflammatory corneal NV and marked inhibition of bFGF-stimulated corneal NV in MMP-2-deficient mice (Kvanta et al., 2000; Kato et al., 2001; Samolov et al., 2005), the precise role of MMPs in modulating corneal NV remains elusive. This is attributed to the dual function of MMPS during angiogenesis, such that the same molecule can have a stimulatory or inhibitory role (e.g. promotion of angiogenesis via ECM degradation or inhibition through production of anti-angiogenic fragments) (Chang et al., 2001; Azar, 2006).

2.2.4.3 Anti-angiogenic factors involved in corneal NV

The cornea is believed to produce and contain numerous anti-angiogenic factors that may be produced endogenously in their active forms (e.g. thrombospondins (TSPs), pigment epithelial–derived factor (PEDF), and tissue

inhibitor of metalloproteinases (TIMPs)) or derived from larger non-angiogenic precursors by proteolytic cleavage (e.g. endostatin and angiostatin) (Table 2).

TSPs: The TSPs are a family of five glycoproteins that regulate multiple ECM functions. Within this family, TSP-1 and -2 constitute a subfamily with strong anti-angiogenic activity. Multiple binding partners have been identified for the TSPs including β1- and β3-integrins, integrin associated protein (IAP or CD47), and the CD36 scavenger receptor (Hiscott et al., 2006). Interactions between TSP-1 and CD36 are known to inhibit angiogenesis by initiating caspase-dependent EC apoptosis (Jimenez et al., 2000) (refer to Section 2.5.5.5) and there is evidence that CD47 ligation may elicit similar apoptotic events (Hiscott et al., 2006). Conversely, TSP-2 suppresses cell-cycle progression in ECs via a caspase-3 independent mechanism (Armstrong et al., 2002).

TSP-1 expression has been consistently reported in the corneal epithelial basement membrane, the corneal endothelium, and posterior Descemet's membrane, while TSP-2 expression is limited to the corneal endothelium (Hiscott et al., 1997; Uno et al., 2004) . Although previous studies postulated that TSP-1 and -2 may contribute to the maintenance of corneal avascularity by creating an anti-angiogenic ''barrier'' around the corneal stroma (Hiscott et al., 1997), Cursiefen and colleagues recently deduced that TSP-1 and -2 are not critical regulators of developmental corneal avascularity (2004a), but that TSP-1, and to a lesser extent TSP-2, are important inhibitors of inflammation- (Cursiefen et al., 2004a) and bFGF- (Volpert et al., 1995; Jimenez et al., 2001; Simantov et al., 2001) induced corneal NV.

PEDF: Initially identified as a neurotrophic factor, PEDF has since been categorized as one of the most powerful natural inhibitors of angiogenesis, more so than TSP-1, angiostatin, or endostatin (Dawson et al., 1999). PEDF shares sequence and structural homology with the serine protease inhibitor (Serpin) family but in itself does not inhibit proteases. In the eye, not only is PEDF abundantly present in the RPE and vitreous, but it is also highly expressed in the cornea, where it has been immunolocalized to the corneal epithelium and endothelium (Ogata et al., 2002). Moreover, PEDF is considered essential for maintaining the avascular states of the cornea and vitreous, while previous studies have demonstrated that recombinant PEDF inhibits bFGF-induced corneal NV (Dawson et al., 1999).

TIMPs: The TIMPs are a family of natural anti-angiogenic factors that control ECM breakdown by counteracting the proteolytic activity of MMPs, as well as other biological functions related to activation of pro-MMPs and regulation of apoptosis (Matthews et al., 2007). Thus far, four members of TIMPs have been characterized and designated TIMP-1, -2, -3 and -4. Expression of TIMP-1 and -3 is inducible; TIMP-2 expression is largely constitutive, while that of TIMP-4 is highly regulated and restricted to certain tissues. Of interest, all four TIMPs have been identified in the cornea (Ma et al., 2006), with TIMP-4 immunoreactivity being observed during the course of suture-induced corneal NV (Ma et al., 2003). Moreover, Matthews and co-workers recently proposed that TIMPs -1 and -3 may play an important role in corneal repair (Matthews et al., 2007).

Angiostatin and endostatin: Since the discoveries of angiostatin, a 38 kDa proteolytic fragment of plasminogen (O'Reilly et al., 1994), and endostatin, a 20 kDa carboxyl-terminal fragment of collagen type XVIII (O'Reilly et al., 1997), it has become apparent that several endogenous anti-angiogenic factors are derived from larger parent molecules (mainly ECM proteins) with no apparent anti-angiogenic activity. Angiostatin and endostatin, in particular, potently suppress tumor angiogenesis by inhibiting EC proliferation and migration and inducing EC apoptosis (Dhanabal et al., 1999; Eriksson et al., 2003). Moreover, their biological effects are mainly attributed to their antagonism of VEGF and bFGF signalling (Eriksson et al., 2003; Ma et al., 2006).

In the eye, implantation of angiostatin and angiostatin-like fragments inhibits corneal NV induced by bFGF (Chang et al., 2001) or inflammation (Ambati et al., 2002), and in the latter study, angiostatin was reported to cause regression of established blood vessels, a clinically important parameter. In this context, others have proposed that angiostatin-related proteins present in tear film may be important for preventing corneal NV in the hypoxic closed eye environment (Sack et al., 1999).

Collagen XVIII is an ECM protein best known by its degradation product endostatin, which is generated following cleavage by MMPs, elastase, and cathepsins (Ma et al., 2006). Importantly, Collagen XVIII/endostatin plays an essential role in ocular development and the maintenance of visual function (Marneros and Olsen, 2005) where it has been localized to the retina (inner limiting membrane and RPE) (Halfter et al., 1998) and cornea (Lin et al., 2001; Ma et al., 2006). Consistent with this, delivery of endostatin by implants or adeno-

associated viral vectors has been shown to inhibit corneal NV in murine models (Lai et al., 2007).

Table 2. Summary of angiogenic and anti-angiogenic factors detected in the cornea (adapted from Chang et al., 2001).

	Corneal micropocket assay	Corneal expression
Angiogenic factors		
Fibroblast growth factor	Yes	+
Vascular endothelial growth factor	Yes	+
Placenta growth factor	Yes	+
Transforming growth factor-α	Yes	+
Transforming growth factor-β	Yes	+
Insulin-like growth factor	Yes	NO
Leptin	Yes	ND
Integrins	Yes	+
Platelet-derived growth factors	Yes	+
Matrix metalloproteinases	ND	+
Angiogenin	Yes	+
Hepatocyte growth factor-scatter factor	ND	+
Tumor necrosis factor α	Yes	+
Connective tissuge growth factor	ND	+
Monocyte chemoattractant protein-1	Yes	+
Interleukin-8	ND	+
Anti-angiogenic factors		
Endostatin	Yes	+
Angiostatin	Yes	+
Prolactin	Yes	Receptor
MMPs	ND	+
TIMP	ND	+
Thrombospondin	Yes	+
Arresten	ND	α 1 IV collagen
Canstatin	ND	α 2 IV collagen
Tumstatin	ND	α 3 IV collagen
Pigment epithelium derived factor	Yes	+
Tumor necrosis factor α	Yes	+
Interleukin-4	ND	+
Interleukin-13	ND	+
VEGF Receptor-1	Yes	Yes
VEGF Receptor-3	ND	Yes

NO, no expression reported; ND, not determined

2.2.5 Immune privilege, angiogenic privilege, and corneal avascularity

Owing to its unique avascular features and the fact that corneal NV is incompatible with optimal vision, the cornea has been described as an immune privileged and angiogenically privileged tissue.

2.2.5.1 Immune privilege

Immune privilege within the eye is an evolutionary adaptation designed to protect the eye against sight-destroying inflammation. Corneal immune-privilege describes the phenomenon that foreign tissue grafts placed into the cornea experience prolonged survival, whereas similar grafts placed at conventional recipient sites are acutely rejected (Streilein, 2003). The existence of this feature is based on the fact that the visual axis is extremely delicate and intolerant of the distortion that often accompanies inflammation. Importantly, corneal structures such as the corneal endothelium, are incapable of replication and cannot regenerate after injury and loss due to inflammation (Streilein, 2003).

2.2.5.2 Angiogenic privilege

Broadly speaking, corneal angiogenic privilege describes the phenomenon that the cornea not only normally lacks both blood and lymphatic vessels, but also actively maintains this avascularity under inflammatory and other angiogenic stimuli. Recent advances have spearheaded the elucidation of mechanisms that are operative in maintaining corneal avascularity (Cursiefen et al., 2004a; Ambati et al., 2006; Cursiefen et al., 2006). While examining eyes from a wide range of

mammalian species, Ambati and colleagues concluded that corneal angiogenic privilege is not a redundant process, but is the consequence of a high concentration of sFlt-1 present in the cornea that is involved in 'entrapping' VEGF and thus inhibiting local vascularization (Ambati et al., 2006). In keeping with this, an intriguing study by Cursiefen et al. postulated that VEGFR-3 expression on the corneal epithelium may promote corneal avascularity and/or blood vessel regression by serving as a 'sink' for its pro-angiogenic ligands, VEGF-C/-D, specifically in the setting of inflammatory corneal NV. The same authors suggest that sFlt-1 may thus be more suitable for blocking hypoxia-driven NV (Cursiefen et al., 2006).

2.2.5.3 Mechanisms governing corneal avascularity

Several mechanisms may contribute to corneal avascularity (Azar, 2006):

- mechanical considerations of corneal anatomy including constant dehydration resulting in tightly packed collagen lamellae interspersed with compact keratocyte networks;
- the angiostatic nature of corneal epithelial cells (Cursiefen et al., 2006);
- barrier function of the limbal epithelium which prevents ingrowth of limbal vessels into the cornea;
- the immune privilege of the cornea;
- low levels (or even absence) of angiogenic factors under homeostatic conditions and during avascular corneal wound healing;
- active production of potent anti-angiogenic factors that counterbalance pro-angiogenic stimuli during homeostasis.

2.2.6 Corneal NV models

Features of corneal NV can be reproduced in several rodent and rabbit species. The basic premise of these models capitalizes on the avascular features of the cornea such that the vascular response following stimulation or suppression by angiogenesis inducing or inhibiting factors can be monitored and quantified by direct observation with a slit lamp or via labelling with fluorescent markers (Auerbach et al., 2003). Commonly used models are the corneal micropocket assay and various inflammation-induced corneal NV models.

The corneal micropocket assay uses standardized slow-release pellets prepared by mixing purified angiogenic growth factors such as bFGF or VEGF with sucralfate (a stabilizer) and Hydron (to allow slow release) in order to screen molecules for potential angiogenic activity (Auerbach et al., 2003; Rogers et al., 2007). This assay is unique because it gives a predictable, persistent, and aggressive neovascular response that is dependent on direct stimulation of blood vessels rather than on indirect stimulation by inflammatory stimuli (Kenyon et al., 1996). Nonetheless, due to its limited pathophysiological relevance, our studies were performed using an inflammatory corneal NV model.

Models of inflammatory corneal NV are characterized by a robust outgrowth of new blood vessels from the limbal arcade and have been demonstrated to be VEGF-dependent, eliciting macrophage recruitment (Joussen et al., 2003; Cursiefen et al., 2004b). Some of these models, such as the one used here, are characterized by corneal and limbal epithelial debridement via chemical (e.g. alkali burn) (Ormerod et al., 1990) and/or mechanical (e.g. corneal scraping,

cauterization) injury. This is proceeded by conjunctivalization, which simply refers to coverage of the corneal surface with conjunctival epithelium accompanied by chronic inflammation and NV (Amano et al., 1998; Lee et al., 1998; Revoltella et al., 2007). Alternative models include the laser-induced corneal NV model (Kvanta et al., 2000) or the suture model which evokes an inflammatory-neovascular response via intrastromal placement of sutures in the cornea (Cursiefen et al., 2004b). Altogether, the advantage of inflammatory models is that they are pathophysiologically relevant, mimicking clinical cases of excessive corneal NV induced by persistent inflammation seen in chemical or thermal burns, infections, trauma, or autoimmune diseases.

2.2.7 Treatment options

Corneal NV is currently managed using several modalities including medication, surgery, and laser therapy which are aimed at targeting actively proliferating and/or established blood vessels (Chang et al., 2001). Corticosteroids are the conventional medical treatment for suppressing actively growing vessels but are not always efficacious and may further be fraught with serious complications such as increased risk of infection, glaucoma, and cataracts (Dorrell et al., 2007a). Non steroidal anti-inflammatory drugs are also prescribed to inhibit synthesis of prostaglandins whose production is typically elevated during corneal wound healing and angiogenesis. Surgical and laser interventions such as argon laser, electro-coagulation, and photodynamic therapy are used to occlude established large vessels however, they may incite tissue destruction and scar formation leading to further functional loss. Another therapeutic alternative is

restoration of the ocular surface with the use of corneal, limbal, or amniotic membrane transplantation with the objectives of treating the stem cell deficiency and diminishing angiogenic stimuli (Chang et al., 2001).

Recently, several natural and synthetic anti-angiogenic agents have entered the clinic with many more in a late phase of preclinical development. Promising anti-angiogenic agents include: (a) naturally occurring inhibitors of angiogenesis, such as angiostatin and endostatin; (b) specific inhibitors of EC growth, such as TNP-470 and thalidomide; (c) agents that neutralize angiogenic peptides including anti-VEGF strategies e.g. Lucentis (Genentech, Inc.) and Macugen (Eyetech Pharmaceuticals) which have shown great promise in slowing and inhibiting new blood vessel growth (Hosseini and Nejabat, 2007).

2.3 Hypoxia

Adaptation of organisms to low oxygen tension, i.e. *hypoxia*, is a fundamental biological process that is required in both physiological and pathophysiological settings. Hypoxia results from a mismatch between oxygen supply and demand, and determines a series of metabolic and systemic adaptations that enable cells to acclimate and recover from this insult. Importantly, these events are accompanied by molecular adaptive responses that involve hypoxia-inducible factors (HIFs) which function as master regulators of oxygen homeostasis. Indeed, HIFs are necessary for the activation of a plethora of genes involved in diverse physiological responses including angiogenesis, glycolysis, and erythropoiesis (Wenger, 2000; Wenger, 2002). This section

briefly reviews the expansive literature on hypoxia and HIFs and attempts to place them in the context of basic ocular biology and pathophysiology.

2.3.1 HIF-1 and HIF family

One of the best characterized responses to hypoxia is the activation of HIF-1, a basic helix-loop-helix (bHLH) Per-Arnt-Sim (PAS) transcription factor discovered by Semenza and co-workers on the basis of its ability to bind to a specific consensus sequence, the hypoxia-response element (HRE), in the erythropoietin gene (Semenza et al., 1991; Semenza and Wang, 1992). HIF-1 is central to the cellular adaptation to low oxygen tension and it is known to control the expression of at least one hundred genes involved in oxygen homeostasis, glucose energy metabolism, and angiogenesis (Schumacker, 2005; Semenza, 2007). Active HIF-1 is a heterodimer composed of two subunits, HIF-1α and HIF-1β, also known as the arylhydrocarbon nuclear translocator (ARNT). HIF-1β is constitutively expressed, while HIF-1α expression is negligible under normoxia and induced under hypoxia.

In normoxia, HIF-1α is hydroxylated by prolyl-hydroxylase domain-containing proteins (PHD) -1, -2, and -3 and an asparaginyl-hydroxylase, FIH-1, that catalyze, respectively, the oxygen-dependent prolyl- and asparaginyl-hydroxylation within the oxygen-dependent degradation domain (Bruick and McKnight, 2001; Mahon et al., 2001; Lando et al., 2002). In addition, a novel HIF prolylhydroxylase, termed PH-4, has been cloned (Oehme et al., 2002). Post-translational modification of HIF-1α by PHDs induces binding to the Von Hippel-Lindau (VHL) protein, an E3-ubiquitin ligase, and targets HIF-1α for subsequent

proteasomal degradation. Conversely, under sustained hypoxia, the PHDs are inactive; HIF-1α degradation is inhibited leading to its accumulation, dimerization with HIF-1β, and subsequent translocation to the nucleus. There, it binds to HREs with the core sequence, 5'-(A/G)CGTG-3', in the promoters of target genes, and recruits coactivator proteins, P300 and CREB-binding protein, all of which leads to increased gene transcription (Semenza et al., 1996; Semenza, 2000; Semenza, 2001; Pagé et al., 2002; Treins et al., 2005).

To date, HIF-2α and HIF-3α have been identified and cloned as HIF-1α paralogues that can dimerize with HIF-1β and bind to HREs in the genes of hypoxia-responsive molecules. HIF-1α and HIF-2α are closely related (Hu et al., 2006) and bind to an overlapping but distinct set of target genes, whereas HIF-3α is the more distantly related isoform and appears to function as an inhibitor of HIF-1α (Makino et al., 2007). Establishing the specific roles of the HIF paralogues in oxygen homeostasis is a major challenge of current research (Semenza, 2007), nonetheless, our primary interest was in the well-characterized HIF-1.

2.3.2 HIFs in ocular disease

While hypoxia is a prominent occurrence during normal development, it also arises as a consequence of numerous ocular disorders accompanied by vascular insufficiency. In such conditions, HIF-1 initiates angiogenesis and expression of key angiogenic factors (e.g. VEGF) that are aimed at re-establishing adequate re-oxygenation.

In the eye, the corneal environment is known to generate conditions of hypoxia following eye closure during sleep (Sack et al., 1999) and notably also following contact lens wear and consequent corneal NV. Interestingly, Makino et al. recently documented high expression of inhibitory PAS (IPAS) in the corneal epithelium that correlated with low level hypoxia inducible VEGF expression and an avascular phenotype in the cornea (Makino et al., 2001).

There are also specific retinal diseases in which hypoxia either contributes to or plays a critical role in promoting neovascularization (Arjamaa and Nikinmaa, 2006). Retinal neovascularization, typically observed in proliferative diabetic retinopathy, retinal vein occlusion or retinopathy of prematurity, is the consequence of an abnormal vascular response to retinal ischemia or hypoxia and one of its major consequences is up-regulation of VEGF. During retinal angiogenesis vascular endothelial cells proliferate through the internal limiting membrane into the vitreous, where they may incite vitreous haemorrhage or retinal detachment (Kvanta, 2006). In diabetic retinopathy, HIF-1α-mediated induction of VEGF results in remodelling of the retinal vasculature with resultant blood retinal barrier breakdown, angiogenesis, or vascular occlusion. Similarly, transgenic mice in which the HRE is deleted from the *Vegf* promoter fail to develop hypoxia-induced retinal angiogenesis following subjection to oxygen-induced retinopathy (Vinores et al., 2006).

2.3.3 Regulation of HIF-1 by reactive oxygen species

The generation of reactive oxygen species (ROS) during hypoxia and their role during oxygen sensing has been a subject of long-lasting debate. Inasmuch as

PHDs are well recognized oxygen sensors, an alternative mitochondrial oxygen sensing model has been proposed by Chandel and colleagues whereby hypoxia increases the generation of ROS from complex III of the mitochondrial electron transport chain and this signal acts to stabilize HIF-1α through inhibition of PHDs (Chandel et al., 1998; Chandel et al., 2000; Chandel and Budinger, 2007); plausible mechanisms by which mitochondrial ROS (mROS) regulate PHD activity have been reviewed (Cash et al., 2007). The controversy regarding this model is attributed to the fact that previous studies relied on pharmacologic tools with the appearance of conflicting reports (Srinivas et al., 2001; Vaux et al., 2001) in addition to technical concerns regarding the measurement of ROS production in living cells. However, several recent reports using genetic and biochemical approaches have provided more conclusive evidence that the hypoxia-induced production of mROS is both necessary and sufficient for hypoxia-dependent HIF-1α accumulation, indicating a crucial role for mROS in oxygen sensing and HIF-1α regulation (Brunelle et al., 2005; Guzy et al., 2005; Guzy and Schumacker, 2006; Lin et al., 2008). Importantly, under anoxia (0% O_2), the mitochondrial oxygen sensor cannot function due to its inability to generate ROS, hence mROS-dependent HIF-1α induction is thought to be solely observed under hypoxic (1.5-5% O_2) conditions (Brunelle et al., 2005; Wenger, 2006).

2.4 ROS, free radicals, and oxidative stress

The eye, with its intense exposure to light and its optical demands requiring exact tissue organization, is highly susceptible to oxidant stress. This is potentiated by the fact that ocular tissue typically regenerates slowly thus

increasing the likelihood of cumulative oxidant-inflicted damage, a chief etiological factor in the pathogenesis of diseases such as cataracts, uveitis, ischemic retinopathies, corneal inflammation, and AMD (Behndig et al., 1998; Imamura et al., 2006). Herein we address the involvement of ROS, free radicals, and oxidative stress in corneal and retinal pathophysiology.

2.4.1 Definition of terms

ROS are highly reactive oxygen-derived small molecules that are continuously generated in all aerobic organisms as products of normal cellular metabolism. The term encompasses several types of reactive oxygen metabolites, including free radicals, which are defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals. This unpaired electron(s) usually confers a considerable degree of reactivity to the free radical. Prominent examples of ROS include superoxide ($O_2^{\bullet}$), hydroxyl ($^{\bullet}OH$), peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO_2^{\bullet}$), and singlet oxygen ($^1O_2$). The term ROS also comprises certain nonradicals that are either oxidizing agents and/or are easily converted into radicals, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), aldehydes, and ozone (O_3). ROS can also include nitrogen-containing oxidants, such as nitric oxide itself ($NO_2^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), dinitrogen trioxide ($N_2O_3^{\bullet}$) and the powerful oxidant peroxynitrite ($OONO^-$), although these are appropriately referred to as reactive nitrogen species (RNS) (Karihtala and Soini, 2007; Valko et al., 2007) and will not be addressed hereafter.

It is well established that ROS play a dual role as both deleterious and beneficial species. Regulation of this delicate balance is a very important aspect of living systems and is achieved by mechanisms termed redox regulation (Valko et al., 2007). Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in host defence against infectious agents and as mediators in cellular signalling pathways. The detrimental effect of ROS resulting in potential biological damage to cellular lipids, proteins or nucleic acid is called oxidative stress, a process that occurs when an overproduction of ROS disrupts the inherent prooxidant/antioxidant equilibrium status (Karihtala and Soini, 2007).

2.4.2 ROS relevant to ocular tissue

2.4.2.1 Superoxide ($O_2^{\bullet-}$)

The superoxide anion is the oxygen radical formed in the greatest quantity during molecular oxygen metabolism (reaction 1, Table 3). By itself, it is not very reactive, but in the presence of transition metals such as iron, superoxide can react directly with hydrogen peroxide to produce the highly reactive hydroxyl radical, which is in turn capable of reacting with almost every type of cell molecule; under these conditions superoxide can also react with lipid peroxides to yield alkoxyl radicals. The production of superoxide occurs mostly within the mitochondria of a cell and, to a lesser degree, via certain enzymes of phagocytic cells, namely NADPH oxidase. Superoxide is produced from both Complexes I and III of the electron transport chain (ETC), and once in its anionic form it poorly penetrates lipid membranes and therefore remains predominantly in mitochondria.

2.4.2.2 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide may be formed either spontaneously from molecular oxygen in peroxisomes or, more commonly, from superoxide by catalytic activity of superoxide dismutases (SOD), the latter being approximately four times more efficient (reaction 2, Table 3). Despite its lesser reactivity (compared to other ROS), hydrogen peroxide is capable of readily diffusing throughout mitochondria and across cell membranes, thereby initiating many forms of cellular injury. Hydrogen peroxide is eventually metabolized to O_2 and H_2O by catalase, glutathione peroxidases or peroxiredoxins.

2.4.2.3 Hydroxyl radical (·OH)

The principal injurious effects of ROS in mammalian cells are mediated by $^{\bullet}OH$, a highly reactive and hence very dangerous radical with a very short half-life of about 10 seconds. Moreover, due to its high reactivity, the hydroxyl radical interacts at the site of its production with the molecules in its immediate surroundings. The majority of the hydroxyl radical is produced in the presence of reduced transition metals (ions of iron [Fe], copper [Cu], cobalt [Co], or nickel [Ni]) mainly via the Fenton reaction when Fe^{2+} reacts with H_2O_2 (reaction 3, Table 3); this can form strong oxidizing agents capable of propagating lipid peroxidation. Inasmuch as Fe and Cu ions are usually tightly bound to the surface of proteins, under stress conditions, excess superoxide releases "free iron" from iron-containing molecules and facilitates hydroxyl radical production by making Fe^{2+} available for the Fenton reaction. Additionally, the hydroxyl radical can also

be generated via the Haber–Weiss reaction in which superoxide is a substrate (reaction 4, Table 3).

2.4.3 Sources of ROS

The mammalian organism is exposed to a large variety of ROS from both endogenous and exogenous sources. Inasmuch as exposure to exogenous ROS sources is extremely high, endogenous sources are more pertinent and extensive due to their continuous production during the life span of the organism. However, irrespective of their origin, because most radicals are short-lived species, they interact quickly with other molecules/biomolecules leading to modification and potentially serious consequences for the cell (Cooke et al., 2003).

Table 3. Formation of biologically important reactive oxygen/nitrogen species (adapted from Karihtala and Soini, 2007).

Reaction	Description
1. $O_2 + e^- \rightarrow O_2^{\cdot -}$	Superoxide formation (ETC, NOX, XO)
2. $2\ O_2^{\cdot -} + 2\ H^+ \rightarrow H_2O_2 + O_2$	Hydrogen peroxide formation, catalyzed by SODs
3. $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$	Fenton reaction
4. $O_2^{\cdot -} + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$	Haber-Weiss reaction (iron-catalyzed)
5. L-arginine $\rightarrow$ $NO^{\cdot}$ + L-citrulline	$NO^{\cdot}$ formation (catalyzed by NOS)
6. $NO^{\cdot} + O_2^{\cdot -} \rightarrow ONOO^-$	Peroxynitrite formation

2.4.3.1 Exogenous sources of ROS

Exogenous sources of ROS exist and include ionizing and UV irradiation. A large variety of xenobiotics (e.g. toxins, pesticides, and herbicides), chemicals

(e.g. mustard gas, alcohol), and food also produce ROS as by-products of their metabolism in vivo (Kohen and Nyska, 2002).

2.4.3.2 Endogenous sources of ROS

- **Mitochondria.** Given that aerobic organisms possess an ETC, they are able to attain far greater energy production efficiency than their anaerobic counterparts. Nevertheless, during oxidative phosphorylation, a small number of electrons "leak" to oxygen prematurely forming superoxide (Valko et al., 2007). Nearly 4% of molecular oxygen consumed by the ETC is converted to superoxide (Melov et al., 1999).

- **Leukocytes.** Neutrophils, eosinophils, and monocytes are major producers of endogenous ROS (Karihtala and Soini, 2007). When exposed to a stimulus, these cells are able to recognize and destroy the foreign pathogen by undergoing a series of reactions called the respiratory burst. The respiratory burst is characterized by a twenty-fold increase in oxygen consumption accompanied by an increase in glucose utilization and NADPH formation. NADPH serves as an electron donor to an activated enzymatic complex in the plasma membrane called the NADPH oxidase, which utilizes electrons to produce the superoxide anion from molecular oxygen (Kohen and Nyska, 2002).

- **Enzymes.** NADPH oxidase, xanthine oxidase, and nitric oxide synthase are also large sources of ROS. The NADPH oxidase enzyme complex is best characterized in neutrophils, although RPE cells have been postulated to express NADPH oxidase which may account for the the phagocytic removal of POS activated by the respiratory burst (Miceli et al., 1994). The nonphagocytic

NADPH oxidases produce superoxide at a fraction (1–10%) of the levels produced in neutrophils and are thought to function in intracellular signalling pathways (Bedard and Krause, 2007; Valko et al., 2007) .

2.4.4 Lipid peroxidation

Lipid peroxidation is causally implicated in a wide variety of disorders. Long chain polyunsaturated fatty acids (PUFAs) such as arachidonic acid (C20:4n-6) and DHA (C22:6n-3) are essential components in higher eukaryotes that not only confer fluidity, flexibility and selective permeability to cellular membranes, but also render them especially vulnerable to oxidative damage. Lipid peroxidation of PUFAs can proceed by enzymatic and non-enzymatic mechanisms. Enzymatically derived lipid peroxidation is mediated by a family of enzymes called lipoxygenases that oxygenate free and esterified PUFAs to the corresponding hydroperoxy derivatives. In contrast, non-enzymatic processes are free radical driven chain reactions in which one radical induces the oxidation of a large number of lipid molecules (Catalá, 2006).

Lipid peroxidation has two major outcomes: (1) the presence of the hydroperoxy group disturbs hydrophobic lipid/lipid and lipid/protein interactions, which leads to structural alterations of biomembranes and lipoproteins; (2) hydroperoxy lipids are sources for the formation of free radicals, which may induce secondary modification of other membrane and/or lipoprotein constituents. Oxidation of the lipid membrane bilayer can further result in loss of its barrier function and thus put the integrity of subcellular organelles or of the entire cell in danger (Kühn and Borchert, 2002).

Lipid peroxidation results in liberation of toxic metabolites such as acrolein, malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), isoprostanes (IsoPs), and isofurans (IsoFs), which are also useful as in vivo biomarkers of lipid peroxidation (Catalá, 2006).

2.4.5 Antioxidant defense systems

Biological systems possess effective defence mechanisms against free radical-induced oxidative stress. Such defence mechanisms may be preventative, reparative, physical, or antioxidant in nature. They are generally categorized as enzymatic or non-enzymatic.

2.4.5.1 Enzymatic antioxidants

Major enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase:

- **SODs.** First characterized antioxidant enzymes and are capable of dismutating two superoxide anions to hydrogen peroxide and molecular oxygen (reaction 2, Table 1). Three isoenzymes of SOD are expressed in humans: (1) copper-zinc SOD (CuZnSOD or SOD1) occurs in cytosol, the intermembrane space of mitochondria, and in the nucleus; (2) mitochondrial manganese SOD (MnSOD or SOD2) is localized to the mitochondrial matrix; and (3) extracellular SOD (ECSOD or SOD3) is secreted to the extracellular space, with a lesser proportion present in plasma and other extracellular fluids. MnSOD is the main enzyme responsible for superoxide degradation. However, if incompletely destroyed in mitochondria, superoxide is able to severely cripple mitochondrial

function by inactivating the [4Fe–4S] centers in the ETC, resulting in cumulative damage and ultimately inducing apoptosis. SODs have important cytoprotective roles in ocular tissue. Indeed, MnSOD prevents retinal thinning (Sandbach et al., 2001; Kasahara et al., 2005) and protects against oxidation-induced apoptosis in the RPE (Kasahara et al., 2005), whereas both MnSOD and CuZnSOD, whose amount and activity is the highest in the human retina, play critical roles in preserving the RPE against AMD (Imamura et al., 2006; Justilien et al., 2007). On the other hand, levels of CuZnSOD and ECSOD are approximately equally abundant in the cornea (Behndig et al., 1998).

- **GPx.** A family of enzymes capable of reducing hydroperoxides to the corresponding hydroxy compounds, using glutathione (GSH) as substrate as indicated: $H_2O_2 + 2\ GSH \rightarrow GSSG + 2\ H_2O$. The oxidized form of glutathione disulfide (GSSG) is again reduced by the specific enzyme glutathione reductase.
- **Catalase.** A tetrameric heme enzyme responsible for detoxification of various phenols, alcohols and hydrogen peroxide as illustrated in the reaction: $2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$. Of all ocular tissues, catalase activity is the highest in the RPE where it appears to suppress the generation of lipid peroxides (Liles et al., 1991; Miceli et al., 1994).

2.4.5.2 Non enzymatic antioxidants

Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, and flavonoids among others. In the eye, ascorbic acid is regarded as a major substrate involved in ocular

protection due to its high concentration in the cornea and vitreous humor (Izzotti et al., 2006). Interestingly, vitamin C is also a potent inhibitor of corneal NV (Peyman et al., 2007).

2.4.6 Oxidative stress and antioxidant systems in the cornea

The primary sources of environmental and oxidative stress for the cornea are solar radiation, specifically in the UV range, as well as continual exposure to molecular oxygen. With respect to corneal morphology, UV exposure can cause a reduction in epithelial cell proliferation, alterations in the epithelial layer thickness, and loss in metabolic capacity (Estey et al., 2007). Fortunately, the healthy cornea is endowed with a unique repertoire of defense mechanisms aimed at minimizing and counteracting such stresses. One such component is aldehyde dehydrogenase (ALDH3) which plays a critical and multifunctional role in the protection of the cornea, and perhaps the entire eye, against UV induced oxidative stress. In fact, ALDH3 is one of the most abundantly expressed proteins in the corneal epithelium, accounting for 20 to 40% of its soluble protein content, and functions to directly absorb UV and remove cytotoxic aldehydes produced by UV-induced lipid peroxidation (Behndig et al., 2001; Estey et al., 2007). Additionally, the cornea is rich in SOD (Behndig et al., 1998; Behndig et al., 2001), catalase, glutathione peroxidase, and glutathione reductase, all of which function to absorb UV and scavenge both ROS and the generated aldehydes.

2.4.7 Oxidative stress and antioxidant systems in the retina

Photoreceptors and RPE cells are constantly subjected to a highly oxidative environment owing to their exposure to intense illumination from focal light. The close proximity between the RPE and choroid further exposes RPE cells to high oxygen tension. As previously noted, RPE cells are uniquely involved in the phagocytosis and digestion of shed photoreceptor outer segments which incidentally possess the highest PUFA content of any human tissue. This provides an additional oxidative burden because RPE phagocytosis in itself is an ROS generating process, while lipid peroxidation of PUFAs may induce RPE damage (Liang and Godley, 2003). Because photoreceptors and the RPE have a close structural and functional association, damage to one cell type may compromise the viability of the other. Nonetheless, the RPE has developed specific defense mechanisms to counteract oxidative damage ensued during normal physiological function or under pathological settings (Cai et al., 2000; Liang and Godley, 2003):

- *Absorption and filtering of light.* The RPE contains a complex composition of various pigments that are specialized to different wavelengths. Most light absorption occurs via melanin, with additional absorption supplemented by the photoreceptors. Photoreceptors also contain carotenoids such as lutein and zeaxanthin that are analogous to biological sunglasses in their ability to absorb blue light which is most damaging to the RPE.

- *Antioxidants.* The RPE contains high amounts of antioxidants such as glutathione, SOD and catalase and accumulates non enzymatic antioxidants including carotenoids, vitamin C, vitamin E, and β-carotene. Being a powerful

electron acceptor, melanin behaves as an antioxidant, diminishing cytotoxic lipid peroxidation, and storing and releasing zinc which is necessary for activation of SOD.

2.5 CD36

CD36, the prototype member of the class B family of scavenger receptors (SRs), is a multiligand glycoprotein involved in a diverse array of biological and pathological processes that include angiogenesis, apoptotic cell uptake, phagocytosis, innate immune response, atherogenesis, and diabetes (Febbraio et al., 2001). CD36 is recognized by many aliases, namely platelet glycoprotein, GPIV, glycoprotein IIIb, GPIIIB, leukocyte differentiation antigen CD36, SRBI, PAS IV, PAS-4 protein, platelet collagen receptor, fatty acid translocase (FAT), and thrombospondin (TSP) receptor (Rać et al., 2007). Here we will focus on CD36 as it relates to ocular physiology including discussion on ligands and functions that are pertinent to this objective.

2.5.1 The scavenger receptor family

As previously mentioned, CD36 belongs to a family of receptors called SRs. SRs were historically classified due to their capacity to recognize and mediate binding of modified lipoproteins such as acetylated low-density lipoprotein (acLDL) and oxidized LDL (oxLDL) (Goldstein et al., 1979; Murphy et al., 2005). Today the SR family has expanded to include eight subclasses (A–H) of receptors that are structurally distinct yet share the defining feature of being able to bind modified forms of LDL (Figure 8). The SRs are aptly named having been

subsequently found to bind and "scavenge" a broad array of other structurally related or even structurally and functionally dissimilar ligands including apoptotic cells, anionic phospholipids, and amyloid and pathogen components (Hanahan and Folkman, 1996; Husemann et al., 2002; Murphy et al., 2005; Adachi and Tsujimoto, 2006). Evolutionarily speaking, SRs are presumed to have evolved with the innate immune system as "pattern recognition receptors" that recognized and eliminated apoptotic cells and foreign pathogens during embryonic development and infection (Febbraio et al., 2001). Thereafter, SRs may have acquired other biologic functions through evolution, perhaps related to their ability to recognize common structural patterns that are expressed on pathogens and apoptotic cells (Silverstein and Febbraio, 2000).

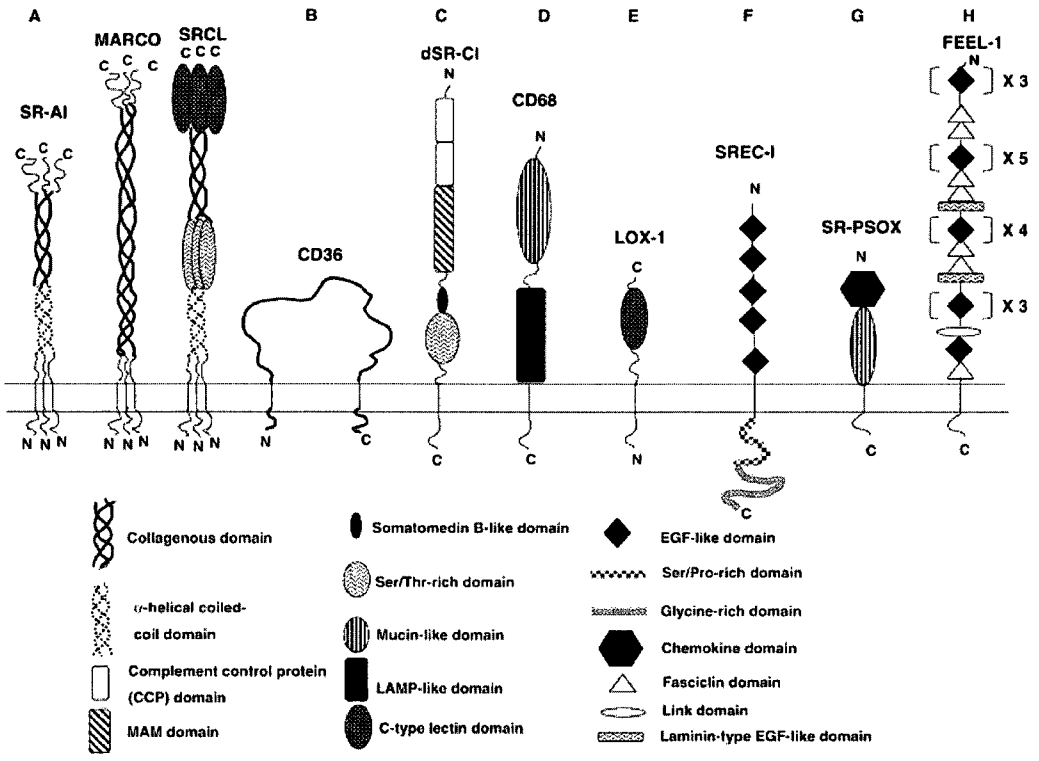

Figure 8. Schematic view of different classes of eukaryote scavenger receptors. The eight different classes are denoted A–H and specific domains are highlighted by the codes indicated within the figure (from Murphy et al., 2005).

2.5.2 Ligands and expression

Initially identified as the platelet integral membrane receptor for the matrix glycoprotein TSP-1 (Okumura and Jamieson, 1976), CD36 has since been shown to bind a remarkably diverse collection of ligands including modified LDL (oxLDL, acLDL), *Plasmodium falciparum*-parasitized erythrocytes, collagen I and IV, anionic phospholipids, long chain fatty acids (LCFAs), and apoptotic cells (Febbraio et al., 2001).

Apart from broad ligand specificity, CD36 possesses the additional characteristic of being expressed by an extensive array of cells and tissues

including microvascular (but not large vascular) ECs, dendritic cells, adipocytes, skeletal and cardiac muscle (including mitochondrial membranes), epithelia of the retina, breast, and intestine, smooth muscle cells, and hematopoietic cells, including erythroid precursors, platelets, monocytes/macrophages, and megakaryocytes. Mutations resulting in absence of CD36 expression in platelets (Type I CD36 deficiency) or in all cells (Type II CD36 deficiency) have been characterized, and are predominantly found in Asian and African populations (Febbraio et al., 2001).

2.5.3 Alternative exons and promoter usage

The CD36 gene is localized to human chromosome 7, rat chromosome 4, and mouse chromosome 5 (Fernández-Ruiz et al., 1993; Aitman et al., 1999; Blake et al., 2000) and is encoded by 15 exons. Exons 1, 2, and 15 are noncoding, whereas exons 3 and 14 encode N-terminal and C-terminal domains, respectively (Armesilla and Vega, 1994). A marked feature of the CD36 gene is the presence of several alternative first exons (exons 1a, 1b, 1c, 1e, 1f) and their promoters, resulting in tissue-specific regulation of CD36 expression. For example, Zingg and colleagues reported on unique expression of exon 1b in atherosclerotic plaques and suggested that CD36 promoter and exon switching may trigger the pathological activation of cells at the atherosclerotic lesion (Zingg et al., 2002). Suffice it to say, the molecular mechanisms regulating the CD36 gene are unusually complex, hence reflecting on the multifunctional aspects of this receptor in diverse tissues and conditions (Andersen et al., 2006).

2.5.4 Protein structure

The primary structure of CD36 is highly conserved across mammalian species and homologs have been described in *Drosophila and C. elegans*. The CD36 nucleotide sequence predicts a transmembrane protein of 471 amino acids with a characteristic "hairpin" topology (Murphy et al., 2005) featuring two short cytoplasmic fragments representing the amino and carboxy termini two transmembrane domains, and a large extracellular loop comprising 7 glycosylation sites and 3 disulfide bridges. The protein is extensively modified post-translationally. Palmitoylation of each of the four cysteines of the intracellular termini supports the hairpin topology model (Tao et al., 1996). The disulfide bridging appears to be necessary for proper folding and trafficking of CD36 to the cell surface (Tao et al., 1996; Gruarin et al., 2000). Heavy glycosylation explains the discrepancy between the protein (53 kDa) and apparent (78-88 kDa) molecular weights of the receptor (Oquendo et al., 1989; Rać et al., 2007), a modification that may protect it from degradation in proteinase-rich environments such as the lysosome and in areas of inflammation or tissue damage (Febbraio et al., 2001). A diagram of the CD36 structure is depicted in Figure 9.

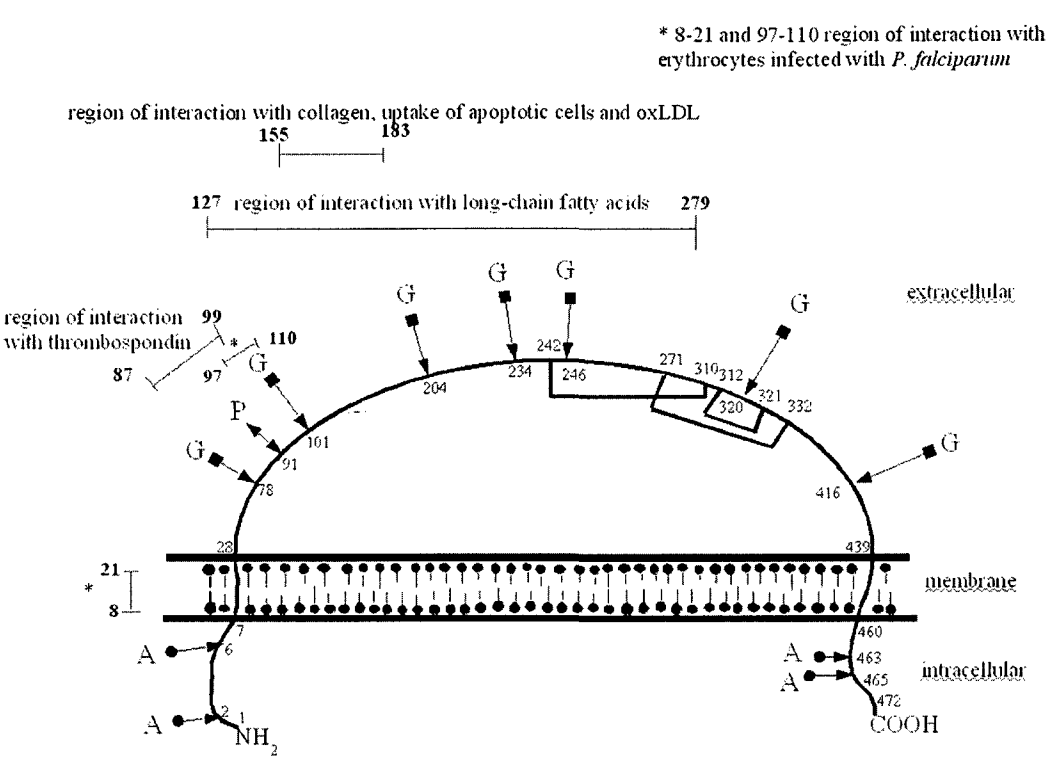

Figure legends: A – site of acylation (palmitoylation), G – site of glycosylation, P – site of phosphorylation

disulfide bonds

Distinguished: two cytoplasmic, two transmembrane fragments and one extracellular fragment.

Figure 9. The major structural features of CD36 (from Rać et al., 2007).

2.5.5 CD36 function

CD36 has been shown to participate in multiple homeostatic and pathological processes. This functional diversity is attributable to the multiple and distinct binding sites present on CD36. Among these functions are uptake of oxLDL, clearance of apoptotic cells, phagocytosis of photoreceptor outer segments (POSs), transport of long chain fatty acids (LCFAs), inhibition of angiogenesis, platelet aggregation, and vascular sequestration of *Plasmodium falciparum*-infected erythrocytes. However we will solely address CD36 functions as they pertain to ocular biology and pathology.

2.5.5.1 Uptake of oxLDL: CD36 in atherogenesis and possible link to age-related macular degeneration

The role of CD36 as the principal SR responsible for macrophage recognition and internalization of oxLDL and its relation to foam cell formation and the pathogenesis of atherosclerosis has been solidly established (Febbraio et al., 2001). Studies have reported that incubation of oxLDL with monocytes/macrophages derived from CD36 deficient individuals results in 40–60% less oxLDL binding, internalization, and cholesterol accumulation compared to CD36-expressing cells. Moreover, macrophages isolated from CD36 null mice are profoundly defective in uptake of oxLDL and foam cell formation (Febbraio et al., 2001). Much interest has hinged on the components of oxLDL that are recognized by and bind to CD36. CD36 has been shown to bind to the oxidized phospholipid (oxPL) component on the outer surface of the LDL particle and more specifically the phosphatidylcholine (PC) headgroup of oxPL (Boullier et al., 2005).

Epidemiological and histopathological studies have revealed an interesting correlation between atherosclerosis and AMD (Vingerling et al., 1995; Mullins et al., 2000; Gordiyenko et al., 2004). Others have hypothesized that the pathogenic mechanisms of AMD share similarities with those of atherosclerosis (Mullins et al., 2000) in which macrophages (expressing SRs) accumulate to ingest oxLDL in the eyes of AMD patients (Kamei et al., 2007). Additionally, it is generally accepted that the clinical symptoms of AMD are precipitated by gradual loss of RPE function with consequent photoreceptor death and/or choroidal

neovascularization. In light of this, Gordiyenko et al. postulated that age-related accumulation of cholesterol and resultant oxLDL may enter the retina via the RPE where they could directly impair RPE function if internalized by surface receptors such as CD36 or other SRs (Gordiyenko et al., 2004)

2.5.5.2 Clearance of apoptotic cells

CD36 plays a prominent role in the clearance of apoptotic cells arising during development, normal homeostasis, and infection, a function that is essential for tissue remodelling and resolution of inflammation. Apoptotic cell engulfment and removal by macrophages is said to be facilitated by phosphatidylserine (PS) displayed at the outer leaflet of the plasma membrane, while CD36 has been shown to facilitate PS recognition on apoptotic cells (Fadok et al., 1998; Savill et al., 2002). In keeping with this, Greenberg et al. recently demonstrated that virtually all CD36-mediated macrophage recognition of apoptotic cells is dependent on oxidative modifications that occur in the PS species (Greenberg et al., 2006), which is in fact analogous to CD36 recognition of PC species in oxidatively modified lipoprotein. As such, the authors postulated that CD36–oxidized PS interactions may prove indispensable for apoptotic cell clearance at sites where lipid peroxidation is enhanced (Greenberg et al., 2006), such as in hypoxic/ischemic or inflammatory settings.

An interesting premise that emerges from these observations is that apoptotic cells and oxLDL possess structural similarities that permit their binding and engulfment by CD36. Indeed, CD36 and other scavenger receptors are thought to recognize ligands in the apoptotic cell membrane that mimic oxLDL structure

(Savill et al., 2002). This supports the view that oxidized PS/PC species represent pattern recognition ligands for CD36 that facilitate its scavenging of apoptotic cells, aberrant lipoprotein, or pathogenic organisms which is consistent with its evolutionarily conserved role in the innate immune system (Boullier et al., 2005).

2.5.5.3 Phagocytosis of shed photoreceptor outer segments

An interesting twist on apoptotic cell clearance is RPE phagocytosis of photoreceptor outer segments (POSs), especially because RPE cells are similar to macrophages in their ability to phagocytose as well as in their expression of surface receptors including CD36 (Ryeom et al., 1996b). POS membranes are known to be abundant in PUFAs, including DHA and anionic phospholipids, the latter being demonstrated ligands of CD36 (Febbraio et al., 2001). Previous work elucidated that αvβ5 integrin is the primary binding receptor for RPE-mediated POS phagocytosis, while CD36 functions primarily as a signalling molecule to activate the POS internalization mechanism (Ryeom et al., 1996a; Finnemann and Silverstein, 2001). More recently, Sun et al indicated that CD36 signalling is not indispensable for OS clearance under basal conditions. However, under conditions of heightened oxidative stress and consequent generation of CD36 ligands, CD36 may prove physiologically important for RPE turnover of light or oxidatively damaged POSs (Sun et al., 2006). In this context, the DHA of 2-lyso-phosphatidylcholine is photooxidized to produce specific PC molecular species possessing a CD36 recognition signal for RPE phagocytosis (Sun et al., 2006; Febbraio and Silverstein, 2007). Taken together, defects in this pathway may

correlate with the increased risk of retinal degenerative diseases, including AMD, that develop under conditions of increased oxidative stress.

2.5.5.4 Fatty acid transport

Cumulative data generated from rodent models of altered CD36 expression have endowed CD36 with the rapidly expanding role of a fatty acid translocase (FAT). This is consistent with knowledge that CD36/FAT is highly expressed at the plasma membrane, as well as in intracellular pools of tissues active in FA metabolism, such as heart, skeletal muscle, and adipose tissue (Campbell et al., 2004). Indeed, numerous studies have documented that CD36 facilitates FA uptake by these tissues and that its deficiency is associated with 60 to 80% defect in FA uptake (Ibrahimi et al., 1999; Brinkmann et al., 2002; Ibrahimi and Abumrad, 2002). Another novel observation is that CD36/FAT exists at the mitochondrial membrane where it participates in the transport of FA into the mitochondria for oxidation in skeletal muscle (Campbell et al., 2004; Bezaire et al., 2006). In humans, polymorphisms in the CD36 gene may underlie defective FA metabolism and some forms of heart disease (Ibrahimi and Abumrad, 2002). To date, the significance of FA transport in the visual system has not been characterized.

2.5.5.5 Inhibition of angiogenesis

Critical studies conducted by Bouck, Silverstein, and colleagues have greatly facilitated our current understanding of the role of CD36 in angiogenesis (Dawson et al., 1997; Simantov et al., 2001). Identification of CD36 as the receptor

mediating the potent anti-angiogenic effects of TSP-1 (Jimenez et al., 2001) has underscored the importance of the CD36/TSP-1 interaction in angiogenesis and vascular biology (Dawson et al., 1997). This concept is further corroborated by evidence that TSP-1 fails to inhibit bFGF-induced corneal angiogenesis in CD36 null mice (Jimenez et al., 2001) and thus is of relevance to the current thesis given that numerous ocular diseases are associated with aberrant neovascularization.

On endothelium, CD36 expression is limited to microvessels, the vessels from which new vessels arise during angiogenesis. Various investigations have elucidated that engagement of CD36 by TSP-1 promotes microvascular EC apoptosis via activation of caspase-3 and Jun N-terminal kinase (JNK) (Jimenez et al., 2001), and down-regulation of VEGFR-2 and VEGF-A induced phosphorylation of MAPK (Primo et al., 2005). However, the interpretation of these effects are complicated by the fact that an abundant plasma protein called histidine-rich glycoprotein interacts with TSP-1 at the same site as CD36 (CSVTCG peptide sequence) and antagonizes binding of TSP-1 to CD36 (Simantov et al., 2001).

There is a prevailing appreciation that the anti-angiogenic effects of CD36 are also induced by oxLDL present in hyperlipidemic states as observed during atherosclerosis and diabetes (Dawson et al., 1997; Febbraio and Silverstein, 2007). However, it should be clarified that the oxLDL and TSP-1 binding domains are separate and independent (Pearce et al., 1995; Pearce et al., 1998).

2.5.6 Intracellular trafficking and signalling

Despite its short cytoplasmic tails, it has become apparent that CD36 is a bona fide signaling molecule that exists in lipid rafts (Febbraio and Silverstein, 2007). CD36 ligation to oxLDL triggers internalization of the receptor-ligand complex via a lipid raft-dependent but clathrin- and caveolin-independent pathway (Zeng et al., 2003). Consistent with this, an elegant study by Malaud et al. used site-directed mutagenesis to demonstrate that a six amino acid motif within the C-terminus is critical for binding, internalization, and degradation of oxLDL (Malaud et al., 2002). While CD36 lacks intrinsic kinase or phosphatase activity, it is known to associate with the Src family protein tyrosine kinases Fyn, Lyn, and Yes (Bull et al., 1994). In this context, Fyn has been reported to participate in CD36-dependent anti-angiogenesis promoted by TSP-1 (Jimenez et al., 2000), while Primo et al. identified residues within the cytoplasmic tail that are required for TSP-1's inhibitory activity (Primo et al., 2005). CD36 residence in lipid rafts has also been hypothesized to facilitate FA uptake where it has been implicated in playing a central role in cellular lipid homeostasis and linked signalling pathways (Febbraio and Silverstein, 2007).

2.5.7 Regulation of CD36 expression

CD36 is regulated at different levels, including gene expression, mRNA stability and protein expression in a cell- and tissue-specific manner, which is in agreement with its wide cellular distribution and the multiple functions ascribed to it. To date, the regulation of CD36 expression has been largely characterized in human monocytes/macrophages and platelets (Zingg et al., 2002).

2.5.7.1 Role of PPARs, growth factors, and cytokines

Critical regulators of CD36 expression are the nuclear transcription factors peroxisome proliferator-activated receptors (PPARs), which modulate CD36 expression in a tissue-specific manner. In macrophages, CD36 expression is upregulated by oxLDL which stimulates transcription of the CD36 gene by activating PPAR-γ and subsequent binding to a regulatory element in the CD36 promoter. In keeping with this, Evans and colleagues postulated the concept of the "proatherogenic feed-forward loop" whereby activation of PPAR-γ by oxLDL initiates a perpetuating positive feedback loop of CD36 expression, uptake of oxLDL and foam cell formation (Tontonoz et al., 1998; Febbraio et al., 2001).

Different growth factors and cytokines are also known to modulate CD36 expression by macrophages. For example, transforming growth factor (TGF)-β1/2 inhibit CD36 expression by a mechanism involving phosphorylation of MAP kinase and subsequent MAP kinase phosphorylation of PPAR-γ and decreased CD36 gene transcription (Han et al., 2000). Similarly, interleukin-4 and 13 stimulate expression of CD36 by induction of PPAR-γ (Yesner et al., 1996; Berry et al., 2007), while TGF-α represses it (Boyer et al., 2007).

2.5.7.2 Role of fatty acids

Consistent with its role in LCFA transport, CD36 expression is regulated by fatty acid levels and energy requirements in adipose tissue and in heart and skeletal muscle. During adipogenesis, CD36 expression is elevated as adipocytes mature to take up and store LCFAs, a process accompanied by PPAR activation (Febbraio et al., 1999). Regulation of CD36-mediated LCFA uptake has been

proposed to occur via a translocation of CD36 from intracellular pools to the plasma membrane (Bonen et al., 2000; Luiken et al., 2002; Chabowski et al., 2006).

2.5.7.3 Role of ROS and oxidative stress

Redox signalling plays a major role in regulating the expression of CD36. Various products derived from lipid peroxidation induce CD36 expression (Cho et al., 2005), while antioxidants such as vitamin E suppress it (Ricciarelli et al., 2000; Cho et al., 2007). Moreover, anti-TNF-α monoclonal antibodies increase CD36 expression via NADPH oxidase activation (Boyer et al., 2007).

CHAPTER 3: GENETIC ABLATION OF CD36 INDUCES AGE-RELATED CORNEAL NEOVASCULARIZATION

Cornea 2008; 27:1037-41

3.1 Preamble

Corneal avascularity and transparency are essential for the proper optical performance of the cornea (Chang et al., 2001). These features are maintained by a homeostatic balance between anti-angiogenic and pro-angiogenic stimuli wherein disruption of the delicate balance can lead to pathological corneal NV. While recent evidence indicates that there is considerable overlap in the molecular mechanisms governing corneal avascularity, this field is rapidly expanding as new mediators are identified (Cursiefen et al., 2004a; Ambati et al., 2006; Cursiefen et al., 2006; Ambati et al., 2007). Because CD36 has long been established as a critical endogenous inhibitor of angiogenesis (Dawson et al., 1997; Jimenez et al., 2000; Febbraio et al., 2001), in the following manuscript we explored whether this receptor serves a major role in maintaining postnatal corneal avascularity.

GENETIC ABLATION OF CD36 INDUCES AGE-RELATED CORNEAL NEOVASCULARIZATION

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3.2 Abstract

Purpose: Corneal avascularity is tightly regulated by a balance between angiogenic and anti-angiogenic factors (angiogenic privilege). In the current study, we tested the hypothesis that the CD36 anti-angiogenic receptor contributes towards the maintenance of corneal avascularity.

Methods: Corneas of CD36 wild-type ($CD36^{+/+}$) and knock-out ($CD36^{-/-}$) mice aged 4, 16, 52 and 78 weeks were histologically evaluated for corneal haze and neovascularization. Quantitative real-time PCR was performed on corneal tissue from $CD36^{+/+}$ and $CD36^{-/-}$ mice aged 4 and 52 weeks to examine the effect of CD36 deficiency on expression of relevant angiogenic factors.

Results: Corneal haze and neovascularization were absent in $CD36^{+/+}$ mice at all ages. Conversely, corneal haze and neovascularization were evident at 52 and 78 weeks in $CD36^{-/-}$ mice, and the latter demonstrated a significant increase in vessel density at 52 and 78 weeks. Interestingly, compared to $CD36^{+/+}$ mice, TSP-1 mRNA was repressed, whereas VEGFA, JNK-1, and c-Jun levels were robustly upregulated in the corneas of 52 week old $CD36^{-/-}$ mice.

Conclusions: $CD36^{-/-}$ mice develop corneal neovascularization that increases in severity with age thus accentuating the role of CD36 in preserving corneal avascularity.

Key words: CD36, corneal avascularity, corneal neovascularization, angiogenic privilege

3.3 Introduction

Corneal avascularity, the absence of blood vessels in the cornea, is necessary for optical transparency and visual acuity. However, the cornea can be secondarily invaded by blood vessels in the event of strong inflammatory and angiogenic stimuli. The resultant opacification, inflammation, scarring, and neovascularization compromises corneal transparency[1,2] and is associated with the leading causes of corneal blindness in the world namely trachoma and herpetic keratitis.[3]

Under normal conditions, corneal avascularity is thought to require low levels of angiogenic factors, such as vascular endothelial growth factor A (VEGFA) and the transcription factor cJun,[4,5] and high levels of anti-angiogenic molecules namely thrombospondins (TSPs), pigment epithelium-derived factor, and angiostatin.[1] Of relevance, it was recently discovered that soluble VEGF receptor-1 (sVEGFR-1), an endogenous VEGFA trap, is crucial for preserving corneal avascularity.[6,7] Nonetheless, knowledge of the mechanisms regulating corneal avascularity is still in its infancy, and whether the CD36 scavenger receptor is an important modulator of this process is yet to be elucidated.

CD36 is an endogenous anti-angiogenic receptor that binds to a variety of ligands including TSP-1, oxidized low-density lipoproteins, collagen, and apoptotic cells. Expression of CD36 is broad and encompasses microvascular endothelial cells, macrophages, and the retinal pigment epithelium.[8] CD36 has been implicated in a wide variety of normal and abnormal biological functions including angiogenesis, phagocytosis, inflammation, lipid metabolism, and

apoptotic cell clearance.[8] Moreover, we recently ascertained that CD36 is constitutively expressed by the cornea and potently inhibits inflammatory corneal neovascularization (NV).[5] Based on this information, we set out to investigate whether genetic deletion of CD36 influences postnatal corneal avascularity.

3.4 Materials and Methods

Animals

Male CD36 deficient ($CD36^{-/-}$) and wild-type ($CD36^{+/+}$) C57BL/6 littermates were generated as previously described[9] and provided by M. Febbraio (Weill Medical College, Cornell University). All animal experiments followed the guidelines of the ARVO Statement for the use of Animals in Ophthalmic and Vision research and were approved by the Animal Care Committee of CHU Sainte-Justine, Montreal, QC.

Corneal haze and histologic evaluation

At the ages of 4 (n=8 animals), 16 (n=4 animals), 52 (+/+ n=4, -/- n=3 animals), and 78 (+/+ n=8, -/- n=5 animals) weeks, $CD36^{+/+}$ and $CD36^{-/-}$ mice were sacrificed by CO_2 asphyxiation and both eyes were enucleated. The eyes were first evaluated for corneal haze which was graded as 0 (clear cornea) or 1 (dense opacity covering the entire cornea). Representative photographs were captured with a Nikon digital camera. Eyes were subsequently fixed in 4% paraformaldehyde and later embedded in paraffin. Serial sections (6 μm) were cut sagittally parallel to the optic nerve and stained with periodic acid and Schiff reagent (PAS; Sigma Aldrich Co.). Sections were histologically examined using a

Nikon eclipse E800 epifluorescent microscope and images were captured with a Nikon digital camera DXM 1200. Neovascularization was quantified by determining the number of vessels observed microscopically per corneal section.

RNA Isolation and Quantitative Real-time PCR Analysis (qRT-PCR)

Due to limitations in tissue availability, our subsequent experiments were performed at the mRNA instead of protein level, as this would require several corneas. Total RNA was isolated from corneal tissue derived from $CD36^{+/+}$ and $CD36^{-/-}$ mice aged 4 and 52 weeks (n=6 corneas per group) using TRIzol reagent (Invitrogen, Inc., Carlsbad, CA) and treated with DNase I (Qiagen, Hilden, Germany). cDNA was synthesized from 1 μg RNA with M-MLV reverse transcriptase (Promega Corp.) according to the manufacturer's instructions and amplified using SYBR Green I (Stratagene, Cedar Creek, TX) in a sequence detection system (MxPro 3000 QPCR Systems, Stratagene). Primers for TSP-1, VEGFA, JNK-1, and c-Jun were synthesized by Alpha DNA, Montreal, Quebec as follows: 5' to 3': TSP-1 5'-AACAAAGGACCTCCAAGCTATCTG-3' (forward) and 5'-GGGAGGCCGCTTCAGC -3' (reverse); VEGFA, 5'-TGCAGGCTGCTGTAACGATG -3' (forward) and 5'-GAACAAGGCTCACAGTGATTTTCT -3' (reverse); JNK-1 5'-TGCCATCATGAGCAGAAGCAAACG -3' (forward) and 5'-TCTGATTCTGAAATGGCCGGCTGA -3' (reverse); c-Jun 5'-TGGGCACATCACCACTACAC-3' (forward) and 5'-AGTTGCTGAGGTTGGCGTA -3' (reverse); 18S (Ambion Inc.). PCR

amplification protocol involved 45 cycles of denaturation at 95°C for 30 s, primer annealing at 62°C, and primer extension at 72°C for 60 s. Each sample was analyzed in triplicate along with RT- and no-template controls. mRNA levels were normalized to 18S which served as an internal control. All qRT-PCR reactions were performed at least 3 times in triplicate.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using the nonparametric Kruskal-Wallis, Mann-Whitney rank sum test, and Chi square analysis. The significance level was set at $p<0.05$.

3.5 Results

Effect of CD36 deficiency and age on occurrence of corneal haze

To address the question of whether CD36 deficiency compromises corneal avascularity, $CD36^{+/+}$ and $CD36^{-/-}$ mice were evaluated at 4, 16, 52, and 78 weeks for opacifaction. Corneal haze was absent in $CD36^{+/+}$ mice at all ages (Fig. 1 A, B). Conversely, $CD36^{-/-}$ mice exhibited corneal haze at 52 weeks (1 out of 3 mice exhibited hazy corneas) and at 78 weeks (4 out of 5 mice developed hazy corneas) (Fig. 1 A, B; $p<0.001$).

Histologic analysis of corneas from $CD36^{+/+}$ and $CD36^{-/-}$ mice at different ages

To further investigate the requirement of CD36 for maintaining corneal avascularity, PAS stained corneal sections of $CD36^{+/+}$ and $CD36^{-/-}$ mice were

examined histologically at 4, 16, 52, and 78 weeks. In wildtype mice at all ages (Fig. 2 A, C, E) and $CD36^{-/-}$ mice aged 4 (Fig. 2B) and 16 weeks (data not shown), the stroma appeared healthy and avascular, comprising flattened spindle-shaped keratocytes scattered between the regularly arranged lamellae. However, at 52 and 78 weeks, compared to $CD36^{+/+}$ (Fig. 2C), $CD36^{-/-}$ mice showed signs of corneal NV, as indicated by neovessel formation and infiltration of inflammatory cells into the corneal stroma (Fig. 2 D, F). Additionally, the epithelial surface of $CD36^{-/-}$ mice was rough, inflamed, and in certain areas appeared to be sloughing off (Fig. 2 D, F). Interestingly, the degree of vascularization was more prominent in the central versus the peripheral cornea (Fig. 2 D, F, G) with apparent scar tissue formation (Fig. 2H). Upon quantification (Fig. 2I), vessel density was significantly increased in an age-specific manner in $CD36^{-/-}$ mice (52 weeks, 45 ± 17 vessels/corneal section, $p<0.01$; 78 weeks, 78 ± 21 vessels/corneal section, $p<0.001$).

Effect of CD36 deficiency on mRNA expression of TSP-1, VEGFA, JNK-1, and cJun

In an attempt to elucidate the molecular mechanisms responsible for the induction of corneal NV in $CD36^{-/-}$ mice, corneal tissue from 4 and 52 week mice was analyzed by quantitative real-time PCR for expression of major angiogenic factors. At 4 weeks there was no significant difference between $CD36^{+/+}$ and $CD36^{-/-}$ mice with respect to their mRNA expression of anti-angiogenic TSP-1[10] and pro-angiogenic VEGFA, c-Jun NH_2-terminal kinase (JNK)-1, and c-Jun (Fig. 3A). On the other hand, at 52 weeks, mRNA expression of TSP-1 was repressed

in $CD36^{-/-}$ mice (0.52 fold vs. $CD36^{+/+}$, 1.2 fold; $p<0.01$; Fig. 3B), whereas mRNA levels of VEGFA ($CD36^{+/+}$, 0.98 fold vs. $CD36^{-/-}$, 2.9 fold; $p<0.001$), JNK-1 ($CD36^{+/+}$, 1.04 fold vs. $CD36^{-/-}$, 3.2 fold; $p<0.001$), and c-Jun ($CD36^{+/+}$, 0.98 fold vs. $CD36^{-/-}$, 3.7 fold; $p<0.001$) were markedly induced (Fig. 3B).

3.6 Discussion

The cornea is normally devoid of blood vessels and actively maintains this avascularity, a phenomenon termed corneal angiogenic privilege.[1,2] In the present study, CD36 deficient mice developed age-dependent corneal NV, an effect correlated with decreased expression of TSP-1 and increased VEGFA, JNK-1, and c-Jun mRNA levels. The age-specific occurrence of corneal NV in $CD36^{-/-}$ mice suggests that early during postnatal life, other anti-angiogenic factors may compensate for the absence of CD36 and that these inhibitors can adequately maintain angiostasis provided that trauma to the ocular surface is negligible. This is plausible given the plethora of anti-angiogenic factors that have been identified in the cornea.[1,2] Nonetheless, corneal cell density and wound healing have been observed to decline with age,[11] a fact that may be attributed to the aging cornea's diminished capacity to respond to environmental or inflammatory insults. Then again, it has been reported that $CD36^{-/-}$ mice fail to efficiently clear apoptotic cells from inflammatory settings.[12] It is therefore conceivable that with age, the protective effects afforded by CD36 become more crucial, thus rendering the cornea more susceptible to injury in its absence.

Corneal opacity is thought to be a consequence of leukocyte infiltration and edema.[1] The age-related progressive increase in corneal opacification in

$CD36^{-/-}$ mice may reflect a poor wound-healing response to accumulated environmental insults. In addition, abnormalities in the corneal epithelium of $CD36^{-/-}$ mice may impair its protective functions and hence contribute to the inflammatory changes. Although we did not specifically address the identity of the inflammatory cells observed in the $CD36^{-/-}$ corneas, we can speculate that they were partly of macrophage lineage given that macrophage involvement in corneal NV is well established.[13] Consistent with this, macrophages are known to provide much of the requisite VEGF that drives inflammatory corneal NV,[13,14] and we demonstrate that VEGFA expression is significantly elevated in the corneas of $CD36^{-/-}$ mice. It is also of particular interest that corneal NV occurs prominently in the central corneas of $CD36^{-/-}$ mice with accompanying corneal scarring. This is indicative of a state of chronic inflammation and extensive stromal neovascularization, thus highlighting the necessity of CD36 for regulating and resolving corneal wound healing.

The mechanisms governing corneal avascularity remain obscure. Several angiogenic factors, including the VEGF family,[13-15] JNK-1 and its downstream transcription factor c-Jun have been implicated in mediating corneal NV.[4,5] As a counterbalancing system, numerous anti-angiogenic factors such as TSP-1 and -2 have been identified in the cornea. Of relevance, Cursiefen et al.[10] failed to detect corneal NV in $TSP\text{-}1^{-/-}$ and $-2^{-/-}$ mice and attributed this to a redundant function of these anti-angiogenic factors. Intriguingly, we show that at 52 weeks, the balance of pro- and anti-angiogenic factors in $CD36^{-/-}$ corneas is shifted towards angiogenesis as evinced by decreased TSP-1 and elevated VEGFA, JNK-1, and c-Jun mRNA expression. These data are underscored by studies demonstrating that

the angiogenesis promoting actions of VEGFA require activation of JNK and subsequent c-Jun phosphorylation.[16] c-Jun has also been shown to induce corneal NV by stimulating matrix metalloproteinase expression and activity.[4] It is well established that binding of TSP-1 to CD36 transduces signals leading to apoptosis-dependent inhibition of neovascularization.[17] Accordingly, we surmise that the decrease in TSP-1 expression in $CD36^{-/-}$ corneas may be accredited to disruption of this positive feedback.[18] Because the transcriptional changes in $CD36^{-/-}$ corneas were evident in old (52 weeks) and not young (4 weeks) mice, we propose that this angiogenic switch is not a primary consequence of CD36 invalidation but manifests secondary to the increasing pathology. Finally, given the recent findings that sVEGFR-1 preserves corneal avascularity by sequestering endogenous VEGF, [6,7] it would be tempting to speculate that in $CD36^{-/-}$ corneas, the increase in VEGFA is paralleled by a concomitant decrease in sVEGFR-1 secretion.

Taken together, the current study supports the view that CD36 is important for preserving corneal avascularity particularly later on in life. We present compelling evidence that genetic ablation of CD36 induces corneal NV which is accompanied by increased expression of angiogenic factors and subsequent induction of a chronic inflammatory response. Additionally, our findings provide valuable insight into the angiogenic privilege of the cornea and support the use of CD36 in preventing or treating corneal neovascularization.

3.7 Acknowledgments

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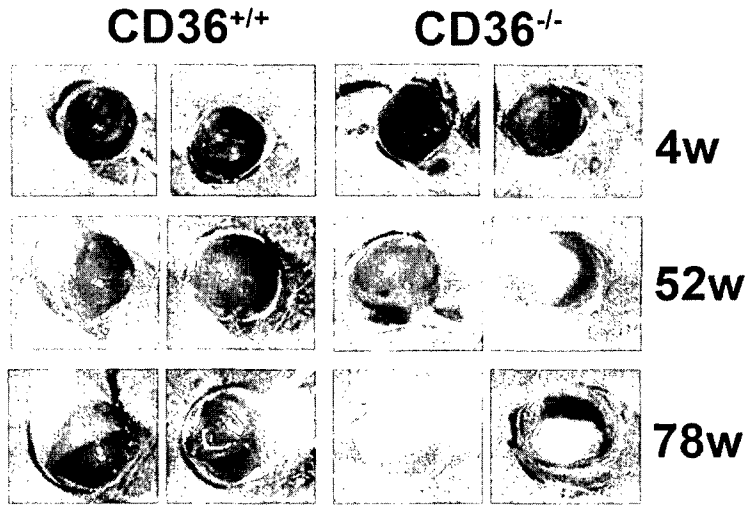
3.9 Figures

A



B

Age (weeks)	CD36+/+ Haze/Total (no. animals)	CD36-/- Haze/Total (no. animals)
4	0/8	0/8
16	0/4	0/4
52	0/4	1/3
78	0/8	4/5***

Figure 1

Figure 1. Analysis of $CD36^{+/+}$ and $CD36^{-/-}$ corneas for occurrence of corneal haze. (**A**) Representative digital images of eyes from CD36 wildtype ($CD36^{+/+}$) and null ($CD36^{-/-}$) mice aged 4 (n=8 animals), (52 (+/+ n=4, -/- n=3 animals) and 78 (+/+ n=8, -/- n=5 animals) weeks. (**B**) Quantification of corneal haze in 4, 16, 52, and 78 week old mice. Corneal haze was scored as 0 (clear cornea) or 1 (corneal opacity). *** $p<0.001$ vs. $CD36^{+/+}$.

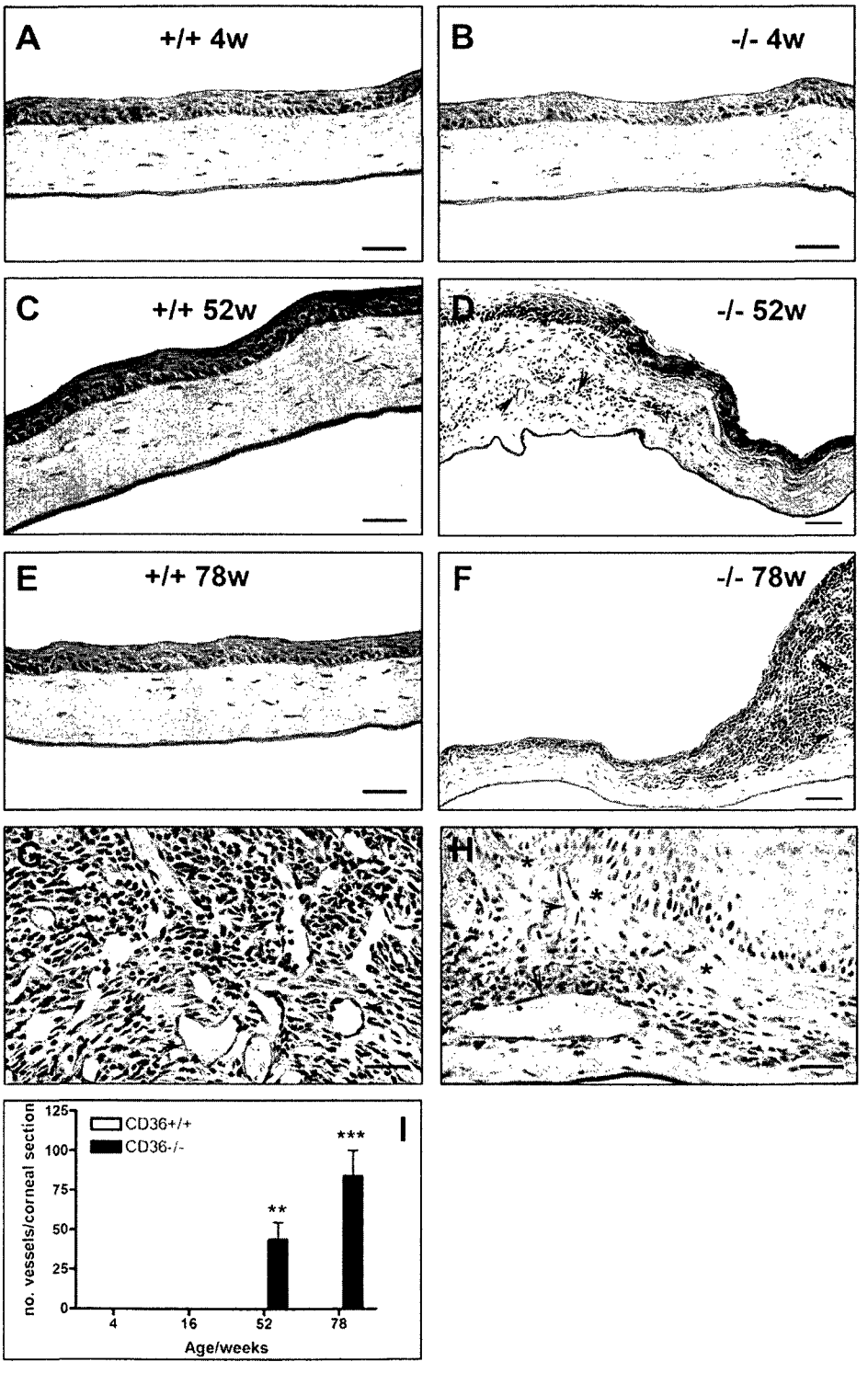

A
+/+ 4w
B
-/- 4w
C
+/+ 52w
D
-/- 52w
E
+/+ 78w
F
-/- 78w
G
H
I
CD36+/+
CD36-/-
no. vessels/corneal section
125
100
75
50
25
0
**
***
4
16
52
78
Age/weeks

Figure 2

Figure 2. Histological evaluation of corneas from $CD36^{+/+}$ and $CD36^{-/-}$ mice for development of corneal neovascularization. Six micrometer sections from eyes of CD36 wildtype ($CD36^{+/+}$) (**A, C, E**) and null ($CD36^{-/-}$) (**B, D, F, G, H**) mice aged 4 (**A, B**), 52 (**C, D**), and 78 (**E-H**) weeks were stained with periodic acid and Schiff reagent and analyzed histologically. Note cellular infiltration in corneal epithelium and stroma (**D, F**). Arrow heads indicate blood vessels, asterisks depict scar tissue formation. Images of the central cornea are illustrated in (**G**) and (**H**). Scale bars 30 µm (**A, B, C, E, G, H**) and 50 µm (**D, F**). (**I**) Quantification of vessel density (number of vessels per corneal section) from $CD36^{+/+}$ and $CD36^{-/-}$ mice. Results represent the mean ± SEM for at least 3 eyes per group. ** $p<0.01$, *** $p<0.001$ vs. $CD36^{+/+}$.

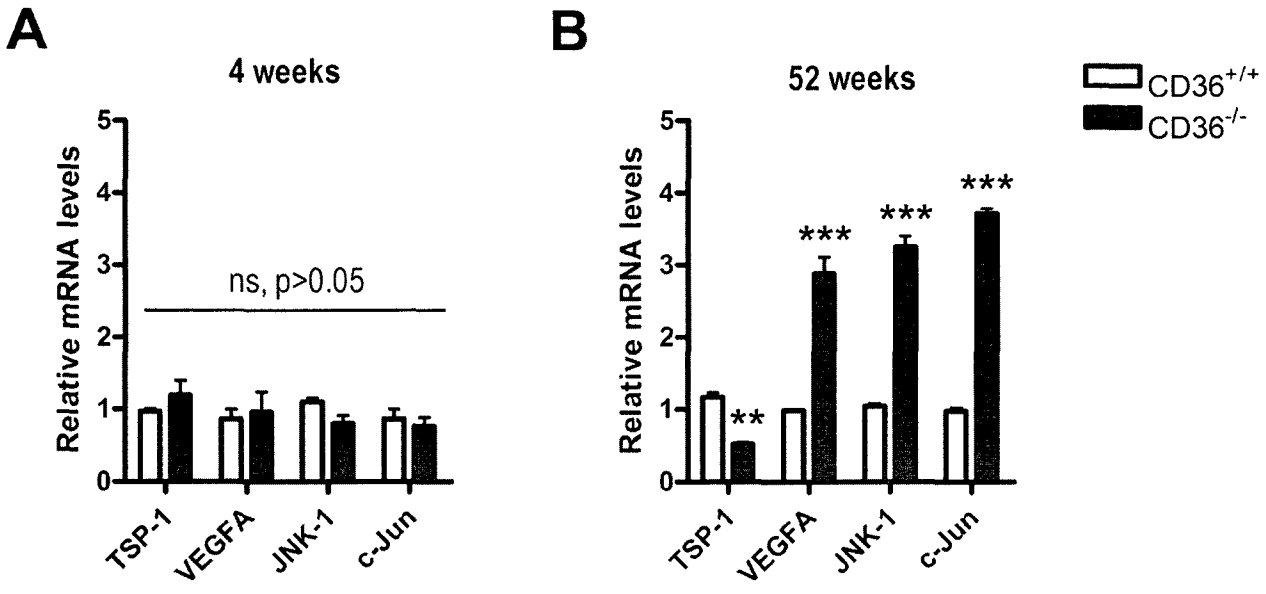


Figure 3. Expression of TSP-1, VEGFA, JNK-1, and c-Jun in CD36 deficient corneas. Corneal tissue from (**A**) 4 week and (**B**) 52 week CD36 wildtype ($CD36^{+/+}$) and null ($CD36^{-/-}$) mice was processed for quantitative real-time PCR and the mRNA levels of TSP-1, VEGFA, JNK-1, and c-Jun were determined. Values represent the mean ± SEM of 3 independent experiments performed in triplicate for n=6 corneas per group. ns, non significant; ** $p<0.01$, *** $p<0.001$ vs. $CD36^{+/+}$.

CHAPTER 4: ACTIVATION OF CD36 INHIBITS AND INDUCES REGRESSION OF INFLAMMATORY CORNEAL NEOVASCULARIZATION

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4.1 Preamble

In the previous chapter we identified an important role for CD36 in maintaining corneal avascularity demonstrating that its absence is associated with the development of age-related corneal NV. Coupled with this observation, and the fact that expression of CD36 in the normal and neovascularized cornea has not yet been characterized, we purposed to examine the role of CD36 in regulating inflammation-induced corneal NV and the mechanisms therein.

ACTIVATION OF CD36 INHIBITS AND INDUCES REGRESSION OF INFLAMMATORY CORNEAL NEOVASCULARIZATION

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4.2 Abstract

Purpose: This study was undertaken to investigate the role of the anti-angiogenic receptor CD36 during inflammatory corneal neovascularization (CNV). **Methods:** Using a murine model of inflammatory CNV, CD36 expression was evaluated by RT-PCR and immunofluorescence. Mice subjected to CNV were treated topically (thrice daily) with CD36 functionally neutralizing antibodies against the oxidized low density lipoprotein (oxLDL) and thrombospondin (TSP)-1 sites (clones JC61.3 and FA6-152 respectively). Neovascularization was analyzed by CD31-immunostained corneal flat-mounts. To elucidate the role of the less characterized oxLDL site during angiogenesis, the CD36 ligand, 1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine (POVPC; 50, 100 μg/mL) was utilized 24 hours after corneal injury for 7 days, whereas for angio-regressive studies, POVPC treatments were initiated 10 days post CNV induction; in this process VEGF expression was also studied. Effects of CD36 activation were further examined *ex vivo* using the mouse aortic ring assay.

Results: CD36 expression was upregulated following corneal injury; CD36 was expressed in corneal epithelium, limbus, invading microvessels, and stromal macrophages. Blocking CD36 activity with FA6-152 significantly increased CNV ($p < 0.001$). Conversely, activating CD36 with POVPC dose-dependently inhibited CNV ($p = 0.003$); this effect was blocked by JC61.3. POVPC also significantly regressed preformed blood vessels ($p < 0.001$). *Ex vivo* experiments on aortic rings confirmed the angio- inhibitory and -regressive effects of POVPC.

Since corneal macrophages express CD36 and may partake in angiogenesis via VEGF-A secretion, we surmised that VEGF-A could be modulated by CD36; indeed, POVPC down-regulated VEGF-A expression in a time-dependent fashion ($p < 0.001$), whereas FA6-152 induced its expression ($p < 0.05$).

Conclusions: CD36 is involved both physiologically and pharmacologically in inhibition and regression of CNV, by direct effect on endothelial cells and partly by negatively regulating VEGF expression in macrophages.

Key words: CD36, corneal neovascularization, oxidized phospholipid, VEGF-A, macrophage

4.3 Introduction

Angiogenic stimulators and inhibitors are the counter-balancing systems which tightly control corneal angiogenesis.[1] The healthy cornea is devoid of vascular elements and is maintained as an immune privileged site.[2] This is thought to be due to the plethora of anti-angiogenic factors present in this tissue.[1] These include pigment epithelium derived factor (PEDF), maspin, thrombospondin, in addition to endostatin, a proteolysis product of type XVIII collagen, and angiostatin, a proteolysis product of plasminogen.[3-8]

Although vascular endothelial growth factor (VEGF) is known to be a potent stimulator of CNV[9, 10] the molecular basis of this condition remains poorly defined. It is well known, however, that transmigrating and invading macrophages are closely associated with neovascularization and provide much of the requisite VEGF that drives this process.[11, 12]

The class B scavenger receptor CD36 is a transmembrane glycoprotein that has been identified as the critical receptor for thrombospondin (TSP)-1, a potent endogenous inhibitor of angiogenesis[13-15] including of the cornea.[16] CD36 also binds to a variety of other ligands including oxidized low density lipoproteins (oxLDLs),[17, 18] oxidized phospholipids (oxPLs),[19-22] *Plasmodium falciparum*-infected erythrocytes,[23] collagen,[24] and apoptotic cells.[25] Expression of CD36 is broad and encompasses microvascular endothelial cells (ECs), monocytes/macrophages, platelets, conjunctival dendriform cells and the retinal pigment epithelium.[25-28] Furthermore, CD36 has been implicated in a wide variety of normal and abnormal biological functions, including angiogenesis,

atherosclerosis, phagocytosis, inflammation, lipid metabolism, and removal of apoptotic cells.[25]

With respect to its angiostatic functions, CD36 is essential for inhibiting *in vitro* EC migration and the formation of capillary-like structures by TSP-1.[15] CD36 plays a critical role *in vivo*, as demonstrated by the inability of TSP-1 to inhibit angiogenesis in CD36 null mice.[29] We have also recently demonstrated the specific involvement of CD36 and TSP-1 in mediating anti-angiogenic signals in ischemic proliferative retinopathy.[30, 31] Nevertheless, the involvement of CD36, and the relative role of its less well characterized oxidized lipid binding site, in regulating pathological corneal angiogenesis has not yet been fully elucidated. We herein report that expression of CD36 in macrophages and microvascular endothelial cells following corneal injury suppresses CNV; this effect can be ascribed to the pro-apoptotic anti-angiogenic property of CD36 on endothelial cells as well as partly due to a downregulation of VEGF from CD36-expressing macrophages.

4.4 Materials and Methods

Animals

Male C57BL/6 mice were purchased from Harlan (Indiana, USA). Animals were given food and water ad libitum, maintained under pathogen-free conditions of 12-hour light/12-hour darkness. All animal experiments followed the guidelines of the ARVO Statement for the use of Animals in Ophthalmic and Vision research and were approved by the Animal Care Committee of Sainte-Justine Hospital, Montreal, QC.

Murine model of corneal neovascularization

The CNV model used in our studies is characterized by the removal of the corneal and limbal epithelium by means of a chemical and mechanical injury to the cornea. This causes resurfacing of the cornea by a conjunctiva-like epithelium replete with blood vessels. The neovascularization persists for at least 8 weeks and is accompanied by inflammation.[9]

All mice used for inflammation-induced CNV experiments were between 6 and 8 weeks of age. Each mouse was anesthetized before all surgical procedures with isoflurane (Abbott, Canada). Topical proparacaine (Alcon, Canada) and 2 μL of 0.15M NaOH were applied to the central cornea of each mouse. The corneal and limbal epitheliums were removed by scraping with a #23 scalpel. Gentamicin sulfate ophthalmic solution (Sabex Inc., Quebec, Canada) was instilled immediately following epithelial denudation 3 times daily for 2 days. Buprenorphine (0.05 mg/kg; Schering-Plough Ltd) was administered post-operatively for analgesia.

Pharmacological treatment of mice with CNV

C57BL/6 mice undergoing inflammation-induced angiogenesis were randomly divided into four groups. Twenty-four hours after corneal injury, one group received treatment with 100 μg/mL of an anti-CD36 monoclonal antibody (mAb) against the oxLDL binding site (clone JC61.3 IgA mouse, Cayman chemical, Ann Arbor MI) or an isotype control antibody (100 μg/mL anti-mouse IgA, Sigma Aldrich Inc.). A second group was treated with 200 μg/mL of an anti-CD36 mAb against the TSP-1 binding site (clone FA6-152 IgG_1 mouse, Beckman

Coulter) or 200 μg/mL anti-mouse IgG_1 (Sigma Aldrich Inc.). The third group was administered vehicle (99% 0.9% NaCl and 1% ethanol), or 50 or 100 μg/mL POVPC (1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine) (Cayman Chemical, Ann Arbor, MI). In the fourth group, vehicle (99% 0.9% NaCl and 1% ethanol) or 100 μg/mL POVPC treatments were conducted 10 days after surgery for angio-regression studies. All treatments were administered topically three times daily for 7 days, after which corneas were harvested for immunostaining. In another set of experiments, one group of mice underwent vehicle (99% 0.9% NaCl and 1% ethanol) or 100 μg/mL POVPC treatments for 2 and 4 days, whereas a second group was treated with 200 μg/mL IgG_1 or 200 μg/mL FA6-152 for 4 days. The corneas were subsequently dissected and processed for RNA extraction and RT-PCR analysis. In all experiments the treatment groups consisted of 10 mice per group and each set of experiments was repeated at least two times.

Labelling and quantification of CNV

Visualization of vascular endothelial cells was performed by immunostaining corneal flat-mounts with FITC-conjugated anti-CD31 as previously described,[6] or with FITC-conjugated ICAM1 to demonstrate activated vascular endothelial cells. Fresh corneas were dissected, rinsed in 0.1M PBS for 30 minutes, and fixed in 100% ice cold acetone for 25 minutes. After washing in 0.1M PBS, non-specific binding was blocked with 0.1M PBS, 2% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature. Incubation with FITC-conjugated anti-CD31 (1:300, BD Pharmingen) or ICAM1-

FITC (1:100, Abcam plc) in 0.1M PBS, 2% BSA at 4°C overnight was followed by subsequent washes in 0.1M PBS at room temperature. Corneas were mounted with an anti-fading agent (Gelmount; Biomeda, Inc, San Francisco, CA) and observed using a Nikon eclipse E800 epifluorescent microscope. Images were captured with a Nikon digital camera DXM 1200 using Nikon ACT 1 version 2.62 software.

The corneal neovascularization was quantified in a masked fashion using Adobe Photoshop 7.0 image analysis software. The entire flat-mounted cornea was analyzed in order to minimize sampling bias. The total cornea surface area was outlined using the innermost vessel of the limbal arcade as the border and the ratio [(neovascularized area/total cornea area) x 100] was used to provide a measure of the percentage vascularized cornea.[6]

Immunostaining of corneal frozen sections

Mice were killed 7 days after corneal injury. Enucleated eyes were fixed in 4% paraformaldehyde, transferred to 30% sucrose/PBS overnight at 4°C, washed with PBS, and subsequently embedded in optimal cutting temperature medium (Sakura Finetek, USA). Sixteen μm frozen sections were washed with 0.1% Triton X-100/PBS and blocked for 1 hour with 2% BSA before overnight incubation with rabbit polyclonal CD36 (1:100, Santa Cruz). The sections were subsequently incubated with a goat anti-rabbit secondary antibody (1:1000; Molecular Probes), proceeded by a one hour incubation with a TRITC-conjugated lectin endothelial cell marker from *Griffonia simplicifolia* (1:100, Sigma-Aldrich). Cell nucleic were labelled with the nucleic acid stain 4',6-diamidino-2-

phenylindole (DAPI, 300 nM; Molecular Probes). In negative control experiments, the CD36 primary antibody was omitted and sections were incubated with 0.1% Triton X-100/PBS followed by the goat anti-rabbit secondary antibody and DAPI. Images were visualized using epifluorescent microscopy.

RNA extraction and RT-PCR analysis

Eyes were enucleated, the corneas dissected and immediately placed in RNA*later*™ stabilization solution (Ambion Inc.). Total RNA (n = 10 per group) was extracted using the standard TRIzol RNA isolation protocol (Invitrogen Corp.). cDNA was synthesized from 1 μg RNA with M-MLV reverse transcriptase (Promega Corp.) according to the manufacturer's instructions. The following primers were used for PCR from 5' to 3': CD36 sense, GATGACGTGGCAAAGAACAG; CD36 anti-sense, AAAGGAGGCTGCGTCTGTG; VEGF-A sense, ACTGGACCCTGGCTTTACTG; VEGF-A anti-sense, TATGTGCTGGCTTTGGTGAG; JNK-1 sense, TGTGGAATCAAGCACCTTCACTCTGCTG; JNK-1 anti-sense, GCAAAC CATTTCTCCCATAATGCACCC; c-JUN sense, ATGCCCTCAACGCCTCGTTCCTCC; c-JUN anti-sense, CTGCTCGTCGGTCACGTTCTTGGG; β-Actin sense, AGCCATGTACGRAGCCATCC; and β-Actin anti-sense, ATGCCACAGGATTCCATACC. 18S (Ambion Inc.) also served as an internal control. PCR (*Taq* DNA polymerase; Invitrogen Corp.) was performed under the following conditions: denaturation at 94°C, annealing at 56°C (CD36, β-ACTIN;

VEGF-A: 65°C; JNK-1, c-JUN: 64°C; 18S: 60°C), and extension at 72°C. The predicted sizes of PCR products are 550, 350, 300, 350, 450, and 315 bp for CD36, VEGF-A, JNK-1, c-JUN, β-Actin, and 18S respectively. Densitometry values were measured in terms of pixel intensity by Image-Pro Plus software (version 4.1; Media Cybernetic, Silver Spring, MD, USA).

Aortic ring angiogenesis assay

This assay was performed as described previously by us and others.[31, 32] In brief, thoracic aortas were removed from 6 week old mice killed by CO_2 asphyxiation and immediately transferred to a culture dish containing ice-cold endothelial cell basal medium (EGM-2) (Cambrex Bio Science, Walkersville MD). The periaortic fibroadipose tissue was carefully removed with fine micro-dissecting forceps and scissors, paying special attention not to damage the aortic wall. One mm-long aortic rings (12 per aorta) were sectioned and rinsed extensively in eight consecutive washes of EGM-2. The rings were then individually embedded in 48 well plates previously coated with 50 µl Matrigel per well. Next an additional 50 µl of Matrigel was placed over each ring. After 1 hour, 500 µl EGM-2 was added to each well and the cultures were incubated at 37°C for 5 days. The culture medium was changed on day 3 and the test compounds added. The test compounds used and their concentrations were: vehicle (99% 0.9% NaCl and 1% ethanol), POVPC (20 µg/mL) in absence or presence of anti-CD36 antibodies (JC61.3, 6 µg/mL; FA6-152, 10 µg/mL). Aortic rings were photographed on day 5 at 4X magnification using a Nikon eclipse TE300 inverted microscope. For neovessel-regression experiments, rings

were cultured without drugs until day 6, after which the rings were treated with the test compound and allowed to grow until day 7. The angiogenic response was determined by measuring the area of neovessel formation using Image Pro Plus software.

Immunostaining of whole mount corneal stromas

Mice were subjected to corneal injury after which corneal and limbal tissue were excised at day 7 and subsequently prepared for staining as whole mounts, as previously described.[33] Briefly, the corneal epithelium was separated following 20 minute incubation at 37°C in 20 mM EDTA (Sigma Chemical Co., St. Louis, MO). The resultant corneal stromas were then fixed for 30 minutes at 4°C in 1% paraformaldehyde-PBS followed by extensive washing in PBS. After fixation, the corneal tissue was blocked for 1 hour in PBS-GEN (PBS containing 3% BSA, 0.25% gelatine, 5mM EDTA, and 0.025% Nonodet-P40) and then processed for double immunofluorescence with rabbit polyclonal CD36 (1:100, Santa Cruz), rabbit anti-mouse vascular endothelial growth factor A (VEGF-A) (1:100, Chemicon International Inc.), and the monocyte/macrophage marker rat anti-mouse F4/80 (1:100, Serotec, Oxford U.K.). Primary antibodies were visualized using appropriately tritrated Alexa Fluor-conjugated secondary antibodies (1:1000, goat anti-rabbit for CD36 and VEGF-A, goat anti-rat for F4/80). Negative control experiments were conducted in parallel by incubating sections with PBS-GEN alone followed by the secondary antibodies. Sections were visualized by epifluorescent microscopy.

Isoprostane measurements

Isoprostanes (8-Iso-PGF2α) were measured in homogenized normal (n = 10) and 4 day injured corneas (n = 10) by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) as previously described.[34] The levels of 8-isoprostane were quantified and normalized to the protein content of the corneal tissue.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using ANOVA with comparison among means being performed by the appropriate post-hoc test unless otherwise stated. Statistical significance was set at $P < 0.05$.

4.5 Results

Expression of CD36 in normal and neovascularized corneas

We examined the expression of CD36 (2 and 4 days after injury) in the normal and neovascularized cornea. As revealed in Fig. 1A, the CD36 gene is transcriptionally active and its expression is substantially induced during the course of neovascularization ($p < 0.001$). CD36 immunoreactivity is largely localized in the corneal epithelium and limbal vessels of the normal cornea (Fig. 1B), and highly expressed by infiltrating vessels in the stroma of the neovascularized cornea (Fig. 1C). We further illustrate that the vascular endothelial cells are activated by immunostaining neovascularized corneas with ICAM-1; this observation has been corroborated by others.[35] Because of the significant induction in CD36 gene expression observed in the injured corneas, we

hypothesized that inflammatory cells such as macrophages also significantly expressed CD36. Immunofluorescence analysis revealed CD36 expression by dendritiform shaped cells in the normal corneal stroma (Fig. 1D). Double staining for CD36 and F4/80, a monocyte/macrophage marker, demonstrated that CD36+ cells were also uniformly F4/80+. Additionally, as early as 3 days after induction of CNV, cells co-expressing CD36 and F4/80 were present in the central areas of the cornea and increased in numbers through day 7, when the maturational state of these cells also changed (Fig. 1D).

Effect of blocking CD36 on CNV

CD36 is a potent anti-angiogenic receptor activated on separate binding sites by oxLDL and TSP-1.[13-15, 17, 18] We postulated that a blocking antibody would exacerbate inflammation-induced CNV. Twenty-four hours after corneal injury, mice treated for 7 days with the anti-TSP-1 binding site antibody (FA6-152) but not with the anti-oxLDL antibody (JC61.3) exhibited a 23% greater vascularized corneal area (72.1% ± 5.2 vs. 55.6% ± 2.2 respectively; $p < 0.001$) and a more pronounced vessel density (Fig. 2); an isotype-control antibody had no significant effect.

Effect of activation of CD36 by oxPLs on CNV and preformed blood vessels

Because the anti-oxLDL binding site antibody did not affect CNV in response to corneal injury (Fig. 2), we postulated that oxidized phospholipids may not be abundantly generated; indeed concentrations of isoprostanes, a marker of oxidized lipid generation, were not increased 4 days after injury (Fig. 3A).

Accordingly, if stimulated with a synthetic oxidized phospholipid, namely 1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine (POVPC), CNV should be decreased. This assumption was confirmed as topical application of POVPC (50 and 100 μg/mL) decreased neovascularization in a concentration-dependent manner ($p = 0.003$; Fig. 3A); as expected, this effect was abrogated by JC61.3 ($p = 0.02$; Fig. 3A).

We also tested if activation of CD36 can regress existing CNV, a clinically desirable effect.[36] Topical application of POVPC (100 μg/mL) in vascularized corneas (10 days after corneal injury) reduced the neovascularized area by 24% ($p < 0.001$; Fig. 3B).

Effect of oxPLs on microvessel formation in an aortic ring angiogenesis assay

To ascertain that effects of POVPC are CD36-dependent we utilized the mouse aortic ring angiogenesis assay. Aortic rings were treated on day 3 with saline, POVPC (20 μg/mL), or a combined treatment of POVPC and JC61.3 (6 μg/mL) or FA6-152 (10 μg/mL). POVPC inhibited neovessel formation ($p < 0.001$; Fig. 4A). This angio-static effect of POVPC was blocked by the anti-oxLDL site antibody ($p < 0.001$); effects of POVPC were hardly affected by the anti-TSP-1 site antibody. POVPC also induced regression of new vessels grown for 6 days ($p < 0.0001$; Fig. 4B).

Effect of activation of CD36 on VEGF-A expressed by macrophages in inflamed cornea

Aortic ring angiogenesis does not involve a role for macrophages. Hence, to elucidate the role of macrophage-expressing CD36 in CNV (Fig. 1D) we surmised that VEGF expression is modulated by CD36. This inference is supported by evidence that macrophages are a significant source of VEGF-A.[11, 37, 38] VEGF-A co-localized with the F4/80 macrophage marker (Fig. 5A). Corneal injury resulting in CNV was associated with an increase in VEGF-A (mRNA) expression (RT-PCR) (Fig. 5B). POVPC significantly diminished VEGF-A expression in a time-dependent fashion ($p < 0.001$), whereas treatment with FA6-152 significantly induced VEGF-A mRNA expression ($p < 0.05$) (Fig. 5C).

We conducted further experiments to explore the mechanisms underlying the FA6-152 induced upregulation of VEGF-A. Activation of the mitogen activated protein kinase (MAPK), JNK-1, has been shown to play a critical role in the inhibition of bFGF induced corneal angiogenesis in a CD36-dependent manner.[39] In our model, the effects of FA6-152 were associated with a marked increase in the mRNA expression of JNK-1 ($p < 0.001$) and its phosphorylation substrate c-JUN ($p < 0.05$) (Fig. 5D).

4.6 Discussion

CD36 is a potent anti-angiogenic receptor, and its angiostatic effects have been widely documented.[15, 25, 29] However, to date, the expression profile and specific involvement of CD36 in the cornea and during CNV are not yet fully

elucidated. Our studies reveal that the CD36 gene is transcriptionally active in the normal cornea, its expression is substantially upregulated during the course of CNV (Fig. 1A), and in this context it is present largely in microvessels and macrophages. Interference of its action enhances neovascularization (Fig. 2), and conversely activation of CD36 diminishes angiogenesis and induces regression of existing corneal microvessels (Fig. 3). The anti-angiogenic effects of CD36 stimulation appear to be dependent upon direct actions on microvessels (Fig. 4) and likely indirectly by diminishing concentrations of macrophage-derived VEGF-A (Fig. 5). Hence, CD36 limits CNV in response to injury, and thus provides a major role in attempting to maintain corneal avascularity, a prominent feature of the normal cornea.

In the pathophysiological setting of corneal injury, stimulation of CD36 seems largely mediated through its TSP-1 site (Fig. 2), consistent with the already reported role of TSP-1 in CNV.[16] However, CD36 can also be activated by a number of oxidized lipids and low density lipoproteins.[14, 15, 17-20] Numerous studies have identified oxidized phospholipids as high affinity CD36 ligands,[19-22] but we could not find increased levels of markers of oxidized lipids, namely isoprostanes, in injured corneas (Fig. 3). On the other hand, activation of the CD36 receptor using an oxPL ligand (POVPC) significantly suppressed CNV and this effect was prevented by a cognate antibody, confirming the specificity of POVPC for CD36 (Fig. 3).[19-22] *In vivo* effects were corroborated using the *ex vivo* aortic ring angiogenesis assay. Collectively, our results together with those reported on TSP-1[16] provide conclusive *in vivo* and *ex vivo* evidence for the efficacy of CD36 as a major target in CNV.

Our *in vivo* observations following corneal injury point to expression of CD36 in macrophages (Fig. 1D). Macrophages play an important role in angiogenesis,[11, 12] such that their selective depletion markedly limits pathological neovascularization.[12, 37] Macrophages are also a significant source of VEGF-A (Fig. 5A) as reported,[38, 40] and abundant evidence points to a dominant role for VEGF in inflammation-induced CNV,[9, 10, 37] VEGF also amplifies inflammatory CNV by further recruiting macrophages/monocytes.[37] We therefore determined if CD36 could modulate VEGF expression in the injured cornea; this was found to be the case as stimulation of CD36 with POVPC diminished VEGF-A expression, whereas blocking CD36 expression with FA6-152 substantially induced VEGF-A mRNA levels (Fig. 5). This is consistent with recently documented effects of another CD36 ligand, TSP-1.[41]

The mechanisms of action of angiogenesis inhibitors have been a subject of considerable attention. The prevailing mode of action of CD36 in inhibition of angiogenesis is believed to be through sequential activation of $p59^{fyn}$, caspase-3-like proteases and p38 MAPKs, by targeting newly formed endothelial cells.[15, 29, 42] In an attempt to explore the signaling pathway activated upon binding of the anti-TSP1 CD36 mAb, we evaluated the expression of the stress-activated MAPK, JNK-1, which was found to be significantly induced in FA6-152 treated corneas. This finding is corroborated by a study reporting an activation of the p38 and p42/44 MAPKs in an inflammatory model of CNV. We propose that the induction of JNK-1 may be attributed to the upregulation of VEGF-A observed following CD36 blockade. Consistent with this hypothesis, studies have reported that VEGF stimulates MAPK activity in various settings.[43] Other signaling

mediators such as the Sonic hedgehog (Shh) pathway, whose inhibition was recently reported to reduce ocular neovascularization,[44] was not activated in our model nor was it implicated in the anti-angiogenic effects of CD36 (data not shown). Of noteworthy mention, it may have been interesting to investigate whether the CLESH containing protein, histidine rich glycoprotein (HRGP), modulates CD36 interactions in our studies, seeing as HRGP has been shown to abrogate the CD36-dependent signaling of TSP-1.[45] Taken together, anti-angiogenic effects of CD36 are mediated not only through a direct effect on microvessels (Fig. 4) but also apparently by inhibiting macrophage-derived VEGF-A expression (Fig. 5).

Current therapies for CNV such as thermal laser or photodynamic therapy induce only temporary closure of blood vessels, [46] whereas a clinically more important aspect of CNV therapy is regression of established blood vessels. In the present study, we investigated this limitation by delaying POVPC treatments until 10 days after corneal injury and observed a significant reduction in existing CNV (Fig. 3B); a similar effect was observed *ex vivo* on aortic ring explants (Fig. 4B). Therefore, activating CD36 not only suppresses but also induces regression of blood vessels. The mechanisms of blood vessel regression are not fully characterized. Nonetheless, certain inferences can be made based on available evidence. For instance, it has been proposed that in the ovary, blood vessel regression involves endothelial cell detachment and blood vessel occlusion.[47] Pericyte loss also determines the susceptibility of vessels to regress.[48] Likewise, increased levels of angiopoietin-2 along with a down-regulation of VEGF can

destabilize mature capillaries and induce their regression.[49] These mechanisms may operate in response to CD36 stimulation.

Taken together, the current findings provide the first demonstration of the protective involvement of CD36 in limiting inflammatory CNV. Other anti-angiogenic factors such as endostatin, thrombospondin, PEDF, and maspin have also been found in the uninjured cornea reaffirming the importance of these types of factors in maintaining transparency of the healthy cornea.[2, 3, 50] Our observations have significant implications for the treatment of ocular neovascularization in that CD36 stimulants would be effective not only in patients with ongoing CNV, but also in those presenting with established CNV. Findings may not only apply to corneal inflammation following injury or infection, but may also be relevant in corneal graft failure which involves an inflammatory immune rejection. Finally, stimulation of CD36 with simple agonists such as oxPLs may provide insights into inexpensive therapies for CNV secondary to commonly encountered infections (such as trachoma) in developing countries.

4.7 Acknowledgments

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4.8 List of abbreviations

CNV	corneal neovascularization
JNK-1	c Jun N-terminal kinase
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
oxPL	oxidized phospholipid
oxLDL	oxidized low density lipoprotein
POVPC	1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine
VEGF-A	vascular endothelial growth factor A
TSP-1	thrombospondin 1

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4.10 Figures

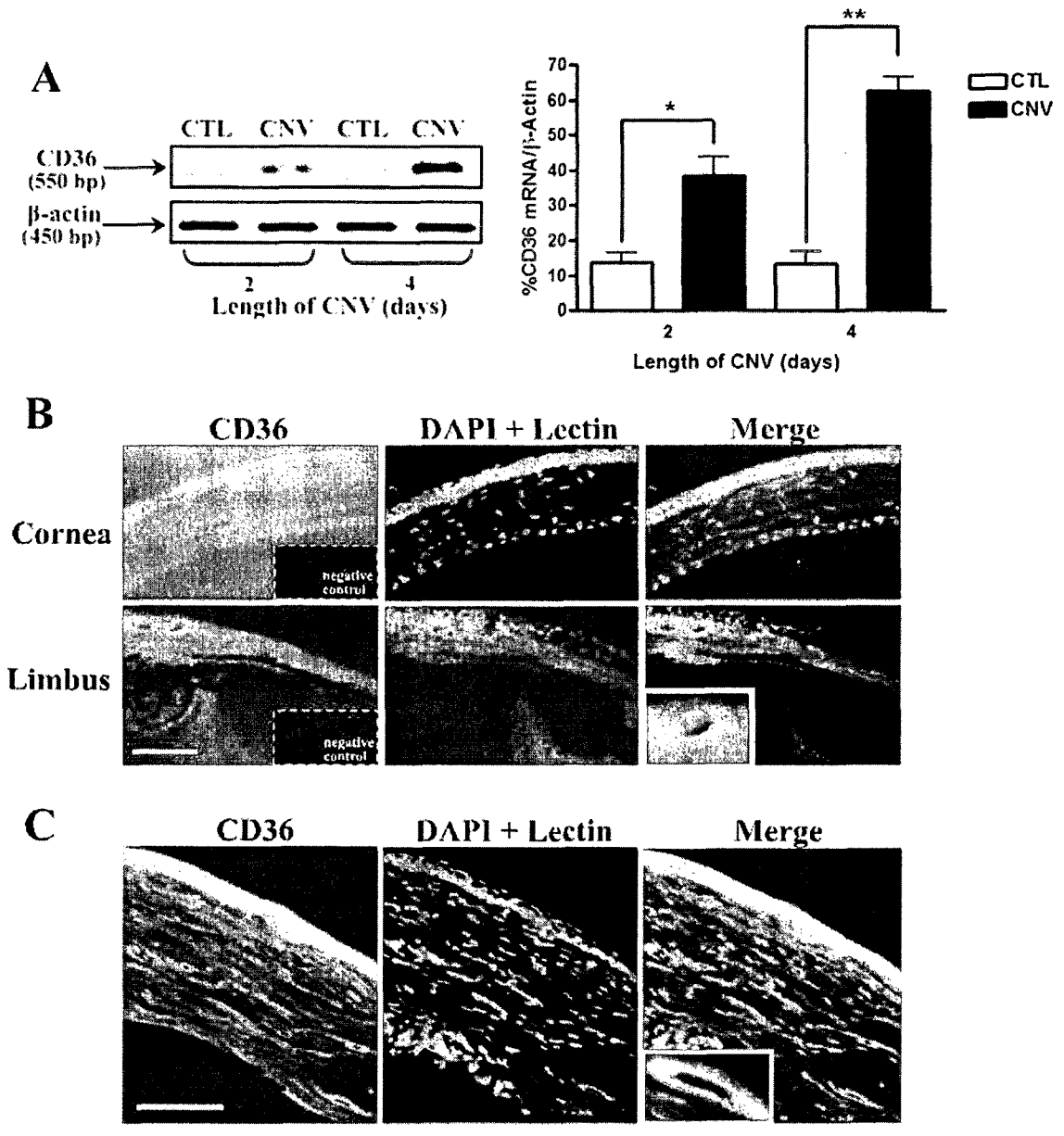

Figure 1

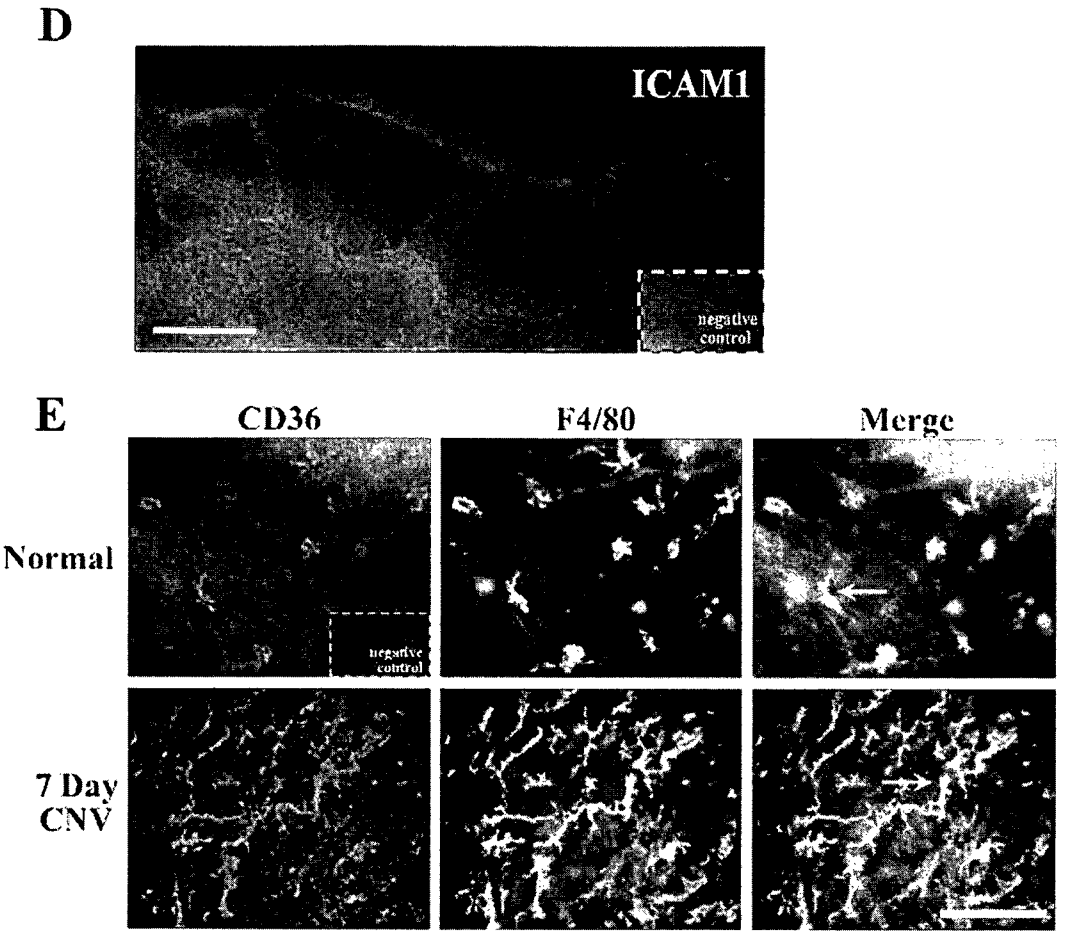

Figure 1 continued

Figure 1. CD36 expression and immunolocalization in the normal and neovascularized cornea. (**A**) RT-PCR analysis of CD36 mRNA expression in normal and injured corneas after 2 and 4 days of injury (*$P < 0.01$ vs. control at day 2; **$P < 0.001$ vs. control at day 4). Immunofluorescence on (**B**) normal and (**C**) inflamed corneas revealed CD36 immunoreactivity (green) in the corneal epithelium, and coexpression with lectin (red) in vessels from the normal limbus, as well as in newly formed vessels in the stroma of the inflamed cornea. (**D**) Vascular endothelial cells of the neovascularized cornea stained positive for ICAM-1, a marker of activated endothelial cells. (**E**) Expression of CD36 by stromal macrophages from normal and injured corneas. Fluorescence micrographs illustrate CD36 (red) coexpression with the microglial/macrophage marker F4/80 (green) in both the normal and 7-day neovascularized cornea as indicated by arrows. Yellow: merged fluorescent labeling. Insets: images taken at higher magnification. Scale bars: (**B**) 50 μm; (**C, D, E**) 30 μm.

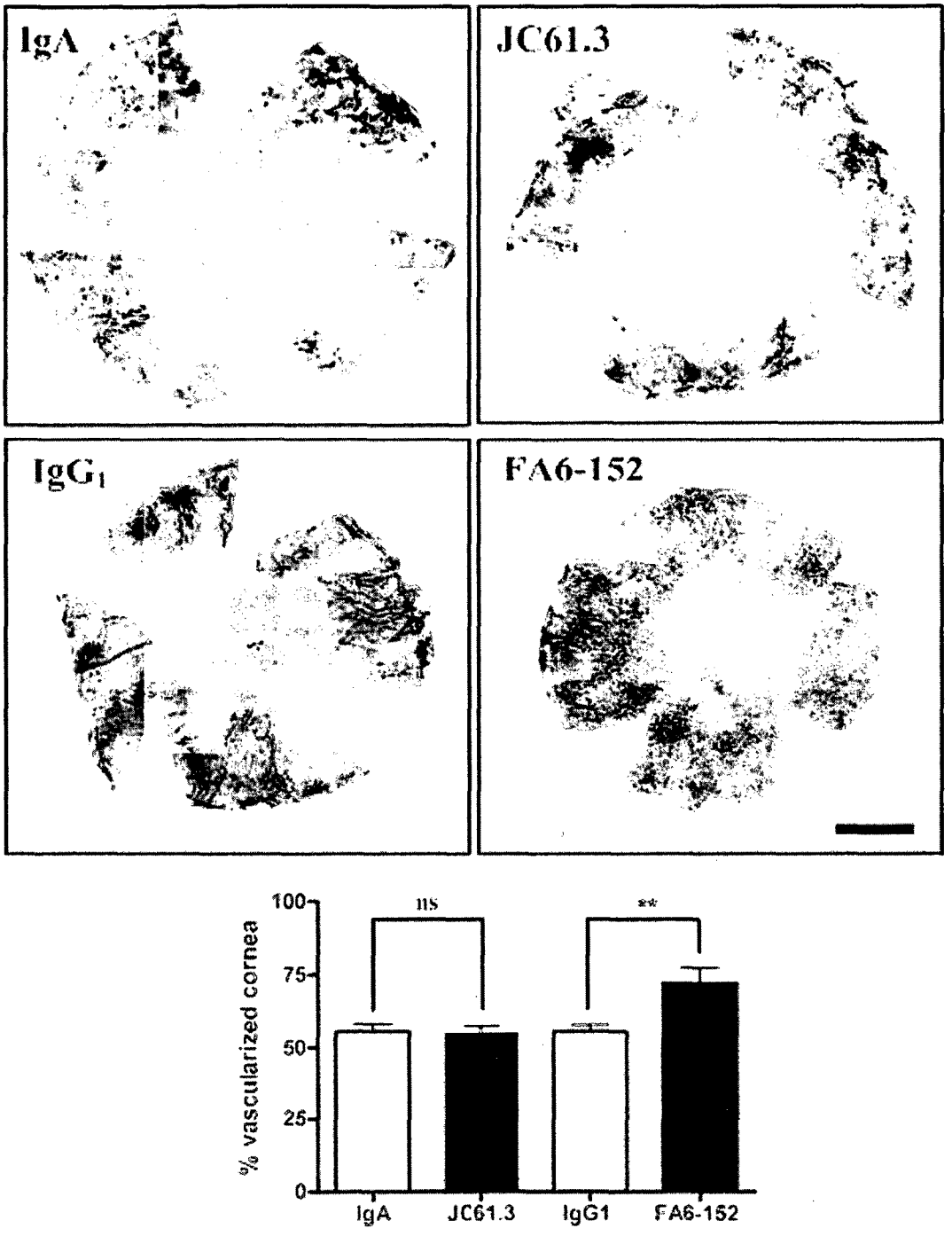
IgA
JC61.3
IgG1
FA6-152
ns
**
% vascularized cornea
100
75
50
25
0
IgA
JC61.3
IgG1
FA6-152

Figure 2

Figure 2. Blocking CD36 activity enhanced CNV. Mice subjected to inflammation-induced CNV were treated three times daily for 7 days with vehicle, 100 μg/mL of an anti-CD36 monoclonal antibody (CD36 mAb) against the oxLDL site (JC61.3), or 200 μg/mL of a CD36 mAb against the thrombospondin-1 binding site (FA6-152). Corneas were harvested and flatmounts were labeled with murine CD31-FITC. Quantification of the vascularized area (**$P < 0.001$ vs. IgG_1). Scale bar, 200 μm.

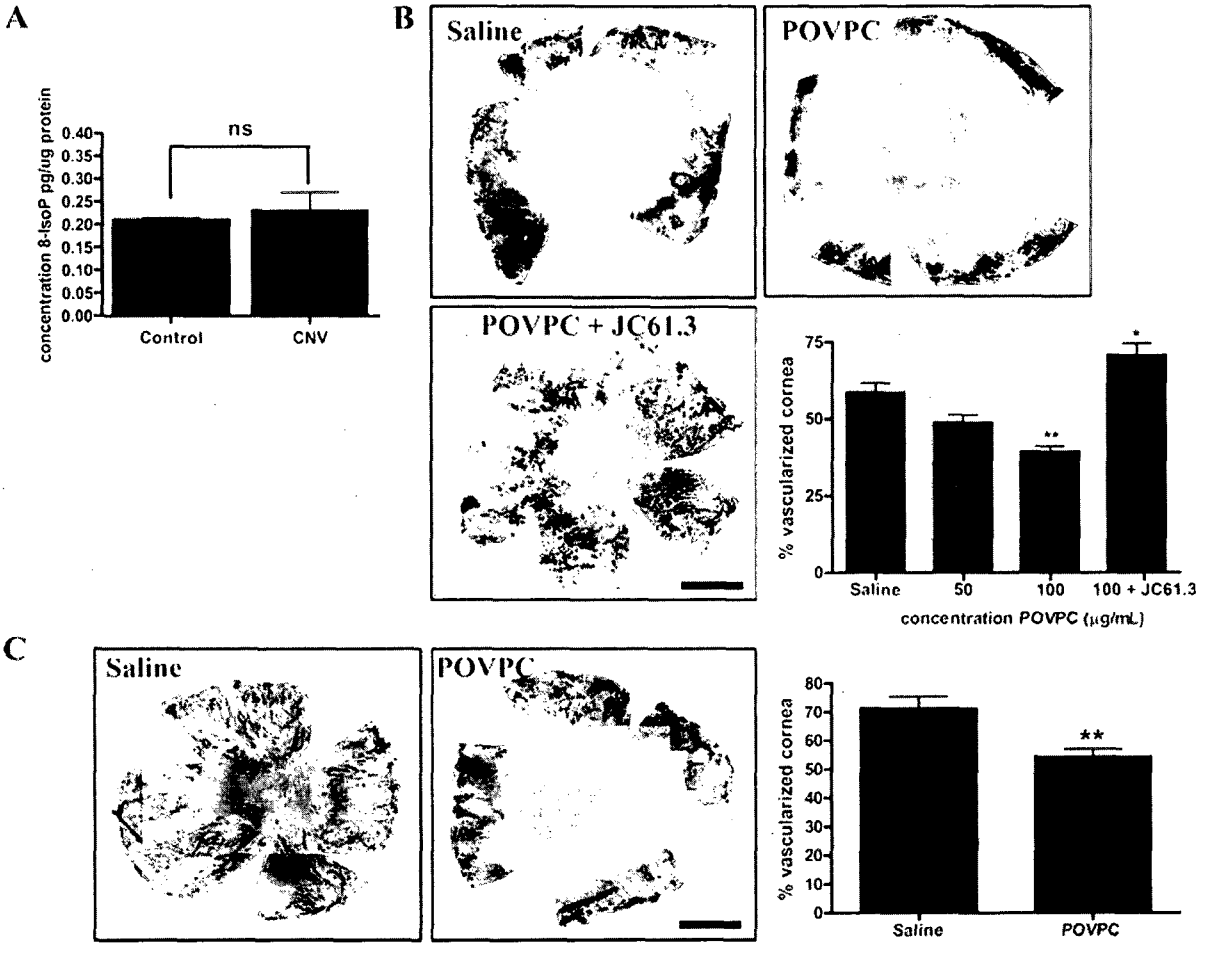
A
concentration 8-IsoP pg/ug protein
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
ns
Control
CNV
B
Saline
POVPC
POVPC + JC61.3
% vascularized cornea
75
50
25
0
**
*
Saline
50
100
100 + JC61.3
concentration POVPC (μg/mL)
C
Saline
POVPC
% vascularized cornea
80
70
60
50
40
30
20
10
0
**
Saline
POVPC

Figure 3

Figure 3. CD36 ligation by an oxidized phospholipid ligand inhibits and induces regression of CNV. (**A**) Levels of 8-isoprostane were measured in normal and 4-day postinjury corneas. (**B**) Mice underwent corneal injury and received three treatments daily of 50 or 100 μg/mL of the oxidized phospholipid 1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine (POVPC), or a cotreatment of POVPC (100 μg/mL) and JC61.3 (100 μg/mL) for 7 days. Corneal flatmounts were labeled with murine CD31-FITC. $*P = 0.02$; $**P = 0.003$ vs. saline. (**C**) Treatments with saline or 100 μg/mL POVPC were initiated 10 days after corneal injury for a period of 7 days, and the vascularized corneal area was subsequently determined and quantified ($**P < 0.001$ vs. saline). Scale bar, 200 μm.

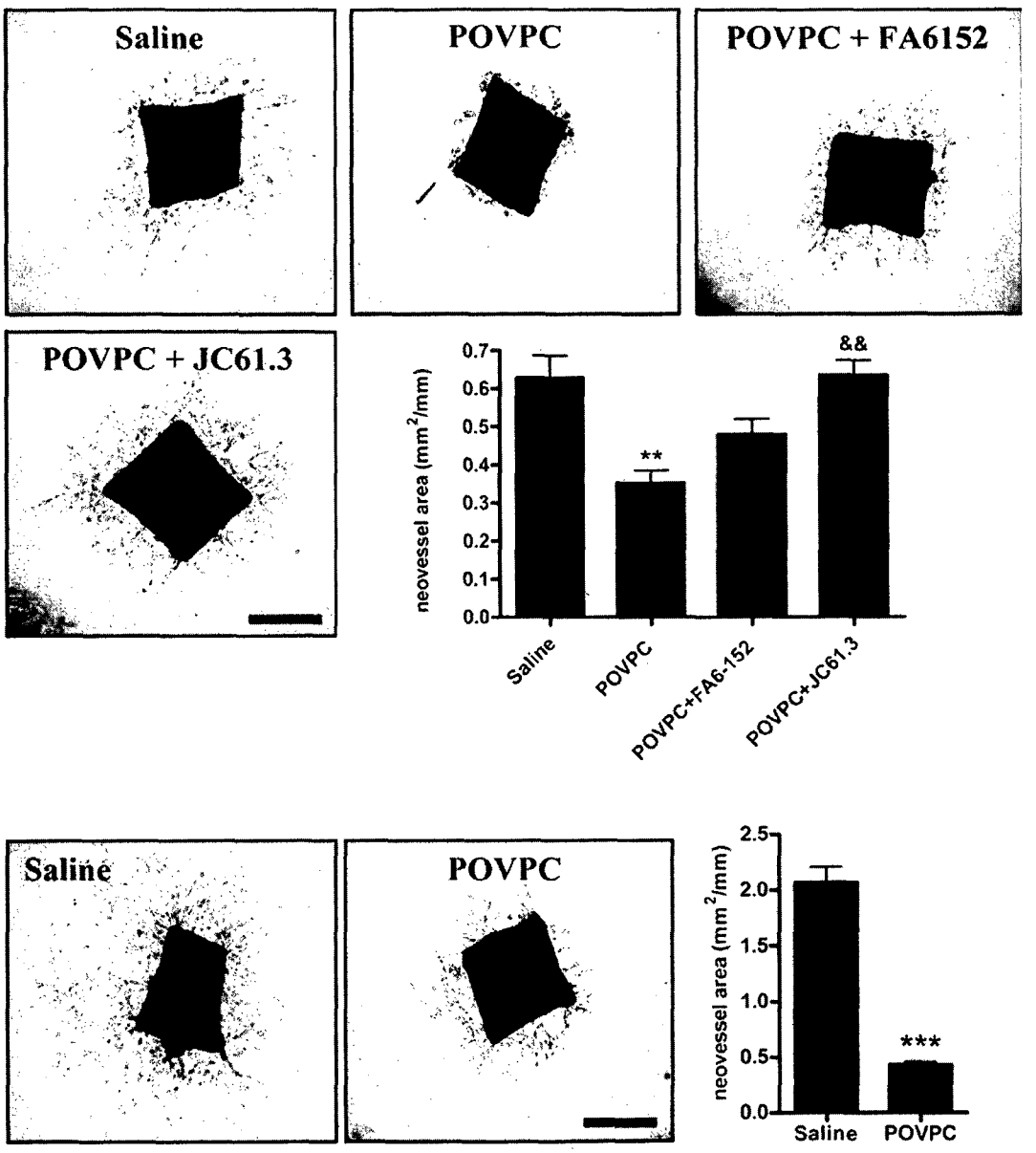
Saline
POVPC
POVPC + FA6152
POVPC + JC61.3
neovessel area (mm²/mm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
&&
Saline
POVPC
POVPC+FA6-152
POVPC+JC61.3
Saline
POVPC
neovessel area (mm²/mm)
2.5
2.0
1.5
1.0
0.5
0.0
***
Saline
POVPC

Figure 4

Figure 4. CD36 activation using POVPC inhibits microvessel outgrowth from aortic ring explants. Mouse aortas were seeded on Matrigel and on day 3 incubated in the presence of (**A**) saline, the oxidized phospholipid 1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine (POVPC; 20 μg/mL), or POVPC plus JC61.3 (6 μg/mL) or FA6-152 (10 μg/mL). Photographs were taken on day 5. (**B**) For regression analysis, aortic rings were treated with POVPC (20 μg/mL) on day 6 and photographs taken on day 7. $^{**}P < 0.001$ vs. saline; $^{\&\&}P < 0.001$ vs. POVPC. Scale bar, 200 μm.

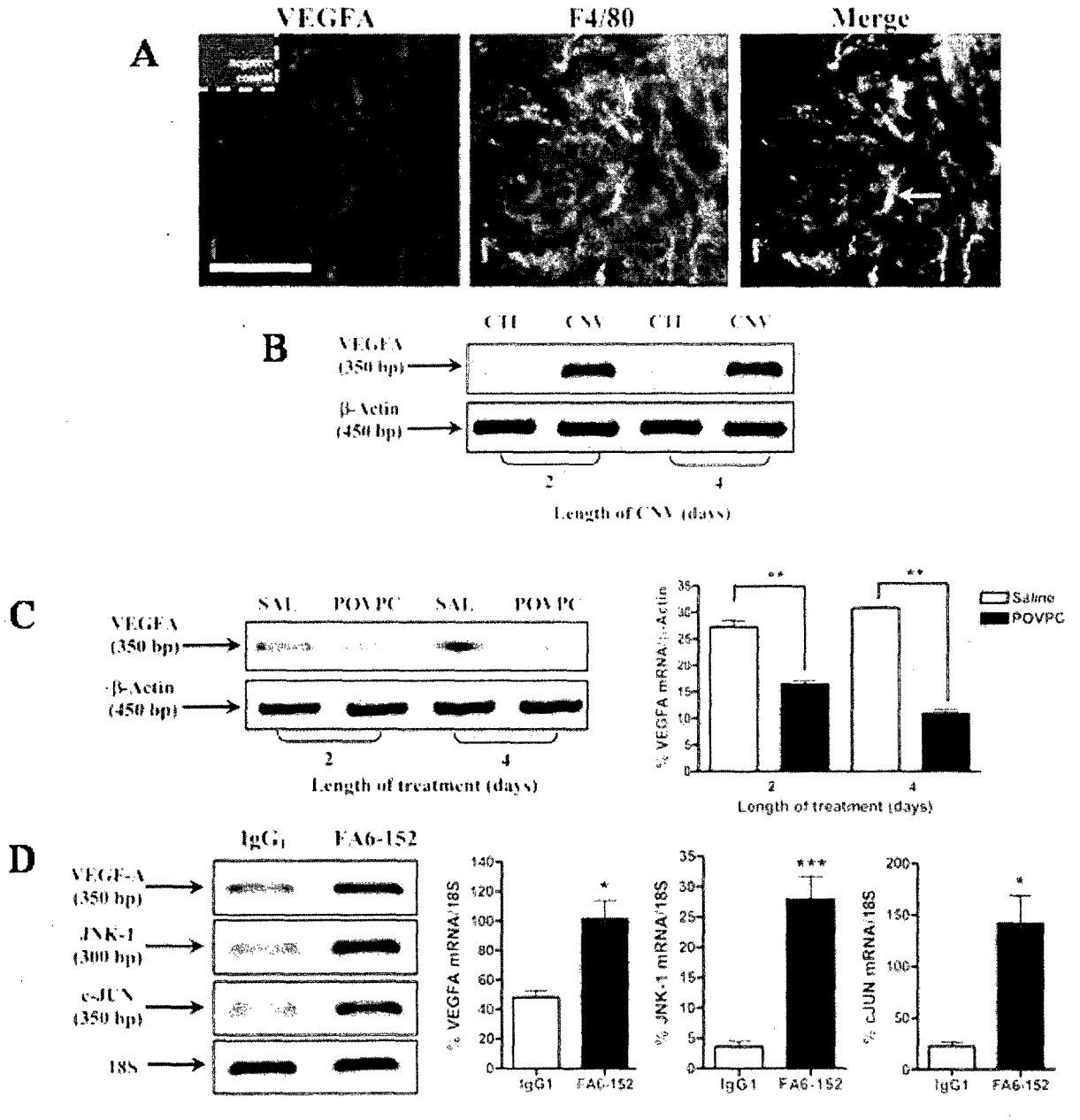
A
VEGFA
F4/80
Merge
B
CTL
CNV
CTL
CNV
VEGFA (350 bp)
β-Actin (450 bp)
2
4
Length of CNV (days)
C
SAL
POVPC
SAL
POVPC
VEGFA (350 bp)
β-Actin (450 bp)
2
4
Length of treatment (days)
Saline
POVPC
% VEGFA mRNA/β-Actin
Length of treatment (days)
D
IgG1
FA6-152
VEGF-A (350 bp)
JNK-1 (300 bp)
c-JUN (350 bp)
18S
% VEGFA mRNA/18S
% JNK-1 mRNA/18S
% cJUN mRNA/18S
IgG1
FA6-152

Figure 5

Figure 5. VEGF-A is expressed by macrophages in the inflamed cornea and is downregulated after activation of CD36. (**A**) Immunofluorescent staining of corneal stromal flatmounts revealed that VEGF-A (*red*) colocalizes with the microglial/macrophage marker F4/80 (*green*) in the inflamed cornea 7 days after injury. *Yellow*: merged image of fluorescent labeling. Scale bar, 30 μm. (**B**) *Top*: VEGF-A mRNA expression 2 and 4 days after corneal injury; *bottom*: ß-actin. (**C**) VEGF-A mRNA expression after 2 and 4 days of POVPC treatment (**$P < 0.001$ vs. saline). (**D**) VEGF-A, JNK-1, and c-JUN mRNA expression after 4 days of FA6-152 treatment (*$P < 0.05$, **$P < 0.001$ vs. IgG_1).

CHAPTER 5: HYPOXIA UPREGULATES CD36 EXPRESSION AND FUNCTION VIA HYPOXIA INDUCIBLE FACTOR-1 AND ROS DEPENDENT MECHANISMS

Submitted for revision in *Free Rad Biol Med.*

5.1 Preamble

In the course of the previous study, we demonstrated strong upregulation of CD36 following inflammatory CNV; similar observations have been reported elsewhere (Cho et al., 2005; Salajegheh et al., 2007). Given that hypoxia is a potent stimulus for neovascularization and that both processes generally occur concomitantly, we explored the hypothesis that hypoxia, and by inference, HIF-1, are critical regulators of CD36 expression and function. To accomplish our objectives, CD36 expression was analyzed in corneal and retinal tissue from hypoxia-exposed animals, meanwhile *in vitro*, RPE cells were utilized to delineate the putative mechanisms involved.

HYPOXIA UPREGULATES CD36 EXPRESSION AND FUNCTION VIA HYPOXIA INDUCIBLE FACTOR-1 AND ROS DEPENDENT MECHANISMS

Running title: Hypoxic regulation of CD36

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5.2 Abstract

Neovascular and degenerative diseases of the eye are leading causes of impaired vision and blindness in the world. Hypoxia or reduced oxygen tension is considered central to the pathogenesis of these disorders. While the CD36 scavenger receptor features prominently in normal ocular function with demonstrated roles during ocular neovascularization, little is known regarding its modulation during hypoxia. Herein we investigated the role and the regulation of CD36 by hypoxia and by the major hypoxia effector, hypoxia inducible factor (HIF)-1. *In vivo*, hypoxia markedly induced CD36 mRNA levels in corneal and retinal tissue. Subsequent experiments on human retinal pigment epithelial cells revealed that hypoxia time-dependently increased CD36 mRNA, protein, and surface expression; this induction was reliant upon ongoing translation, but not on elevations in either mRNA or protein stability. Inhibition of the major cellular sources of reactive oxygen species (ROS), the NADPH oxidase and mitochondria, abrogated the CD36 hypoxic response, as did inhibitors of the ROS-dependent phosphatidylinositol-3-kinase translational pathway. Importantly, the hypoxic induction of CD36 was abolished when HIF-1α was downregulated by siRNA and a HIF-1α inhibitor. The functional ramifications of CD36 hypoxic accumulation were evinced by CD36 specific augmentations in oxLDL uptake and apoptotic cell phagocytosis. Collectively, the present findings provide unique insight into a previously undisclosed induction of CD36 by hypoxia through HIF-1 dependent mechanisms and associated increases in CD36 scavenging activity. As such, augmentations of CD36 by hypoxia may represent a cellular adaptive response

aimed at protecting the susceptible ocular environment from the deleterious consequences of hypoxia.

Key words: CD36; hypoxia; HIF-1; reactive oxygen species; RPE; ocular

5.3 Introduction

Hypoxia, a reduction in cellular oxygen tension, is a key determinant of tissue pathology and survival during tumor development and ischemic diseases including retinopathies, myocardial infarction, and atherosclerosis. In response to hypoxia, mammalian cells express a variety of gene products important for erythropoiesis, angiogenesis, and glycolysis, thereby improving tissue oxygenation and facilitating metabolic demands [1]. These adaptive responses are mediated by a heterodimeric transcriptional complex termed hypoxia inducible factor (HIF), which is generally considered the master regulator of oxygen homeostasis. HIF is comprised of an oxygen regulated α subunit (HIF-1α) and the ubiquitous aryl hydrocarbon receptor nuclear translocator (ARNT or HIF-1β) [2-4]. HIF-1α protein turnover in normoxia is very rapid due to the action of HIF-1α prolyl hydroxylases (PHDs). These oxygen-dependent enzymes hydroxylate two conserved proline residues of HIF-1α, promoting binding of the Von Hippel-Lindau protein, ubiquitination, and subsequent proteosomal degradation. Under hypoxic conditions the hydroxylases are inhibited, and transcriptionally activated, leading to potent induction of target genes [2, 4-7]. Of relevance, it has become increasingly evident that reactive oxygen species (ROS) play a crucial role in regulating HIF-1 activity [8] such that ROS efficiently stabilize HIF-1α under hypoxia [9, 10]. Because hypoxia, ROS production, and inflammation typically occur concurrently, it is conceivable that scavenger receptors specifically CD36, may contribute towards hypoxia-induced adaptive responses. As such, CD36 is an

important participant of inflammatory, angiogenic, and oxidative processes [11, 12].

CD36 is a heavily glycosylated transmembrane protein that belongs to an evolutionarily conserved family of scavenger receptors [11]. This multifunctional receptor is expressed on the surface of microvascular endothelial cells, macrophages, dendritic cells, platelets, smooth muscle cells [11] and specialized epithelial cells of the cornea [13] and retina [14]. CD36 functions in scavenger recognition of oxidized low density lipoproteins (oxLDL), oxidized phospholipids (oxPL), apoptotic cells, and photoreceptor rod outer segments [11, 14, 15], in addition to having established roles as a fatty acid transporter [16, 17] and a potent inhibitor of angiogenesis [11, 13, 18]. Nevertheless, despite the multiple roles ascribed to CD36, limited data exists regarding its regulation by hypoxia, albeit several lines of evidence would support this inference.

It was recently described that CD36 expression was upregulated during chronic lung hypoxia, with the identification of a putative hypoxia response element in the CD36 gene [19]. Consistent with this, we have reported that inflammatory corneal neovascularization induces elevated levels of CD36 [13], whereas others have documented an upregulation of CD36 during cerebral ischemia [20] and an attenuation of its expression and activity following antioxidant treatment [21, 22]. Taken together, these studies led us to hypothesize that hypoxia modulates CD36 expression and function, with the objective of characterizing the mechanisms behind its regulation. Indeed, we report that hypoxia augments CD36 mRNA and protein levels through mechanisms

implicating the HIF-1 pathway, mRNA translation, ROS generation, and associated increases in CD36 functional activity.

5.4 Materials and Methods

Compounds and antibodies

Actinomycin D, allopurinol, angiotensin II, cobalt chloride, myxothiazol, rotenone, isotype control antibodies anti-human IgA, anti-mouse IgG_1, and anti-mouse IgM (Sigma Aldrich); cycloheximide, rapamycin (Calbiochem); monoclonal β-Actin, CD36 monoclonal antibody (mAb) clone SMO (Abcam plc); CD36 rabbit polyclonal antibody and horseradish peroxidase linked anti-rabbit IgG (Santa Cruz Biotechnology); monoclonal CD36-FITC (Serotec, Oxford, UK); CD36 mAb clone JC63.1 and YC-1 (Cayman Chemical, Ann Arbor, MI); U74389G (Biomol, PA, USA); Tempol (Fluka Biochemika); DiI-oxLDL (Intracel, Frederick, MD); wortmannin and LY294002 (Alomone Labs Ltd.); CD36 mAb clone FA6-152 (Beckman Coulter, Fullerton, CA); dichlorofluorescein diacetate (DCFDA), annexin V-FITC/PI staining (Molecular Probes, Eugene, OR); CellTracker Green 5-fluoromethylfluorescein diacetate (CMFDA; Cambrex Corp.); Lipofectamine 2000 (Invitrogen Corp.).

Animals and *in vivo* hypoxia model

Six week old male C57BL/6 mice purchased from Charles River (St-Constant, Quebec, Canada) were used according to a protocol approved by the CHU Sainte-Justine Research Center Animal Care Committee. Mice were exposed to ambient room air or placed in an OxyCycler (BioSpherix, Ltd.) and

exposed to hypoxia (8% O_2) for 6 hours. At the end of the experiments, animals were immediately sacrificed and tissues were processed for quantitative real-time PCR (qRT-PCR) analysis.

Cell culture and hypoxia

Human dermal microvascular endothelial cells (HMVECs) and human pulmonary artery smooth muscle cells (PASMCs) (Cambrex, Walkersville, MD), and human retinal pigment epithelial (ARPE-19) and human promyelocytic leukemia (HL60) cells (ATCC, Manassas, VA) were maintained according to standard procedures. For the majority of experiments, ARPEs were used because of their relevance to our ocular studies. For hypoxia exposure, culture dishes were placed in a hypoxia chamber (Billups Rothenburg Inc.) allowing the establishment of a hypoxic environment of 2% O_2, 5% CO_2, 93% N_2; unless otherwise stated, this hypoxic level (for 24 hours) was used in all experiments. The airtight incubator was kept at 37°C for preset time periods whereas normoxic cells were placed at 37°C in a 21% O_2, 5% CO_2 and 74% N_2 humidified incubator.

Downregulation of HIF-1α with silencing RNA

ARPEs were grown to 40% confluence and transfected using Lipofectamine 2000 (Invitrogen) with 50 nM scrambled Silencer Negative Control 1 siRNA (Ambion) or sequence-specific siRNA targeting HIF-1α (Ambion; pre-designed siRNA ID#106498 and #106500, both showed similar knock-down effectiveness and the former was used in all experiments). Cells were incubated with HIF-1α siRNA for 24 h after which they were subjected to

hypoxia followed by analysis by quantitative real-time polymerase chain reaction (qRT-PCR).

RNA isolation and qRT-PCR

Total RNA was extracted using the standard TRIzol RNA isolation protocol (Invitrogen Corp.) and treated with DNase I (Qiagen, Hilden, Germany). cDNA was synthesized from 1 μg RNA with M-MLV reverse transcriptase (Promega Corp.) according to the manufacturer's instructions and amplified using SYBR Green I (Stratagene, Cedar Creek, TX) in a sequence detection system (MxPro 3000 QPCR Systems, Stratagene). Primers for CD36, HIF-1α, and β-Actin were synthesized by Invitrogen Corporation as follows: human CD36, forward 5'-TCTTTCCTGCAGCCCAATG-3', reverse 5'-AGCCTCTGTTCCAACTGATAGTGA-3'; human HIF-1α, forward 5'-TGCTTGGTGCTGATTTGTGA-3', reverse 5'-GGTCAGATGATCAGAGTCCA-3'; human β-actin, forward 5'-GGGRCAGAAGGATTCCTATG-3', reverse 5'-GGTCTCAAACATGATCTGGG-3'; mouse CD36, forward 5'-TCCTCTGACATTTGCAGGTCTATC-3', reverse 5'-AAAGGCATTGGCTGGAAGAA-3'; mouse β-actin, forward 5'-ACTATTGGCAACGACCGGTTT-3', reverse, 5'-AAGGAAGGCTGGAAAAGAGGG-3'. PCR amplification protocol involved 40 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C, and primer

extension at 72°C for 60 s. Each sample was analyzed in triplicate along with RT- and no-template controls.

Determination of total CD36 expression by Western blot analysis

Protein extraction from cells and Western blots were performed as previously described [23] using 40 μg total protein and anti-CD36 (1:400) and anti-β-Actin (1:10000) antibodies. Protein expression was quantified via densitometry (Image-Pro Plus software, ver. 4.1; Media Cybernetics, Silver Spring, MD).

Determination of CD36 surface expression

ARPEs at 70 % confluence were exposed to normoxia or hypoxia followed by analysis of CD36 expression via fluorescence-activated cell sorting (FACS) (FACScan; Becton-Dickinson) using an anti-CD36-FITC antibody. A minimum of 10, 000 cells/sample was assessed. Data was acquired and analyzed using CellQuest software.

Immunofluorescence

ARPEs were seeded onto 15 mm glass coverslips and exposed to normoxia or hypoxia. Cells were fixed with 4% paraformaldehyde, permeabilized with PBS/0.1% Triton X-100, blocked with PBS/5% goat serum/5% calf serum, and incubated overnight at 4°C with an anti-CD36 (1:100) antibody followed by IgG-FITC (1:300). Stained cells were observed using a Nikon eclipse E800 epifluorescent microscope with Nikon digital camera DXM 1200.

Measurement of mRNA stability, mRNA translation, and protein stability

ARPEs were pre-exposed to normoxia or hypoxia followed by addition of actinomycin D (ActD; 4.5 μg/mL) to block transcription and subsequent return to normoxia or hypoxia. Cells were harvested at various time points after ActD treatment and processed for qRT-PCR. CD36 mRNA stability was determined as the percentage of initial mRNA remaining after ActD exposure. To elucidate the contribution of ongoing protein synthesis and protein stability, cycloheximide (CHX) was added to block translation before (25 μM) or after (100 μM) hypoxia. CD36 protein stability was assessed as the proportion of initial protein remaining after CHX exposure.

ROS and 8-Isoprostane measurements

Intracellular ROS generation was measured using the fluorescent probe DCFDA. ARPEs were cultured in 24-well plates and exposed to hypoxia at indicated time points or angiotensin (Ang II; 100 nM) for 45 minutes. Cells were subsequently incubated with DCFDA (10 μM) for 30 minutes at 37°C followed by measurements using a multi-well fluorescent plate reader (Wallac 1420 VICTOR Multilabel Counter) set at 485 nm excitation and 535 nm emission wavelengths. 8-Isoprostanes (8-Iso-PGF2α) were measured in normoxia and hypoxia exposed ARPEs by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) as previously described [13].

Uptake of DiI labelled oxLDL

Analysis of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-oxidized LDL (Intracel, Frederick, MD) uptake was performed according to the manufacturer's protocol with minor modifications. Briefly, cells were pre-treated for 2 hours with IgA or 10 μg/mL anti-CD36 mAb (clone JC63.1) prior to normoxia or hypoxia exposure. Next, cells were incubated for 6 hours with DiI-oxLDL (20 μg/ml) in serum free-media at 37°C, washed extensively with PBS/0.1% BSA, pelleted, and resuspended in 400 μL FACS flow for immediate FACS analysis.

Preparation of apoptotic HL60 cells and phagocytosis assay

A modified version of the phagocytic assay conducted by Greenberg et al. (2006) was used. HL60 cells were pre-labelled with 5 μM CellTracker Green (CMFDA) for 45 min at 37°C according to the manufacturer's instructions (Cambrex Corp.). Cells were subsequently rendered apoptotic after treatment with 5 μM camptothecin (4 hours), washed with media, and cultured for an additional 6 hours before use. Apoptosis was confirmed by DNA laddering and annexin V-FITC/PI staining (> 50% annexin V positive). Meanwhile, ARPEs were pre-treated for 2 hours with IgA or JC63.1 (10 μg/mL) and exposed to normoxia or hypoxia. $5x10^5$ apoptotic cells were then allowed to incubate for 3 hours on ARPE monolayers in serum free-media. After incubation, unbound apoptotic cells were removed by extensive washing with media. Bound or ingested HL60 cells were detected by their green fluorescence and analyzed by immunofluorescence.

Aortic ring angiogenesis assay

This assay was performed as described previously [13]. The aortic ring culture media was changed on day 4 with addition of the following test compounds: CD36 mAbs clones FA6-152 (10 μg/mL) and SMO (5 μg/mL) and their respective isotype controls IgG_1 and IgM. Photographs were taken before (day 4) and after treatment (day 5) using an inverted microscope (Eclipse TE300; Nikon). The area of neovessel formation was determined using Image Pro Plus software.

Statistical analysis

All experiments were repeated at least three times and values are presented as means ± SEM. Data were analyzed by Student's *t* test, one-way or two-way ANOVA followed by post-hoc Bonferroni tests for comparison among means. Statistical significance was set at $p<0.05$.

5.5 Results

Hypoxia upregulates CD36 mRNA levels *in vivo*

As previously mentioned, we reported that inflammatory corneal neovascularization potently elevates CD36 expression [13]. Because inflammation and hypoxia occur concomitantly, we surmised that CD36 expression is modulated by hypoxia. To test this hypothesis, CD36 mRNA levels were determined in corneal and retinal tissue following mouse subjection to ambient room air or whole body hypoxia (8% O_2, 92% N_2 for 6 h). We focused our attention on the cornea and retina due to their abundant CD36 expression [13,

14, 23]. As shown in Fig. 1, CD36 expression was markedly increased by 3.6 and 6.0 fold in corneal and retinal tissue derived from hypoxic mice.

Effect of hypoxia on CD36 mRNA, protein, and surface expression *in vitro*

Given the ability of hypoxia to potently induce CD36 mRNA levels *in vivo*, we explored the kinetics of the hypoxic regulation of CD36 in various cell types that are known to highly express CD36. HMVECs, ARPEs, and PASMCs were exposed to hypoxia (2% O_2) from 0 to 24 h and CD36 expression was assessed by qRT-PCR. Exposure to hypoxia resulted in a time-dependent increase in CD36 mRNA in all cell types that was evident at 3 h and sustained up to 24 h ($p<0.01$; Fig. 2A). Based on these findings and the demonstrated induction of CD36 in hypoxic retinal tissue, subsequent experiments were conducted using ARPEs.

Accordingly, we next performed Western blot analysis on ARPEs exposed to hypoxia for various time-points and observed a parallel increase in CD36 protein as early as 6h (Fig. 2B). Comparable results were obtained by flow cytometry ($p<0.001$; Fig. 2C) and immunofluorescence ($p<0.001$; Fig. 2D) whereby a significant increase in CD36 surface expression was observed following hypoxia.

Implication of translational activity in the hypoxic regulation of CD36

Accumulation of CD36 during hypoxia could result from increases in mRNA stability, mRNA translation, or protein stability. To test the possibility that CD36 mRNA was post-transcriptionally stabilized in response to hypoxia, ARPEs were maintained in normoxia or hypoxia for 24 h, transcription was blocked with

ActD (4.5 μg/mL), and cells were harvested at different time points for qRT-PCR anlaysis. The stability of CD36 mRNA remained unchanged by hypoxia; the percentage of initial CD36 mRNA was 68.8 ± 2.6 % (normoxia) and 70.5 ± 0.9 % (hypoxia) ($p>0.05$; Fig. 3A) following 6h of ActD treatment.

To elucidate the importance of ongoing protein synthesis on the hypoxic regulation of CD36, ARPEs were pre-treated with the translation inhibitor cycloheximide (CHX; 25 μM) before hypoxic exposure. CHX potently abrogated the upregulation of CD36 protein observed during hypoxia (hypoxia, 148 ± 8 % vs. hypoxia + CHX, 94 ± 6 %; $p<0.01$; Fig. 3B).

The effect of hypoxia on CD36 protein stability was determined by investigating the time-course of CD36 protein decay in normoxia or hypoxia pre-exposed cells treated with CHX (100 μM). Hypoxia dramatically reduced the CD36 protein levels such that after 240 mins, 28 ± 4 % of the initial protein remained compared to 64 ± 3.2 % under normoxia (Fig. 3C, D).

Reactive oxygen species are involved in the hypoxic regulation of CD36 mRNA

Studies have demonstrated that ROS generated during cerebral ischemia are markedly attenuated in CD36-null mice and that treatment with antioxidant peptides downregulates ischemia-induced CD36 expression [20, 21]. Similarly, the antioxidant α-tocopherol has been shown to diminish CD36 overexpression in monocytes stimulated by oxLDL [24]. Consequently, we sought to determine whether ROS activity was implicated in the hypoxic regulation of CD36. Given the controversial relationship between ROS production and hypoxia [1, 25, 26],

we first measured the levels of ROS and 8-IsoP (well established markers of lipid peroxidation) [27-29] in normoxia and hypoxia exposed cells. As indicated in Fig. 4, after 24 hours, hypoxia caused a 93 % and 30 % increase in intracellular ROS ($p<0.001$; Fig. 4A) and 8-IsoP ($p<0.05$; Fig. 4B) levels respectively. Intriguingly, correlation analysis revealed that CD36 mRNA levels were significantly correlated with ROS levels during hypoxia ($r = 0.9622$; $p < 0.01$; Fig. 4C). Consistent with this, the superoxide dismutase mimetic tempol (1 mM) and inhibitors of lipid peroxidation (U74389G, 50 μM) and the superoxide generating enzyme NADPH oxidase (apocynin, 50 μM), abrogated the hypoxia-induced increase in CD36 mRNA (Fig. 4D). Conversely, inhibition of xanthine oxidase (allopurinol, 50 μM), another major ROS generating enzyme, failed to attenuate the CD36 hypoxic signal (Fig. 4D).

Recent evidence has implicated mitochondria in the O_2 sensing underlying functional responses to hypoxia [1] and has shown that the rate of mitochondrial ROS production is elevated during hypoxia [30]. Accordingly, we examined the effects of mitochondrial ROS inhibitors on CD36 mRNA levels under hypoxia. Inhibition of mitochondrial electron transport complexes (ETC) I and III with rotenone (100 nM) and myxothiazol (200 nM) respectively, markedly suppressed the induction of CD36 by hypoxia ($p<0.001$; Fig. 4E).

The PI3K/mTOR pathway is implicated in the hypoxic induction of CD36 transcripts

Our findings thus far suggested that ongoing protein synthesis (Fig. 3B) and ROS production (Fig. 4) were essential for the CD36 hypoxic response.

Because ROS-dependent activation of the phosphatidylinositol-3-kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling pathway has been linked to the translational regulation of several hypoxia inducible genes [31, 32], we evaluated the contribution of this cascade. ARPEs were pre-treated for 1 h before hypoxic exposure with two structurally distinct PI3K inhibitors wortmannin (200 nM) and LY294002 (20 μM), as well as rapamycin (100 nM), an inhibitor of the downstream PI3K effector mTOR. As seen in Fig. 5, the hypoxic response (4.7 fold) was significantly ablated when wortmannin, LY294002, and rapamycin were applied (wortmannin pre-exposed + hypoxia, 0.3 fold; LY294002 pre-exposed + hypoxia, 0.2 fold; rapamycin pre-exposed + hypoxia, 0.3 fold).

Role of HIF-1α in CD36 hypoxic regulation

Important insights into the mechanism of oxygen sensing have been garnered using pharmacological hypoxia mimetics such as $CoCl_2$ [33], which is clearly documented to result in upregulation of HIF-1, a ubiquitous transcription factor responsible for the hypoxic activation of multiple genes [34-36]. Incidentally, our experiments focused on HIF-1α because this subunit is the key component of HIF-1 and its expression determines the cellular HIF-1 activity. When ARPEs were treated with $CoCl_2$ (200 μM, 6 h), a 3.3 fold rise in CD36 mRNA was observed and this was comparable to HIF-1α whose expression was induced by 4 fold (Fig. 6A). To further pursue the involvement of HIF-1, ARPEs were exposed to hypoxia in the presence or absence of HIF-1α siRNA (50 nM) or YC-1 (5 μM), a HIF-1 inhibitor that has been demonstrated to exert a novel inhibitory effect on

HIF-1α accumulation [33-35]. YC-1 potently suppressed the hypoxic induction of CD36 expression at both the mRNA (hypoxia, 2 fold vs. hypoxia + YC-1, 0.4 fold; $p<0.01$; Fig. 6B) and protein (hypoxia, 179 ± 8 % vs. hypoxia + YC-1, 63 ± 8 %; $p<0.001$; Fig. 6C) level [37-39]. Importantly, these observations were corroborated when knock-down of HIF-1α by siRNA abolished the induction of CD36 mRNA (hypoxia, 4.6 fold vs. hypoxia + HIF-1α siRNA, 0.4 fold; $p<0.001$; Fig. 6D).

Role of CD36 in mediating the uptake of oxLDL, phagocytosis of **apoptotic cells, and inhibition of angiogenesis during hypoxia**

Although we provide abundant data corroborating the hypoxic regulation of CD36, we considered it pertinent to correlate these findings with a functional consequence. Of relevance, a growing body of evidence has implicated CD36 in the recognition and uptake of oxLDLs and apoptotic cells [11, 40], while we and others have demonstrated that hypoxia promotes lipid peroxidation (Fig. 4B) [41, 42] and apoptosis [43]. Accordingly, we investigated the functional ramifications of the hypoxia-induced CD36 overexpression by evaluating the uptake of oxLDLs and apoptotic cells during hypoxia. First, ARPEs were pre-exposed to hypoxia for 16 hours followed by incubation with DiI-oxLDL (20 μg/mL, 6 h) and FACS analysis. Our results demonstrate that hypoxia caused an increase in DiI mean fluorescence (normoxia, 100 ± 0.1 % vs. hypoxia, 105 ± 0.8 %; $p<0.001$) that was blocked by JC63.1, an anti-oxLDL binding site CD36 mAb (90.0 ± 0.2 %; $p<0.001$) (Fig. 7A).

To explore the influence of hypoxia on CD36 mediated engulfment of apoptotic cells, confluent monolayers of normoxia and hypoxia exposed ARPEs were co-cultured with viable versus apoptotic (5 μM camptotecin) HL60 cells labelled with the fluorescent probe CellTracker green. After incubation, nonadherent/nonphagocytosed cells were removed by vigorous washing, and phagocytosis of apoptotic cells was determined as the ratio of bound HL60 cells to the total number of adherent ARPEs. Relative to normoxia, hypoxia caused a pronounced increase in apoptotic cell phagocytosis (26 ± 3.2 % vs. 63.5 ± 8 % respectively, $p<0.01$) that was significantly abrogated in the presence of JC63.1 (31.5 ± 9.6 %, $p<0.05$) (Fig. 7B).

Because it is well established that CD36 transduces signals leading to apoptosis dependent inhibition of angiogenesis [44], we next tested the outcome of blocking or activating CD36 in a hypoxia-driven model of aortic ring angiogenesis. As expected, compared to normoxia (0.91 ± 0.04 mm^2), hypoxia (1.2 ± 0.05 mm^2) increased aortic ring microvessel sprouting. In contrast, incubation of hypoxia-exposed aortic rings with CD36 functionally blocking (FA6-152) or activating (SMO) mAbs, respectively exacerbated (1.5 ± 0.1 mm^2) or suppressed (0.4 ± 0.04 mm^2) the area of microvessel formation (Fig. 7C).

5.6 Discussion

The CD36 scavenger receptor has emerged as a pivotal player in diverse homeostatic and pathological settings including those affecting ocular tissue [11, 13-15, 23]. Although hypoxia is considered a major stimulus of numerous disease

states including ischemic retinopathies and ocular neovascularization, the molecular mechanisms and pathways governing the regulation of CD36 by hypoxia have yet to be elucidated. Herein, we demonstrate novel hypoxic induction of CD36 that is mediated by the HIF-1 transcription factor and ROS-dependent mechanisms.

Hypoxic injury to ocular tissue is known to elicit lipid peroxidation and subsequent generation of oxPL and oxLDLs both of which are prominent CD36 ligands [11, 41]. Along these lines, the cornea and retina express abundant surface CD36 [13, 14, 23] and are rich in polyunsaturated phospholipids [45-47] which renders them particularly susceptible to oxidant stress. It was therefore intriguing to observe a substantial rise in CD36 corneal and retinal mRNA expression following hypoxia (Fig. 1), a finding that was confirmed by comparable augmentations in CD36 expression in microvascular endothelial cells, RPE cells, and smooth muscle cells (Fig. 2A). Based on this evidence, we surmise that the hypoxic induction of CD36 is not cell or tissue type specific, but may represent a universal adaptive response to hypoxia.

Ample studies have documented that oxidative stress stimulates CD36 expression and that antioxidants reduce its expression and function [20-22]. Indeed, our results link the CD36 hypoxic response to the cellular redox state given that lipid peroxidation and ROS production were increased during hypoxia and in this process CD36 mRNA and ROS levels were positively correlated (Fig. 4A-C). Experimental data has also revealed that the NADPH oxidase [48, 49] and mitochondrial ETC complexes I and III are major cellular sources of ROS principally in the form of the superoxide anion and hydrogen peroxide [9, 25, 50].

Our observations are in accordance with this data since inhibition of the aforesaid ROS sources abrogated the CD36 hypoxic signal (Figs. 4 D, E) and is substantiated by a study reporting that induction of CD36 expression in human monocytes involves redox signaling via the NADPH oxidase [49].

The current data provide presumptive evidence of translational modulation of CD36. First, we demonstrate that cycloheximide, a protein synthesis inhibitor, ablated the CD36 hypoxia evoked induction (Fig. 3B). Second, both CD36 mRNA and protein were not stabilized by hypoxia (Figs. 3A, C), suggesting that the CD36 accumulation may be dependent on continuous translation of CD36 mRNA. Third, the CD36 hypoxic response was sensitive to inhibitors of the PI3K/mTOR signaling cascade which is known to regulate the translation rate of particular mRNAs (Fig. 5) [51-54]. In this context, we propose a mechanism whereby overproduction of ROS during hypoxia activates the PI3K/mTOR pathway thus initiating a translation stimulation signal leading to enhanced CD36 expression. Indeed, similar models depicting translational modulation of HIF-1α through the PI3K/mTOR signaling pathway have been proposed [6, 54].

The novel contribution of our work regarding hypoxia-induced transcriptional regulation of CD36 is that, like many hypoxia-inducible genes, this receptor is regulated by the transcriptional activator HIF-1. Along these lines, we attained an increase in CD36 mRNA levels following administration of $CoCl_2$, a pharmacological activator of HIF-1 (Fig. 6A), and a significant attenuation when the HIF-1 inhibitor, YC-1 [33-39] and HIF-1α siRNA were employed (Fig. 6B, C, D). Moreover, we (data not shown) and others [19] have identified a putative HIF consensus site in the CD36 promoter; whether this site is functional remains to be

determined in the future by reporter construct or mutational studies. Collectively, these data strongly support a role for HIF-1 in the transcriptional modulation of CD36, although it is conceivable that other transcription factors that act synergistically with HIF-1 are involved.

Recent efforts have demonstrated that a variety of stimuli including hypoxia trigger translocation of CD36 from intracellular pools to the plasma membrane with consequent elevations in fatty acid transport [55]. Though our data reveal analogous hypoxia-elicited increases in CD36 surface expression (Fig. 2C, D), unlike in the aforementioned study, we observed a parallel increase in CD36 total protein which was not accompanied by subcellular redistribution of the receptor (data not shown). Then again, in agreement with our findings, Chabowski et al. [55] show that increased CD36 plasmalemmal expression is associated with enhanced functional activity.

It is intriguing to speculate on the functional relevance of CD36 overexpression in oxygen-reduced environments. Hypoxia is reported to trigger lipid peroxidation (Fig. 4B) [27-29, 42] and apoptosis [56], while CD36 is one of several receptors implicated in mediating oxLDL uptake and apoptotic cell clearance [11, 40], functions that we demonstrated to be markedly enhanced during hypoxia in a CD36 specific manner (Figs. 7A, B). Furthermore, the aforementioned processes may occur via similar mechanisms because CD36 recognizes oxPL species in the apoptotic cell membrane that mimic the oxLDL structure [40]. By extension and of pathophysiological relevance, it is conceivable that the hypoxic induction of CD36 in the retina (Fig. 1B), particularly the RPE (Figure 2A), is associated with increases in clearance of effete photoreceptor outer

segments (POS), a process that is critical for normal visual function [14, 15, 45]. Studies have also affirmed that CD36 signaling may be indispensable for RPE POS phagocytosis under conditions of heightened oxidative stress [45].

Present dogma states that angiogenesis, a critical physiological response to hypoxia, is controlled by a dynamic balance between pro- and anti- angiogenic factors [57-59]. Our findings that hypoxia augmented CD36 expression in microvascular endothelial cells (Fig. 2A) and modulated hypoxia-driven aortic ring sprouting (Fig. 7C) was somewhat surprising given the angio-inhibitory nature of this receptor [11, 13, 44]. Nonetheless, studies have reported overexpression of the CD36 ligand, TSP-1, in hypoxia exposed endothelial cells [60] and leg ischemia, where it is inferred that such an overexpression exerts an anti-angiogenic effect aimed at counteracting the hypoxia-induced pro-angiogenic drive [61]. A similar mechanism could thus be operative regarding CD36 accumulation during hypoxia.

In conclusion, our studies provide the first characterization of CD36 as a hypoxia responsive gene that is rapidly activated at the mRNA and protein level via HIF-1, translational, and ROS mechanisms that culminate in enhanced CD36 functional activity, as proposed by the schematic model in Fig. 8. Given the importance of CD36 in cellular biology and pathophysiology, as well as the central role of hypoxia in many disease states, delineating the mechanisms underlying hypoxic regulation of CD36 may provide a valuable framework for future studies investigating the regulatory elements of this receptor. As such, the hypoxic induction of CD36 may serve as a universal adaptive and protective response; this may have notable implications for the development of therapeutic

strategies against eye diseases involving abnormal neovascularization or retinal degeneration.

5.7 Acknowledgments

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5.8 Abbreviations

ActD	actinomycin D
CHX	cycloheximide
$CoCl_2$	cobalt chloride
ETC	electron transport chain
HIF	hypoxia inducible factor
mAb	monoclonal antibody
mTOR	mammalian target of rapamycin
oxLDL	oxidized low density lipoprotein
oxPL	oxidized phospholipid

PI3K	phosphoinositide-3-kinase
POS	photoreceptor outer segment
qRT-PCR	quantitative real-time polymerase chain reaction
ROS	reactive oxygen species
RPE	retinal pigment epithelium

5.9 References

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5.10 Figures

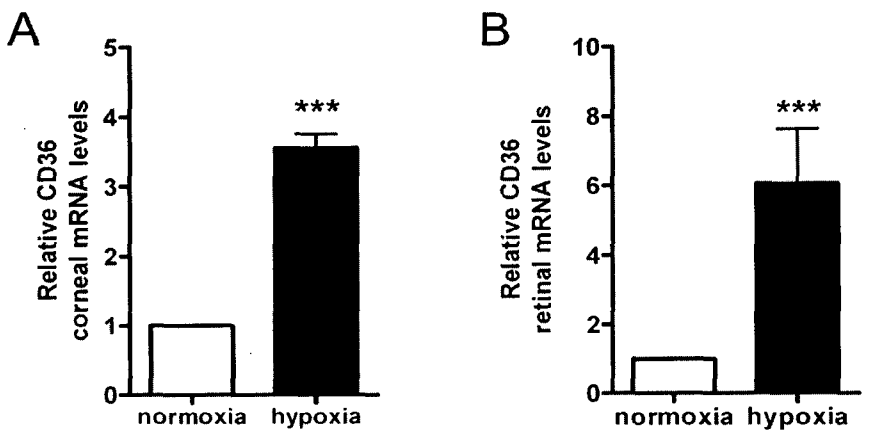
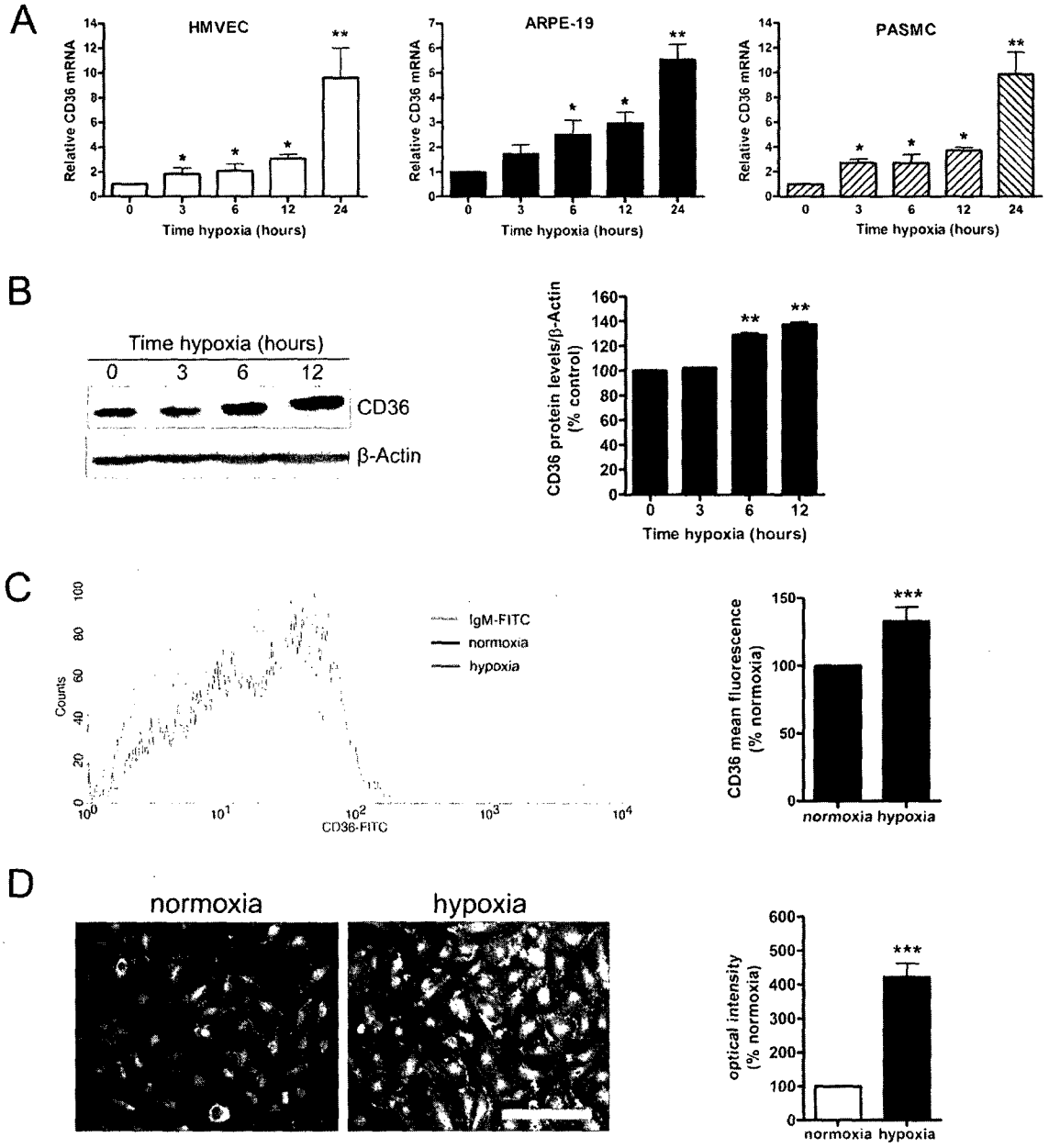


Figure 1. Effect of hypoxia on CD36 mRNA levels in an in vivo hypoxia model. C57BL/6 mice were subjected to ambient air or hypoxia (constant flow of 8 % O_2, 92 % CO_2 in an oxycycler) for 6 h (n = 6 animals per group). At the end of the experiment, animals were sacrificed and corneal (**A**) and retinal (**B**) tissue was harvested, total mRNA was isolated, and the relative CD36 mRNA levels were determined by quantitative real-time PCR. Values depicted as means ± SEM (3 independent experiments) were normalized to β-actin and expressed relative to normoxia (set to 1). ***$p<0.001$ vs. normoxia by Student's t test.


A
HMVEC
ARPE-19
PASMC
Relative CD36 mRNA
Time hypoxia (hours)
B
Time hypoxia (hours)
0 3 6 12
CD36
β-Actin
CD36 protein levels/β-Actin (% control)
C
IgM-FITC
normoxia
hypoxia
Counts
CD36-FITC
CD36 mean fluorescence (% normoxia)
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optical intensity (% normoxia)
normoxia hypoxia

Figure 2

Figure 2. Effect of hypoxia on CD36 mRNA and protein levels *in vitro*. (**A**) HMVEC, ARPE, and PASMC were grown under normoxia (21 % O_2) or hypoxia (2 % O_2) for indicated times up to 24 h and CD36 mRNA was determined by quantitative real-time PCR (qRT-PCR) expressed relative to control (set to 1). Representative images and histograms evincing ARPEs exposed to normoxia or hypoxia for up to 24 h and analyzed by Western blot (**B**), flow cytometry (**C**), or immunocytochemistry (scale bar, 50 μm) (**D**) with values depicted as a percentage of the appropriate control (set to 100 %). See Materials and Methods for detailed procedures. For each experiment, results shown represent the mean ± SEM of 3 independent experiments performed in triplicate. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. 0 h, normoxia, or control by Student's t test or one-way ANOVA.

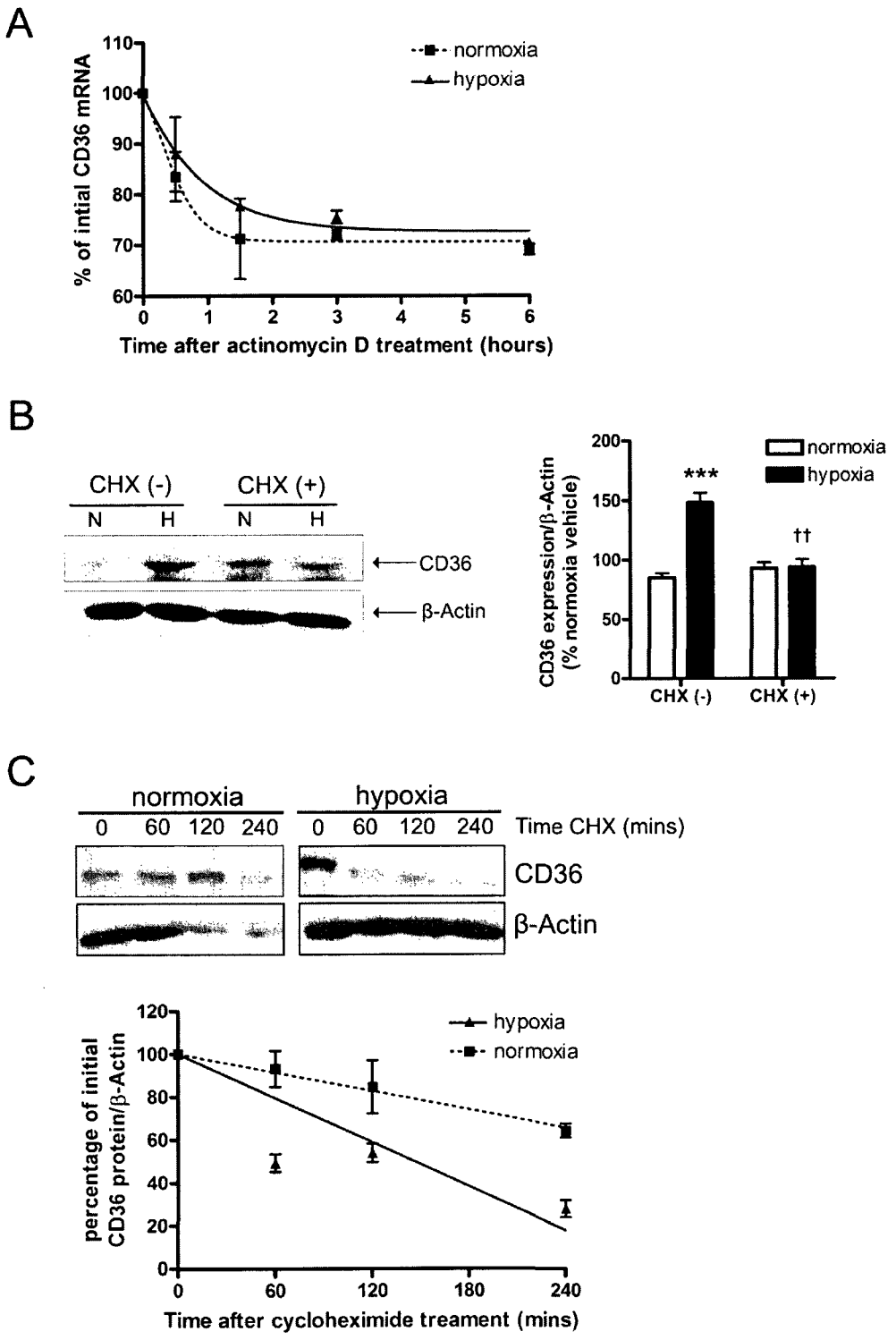
A
normoxia
hypoxia
% of intial CD36 mRNA
Time after actinomycin D treatment (hours)
B
CHX (-)
CHX (+)
N
H
N
H
CD36
β-Actin
CD36 expression/β-Actin (% normoxia vehicle)
normoxia
hypoxia
***
††
CHX (-)
CHX (+)
C
normoxia
hypoxia
0 60 120 240 0 60 120 240 Time CHX (mins)
CD36
β-Actin
hypoxia
normoxia
percentage of initial CD36 protein/β-Actin
Time after cycloheximide treament (mins)

Figure 3

Figure 3. Effect of hypoxia on CD36 mRNA stability, ongoing translation, and protein stability. (**A**) ARPEs were subjected to normoxia (21% O_2) or hypoxia (2 % O_2) for 24 h followed by treatment with actinomycin D (ActD, 4.5 μg/mL) and harvesting of cells at the indicated times. CD36 mRNA was analyzed by quantitative real-time PCR (qRT-PCR). The value obtained at time 0 h was set at 100 % and used to determine the percentage of CD36 mRNA remaining after ActD addition. ARPEs were pre-incubated in (**B**) the absence (-) or presence (+) of cycloheximide (CHX, 25 μM, 1h) and exposed to normoxia or hypoxia for 16 h, or (**C**) pre-exposed to normoxia or hypoxia for 24 h, followed by incubation with CHX (100 μM) for the indicated times. In (**B**) and (**C**) total protein lysates were analyzed by Western blot for CD36 expression with β-actin serving as a loading control. Representative blots (N = normoxia, H = hyoxia) and histograms (quantifications) demonstrate CD36 protein levels expressed as a percentage of normoxia control in (**B**) or as a percentage of the point before CHX addition in (**C**). In each case, experiments were repeated at least 3 times and values are expressed as means ± SEM. **$p<0.01$ vs. normoxia, $^{\dagger\dagger}p<0.01$ vs. hypoxia control.

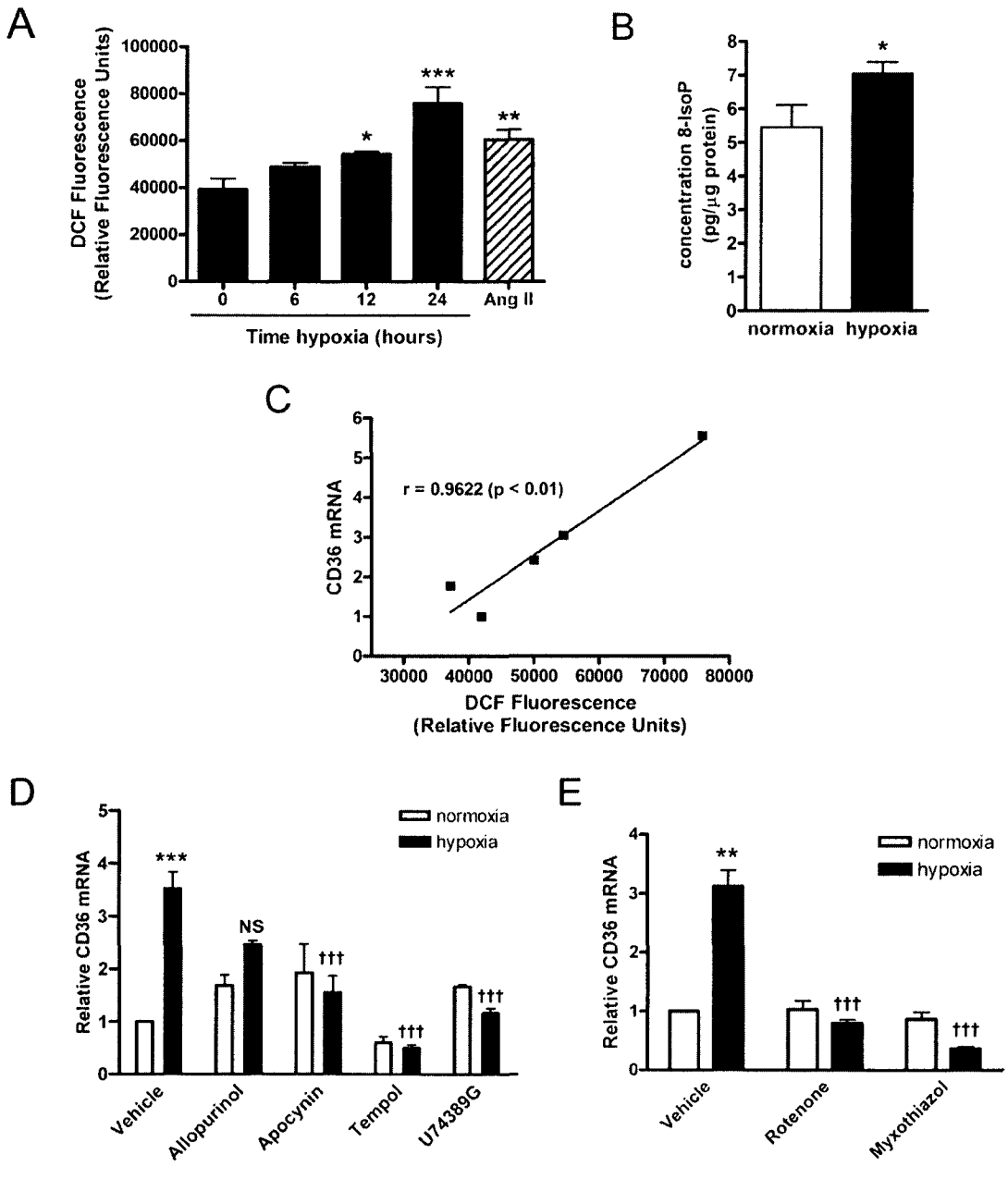
A
DCF Fluorescence (Relative Fluorescence Units)
100000
80000
60000
40000
20000
0
*
***
**
0
6
12
24
Ang II
Time hypoxia (hours)
B
concentration 8-IsoP (pg/μg protein)
8
7
6
5
4
3
2
1
0
*
normoxia
hypoxia
C
CD36 mRNA
r = 0.9622 (p < 0.01)
6
5
4
3
2
1
0
30000
40000
50000
60000
70000
80000
DCF Fluorescence (Relative Fluorescence Units)
D
Relative CD36 mRNA
5
4
3
2
1
0
normoxia
hypoxia
***
NS
†††
†††
†††
Vehicle
Allopurinol
Apocynin
Tempol
U74389G
E
Relative CD36 mRNA
4
3
2
1
0
normoxia
hypoxia
**
†††
†††
Vehicle
Rotenone
Myxothiazol

Figure 4

Figure 4. Role of ROS in the hypoxic regulation of CD36. Levels of ROS (positive control angiotensin II, 100 nM) (**A**) and 8-isoprostanes (**B**) were evaluated using the peroxide-sensitive fluorescent probe 2', 7' dichlorofluorescein diacetate or enzyme immunoassay respectively in ARPEs cultured under normoxic (21% O_2) or hypoxic (2 % O_2) conditions. (**C**) Representative plot of a correlation analysis (Pearson r) between CD36 mRNA and ROS levels generated during hypoxia. ARPEs were incubated for 1 h with cellular (allopurinol, 50 μM; apocynin, 50 μM; U74389G, 50 μM; tempol, 1 mM) (**D**) or mitochondrial electron transport chain complex I (rotenone, 100 nM) and III (myxothiazol, 200 nM) (**E**) inhibitors prior to 24 h hypoxic exposure and quantitative real-time PCR analysis. For each experiment, results are expressed as means ± SEM of 3 to 6 independent experiments performed in quadruplicate. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. 0 h or normoxia, $^{\dagger\dagger\dagger}p<0.001$ vs. hypoxia control, NS = non significant.

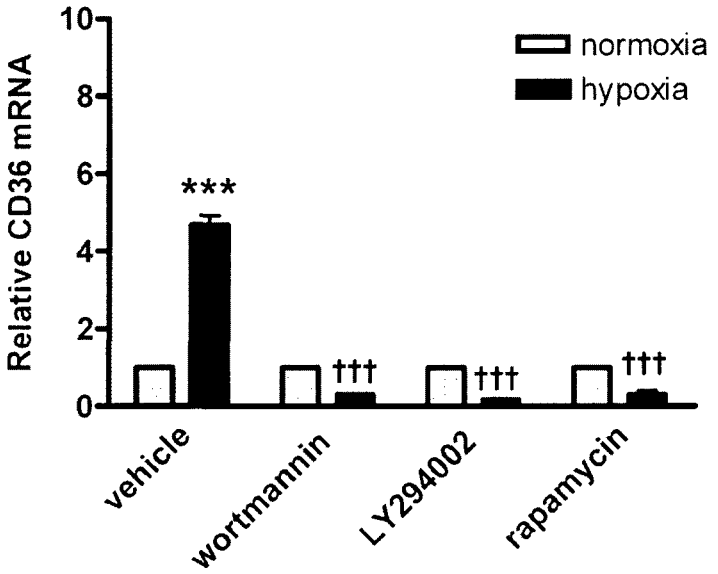

Figure 5. Implication of the PI3K/mTOR pathway in the hypoxic regulation of CD36. Selective inhibitors of PI3K (wortmannin, 200 nM; LY294002, 20 μM), and mTOR (rapamycin, 100 nM) were administered for 1 h prior to normoxia (21% O_2) or hypoxia (2 % O_2) exposure and CD36 expression was determined by quantitative real-time PCR. Data are representative of 3 independent experiments performed in triplicate and values are expressed as means ± SEM. ***$p<0.001$ vs. normoxia, $^{\dagger\dagger\dagger}p<0.001$ vs. hypoxia control.

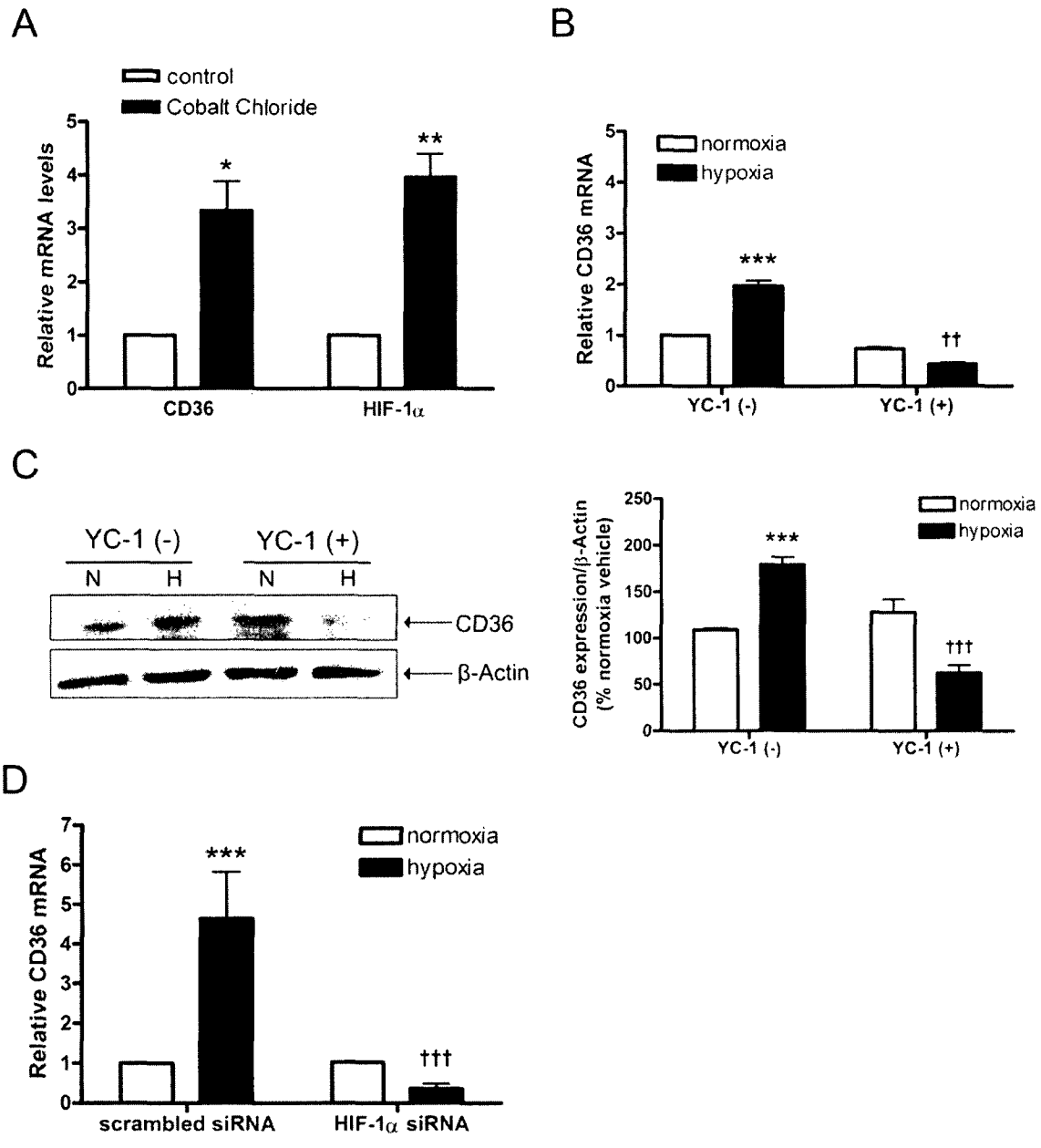
A
control
Cobalt Chloride
Relative mRNA levels
CD36
HIF-1α
B
normoxia
hypoxia
Relative CD36 mRNA
YC-1 (-)
YC-1 (+)
C
YC-1 (-)
YC-1 (+)
N
H
CD36
β-Actin
CD36 expression/β-Actin (% normoxia vehicle)
D
Relative CD36 mRNA
scrambled siRNA
HIF-1α siRNA

Figure 6

Figure 6. Involvement of HIF-1α in CD36 hypoxic regulation. ARPEs were incubated with cobalt chloride (200 μM, 3h) subsequent to analysis of CD36 and HIF-1α mRNA expression by quantitative real-time PCR (qRT-PCR) (**A**), or exposed to normoxia (21% O_2) or hypoxia (2 % O_2) in the absence (-) or presence (+) of YC-1 (5 μM) (**B**, **C**) or HIF-1α siRNA (50 nM) (**D**). A representative blot (N = normoxia, H = hyoxia) and histogram depicting CD36 protein levels (relative to β-actin) as a percentage of control is provided in (**C**). In each scenario, results shown represent the mean ± SEM of 3 independent experiments performed in triplicate. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control or normoxia; $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ vs. hypoxia control.

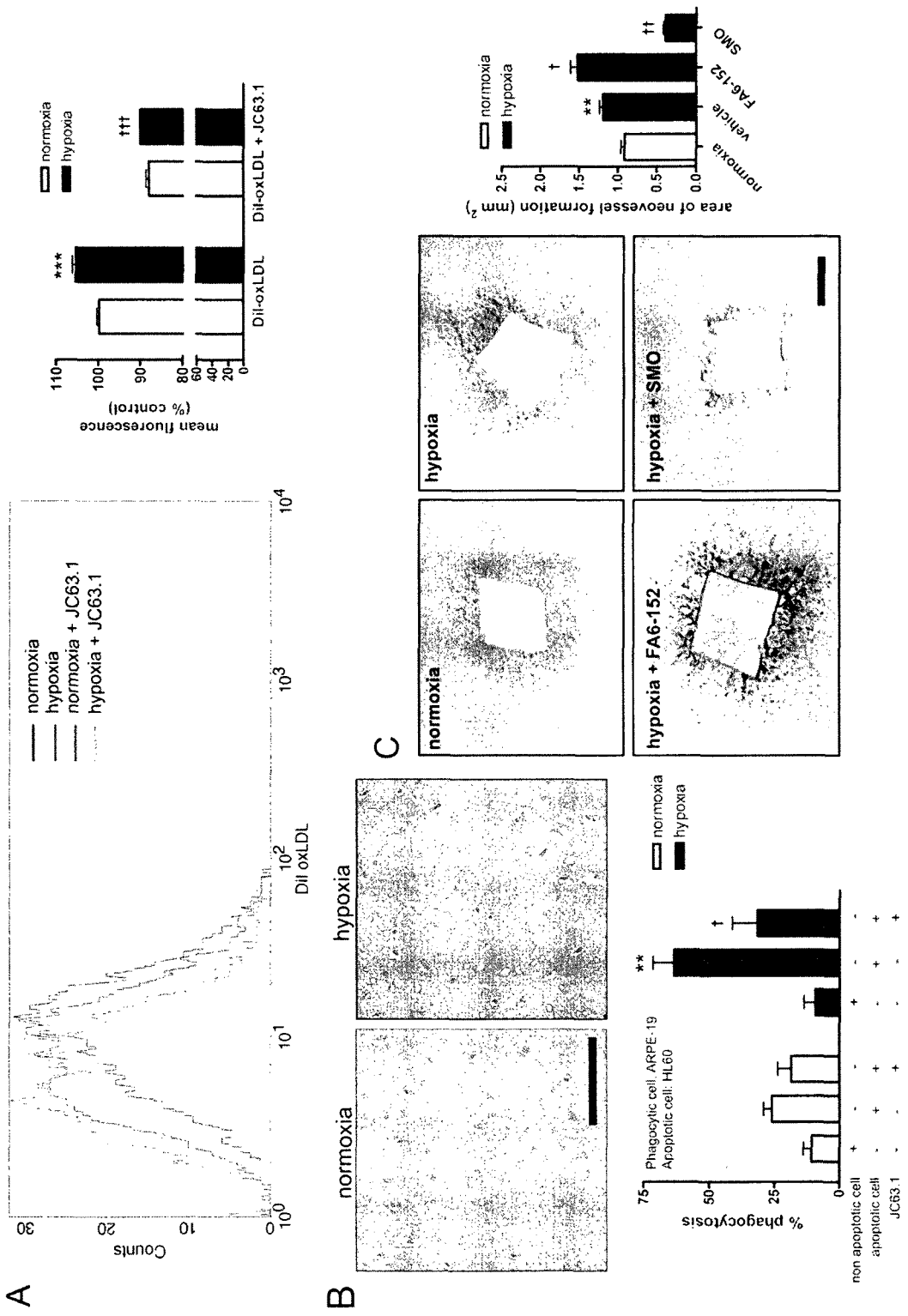
A
normoxia
hypoxia
normoxia + JC63.1
hypoxia + JC63.1
Counts
Dil oxLDL
mean fluorescence (% control)
Dil-oxLDL
Dil-oxLDL + JC63.1
B
normoxia
hypoxia
% phagocytosis
Phagocytic cell: ARPE-19
Apoptotic cell: HL60
non apoptotic cell
apoptotic cell
JC63.1
C
normoxia
hypoxia
hypoxia + FA6-152
hypoxia + SMO
area of neovessel formation (mm²)
vehicle
FA6-152
SMO

Figure 7

Figure 7. Effect of hypoxia on CD36 mediated uptake of oxLDL and phagocytosis of apoptotic cells. Represenative images and histograms (quantifications) of ARPEs that were pre-treated with vehicle (anti-human IgA) or an anti-oxLDL binding site CD36 monoclonal antibody (mAb) (JC63.1, 10 μg/ml) for 2 h, exposed to normoxia (21% O_2) or hypoxia (2% O_2) for 16 h, and incubated with DiI-labeled oxLDL (20 μg/ml) followed by flow cytometry (**A**), or viable or apoptotic (camptotecin, 5 μM, 4h; as monitored by DNA laddering assay and annexin V staining) HL60 cells fluorescently labeled with CellTracker Green CMFDA for 3 h prior to immunofluorescence (scale bar, 50 μm) (**B**). In (**B**), the rate of phagocytosis was determined as the ratio of bound apoptotic HL60 to the total number of phagocytic ARPEs. (**C**) Mouse aortas were seeded on Matrigel and on day 4 incubated in the presence of vehicle (anti-mouse IgG_1 or anti mouse IgM), an anti-TSP-1 binding site CD36 mAb (FA6-152, 10 μg/mL), or a CD36 mAb (SMO, 5 μg/mL) prior to 24 h hypoxia. Photographs were taken on day 5 (scale bar, 200 μm) and the neovessel area quantified in the histogram. In all cases, results are representative of 3 to 4 independent experiments performed in quadruplicate. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. normoxia or appropriate control, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ vs. hypoxia control.

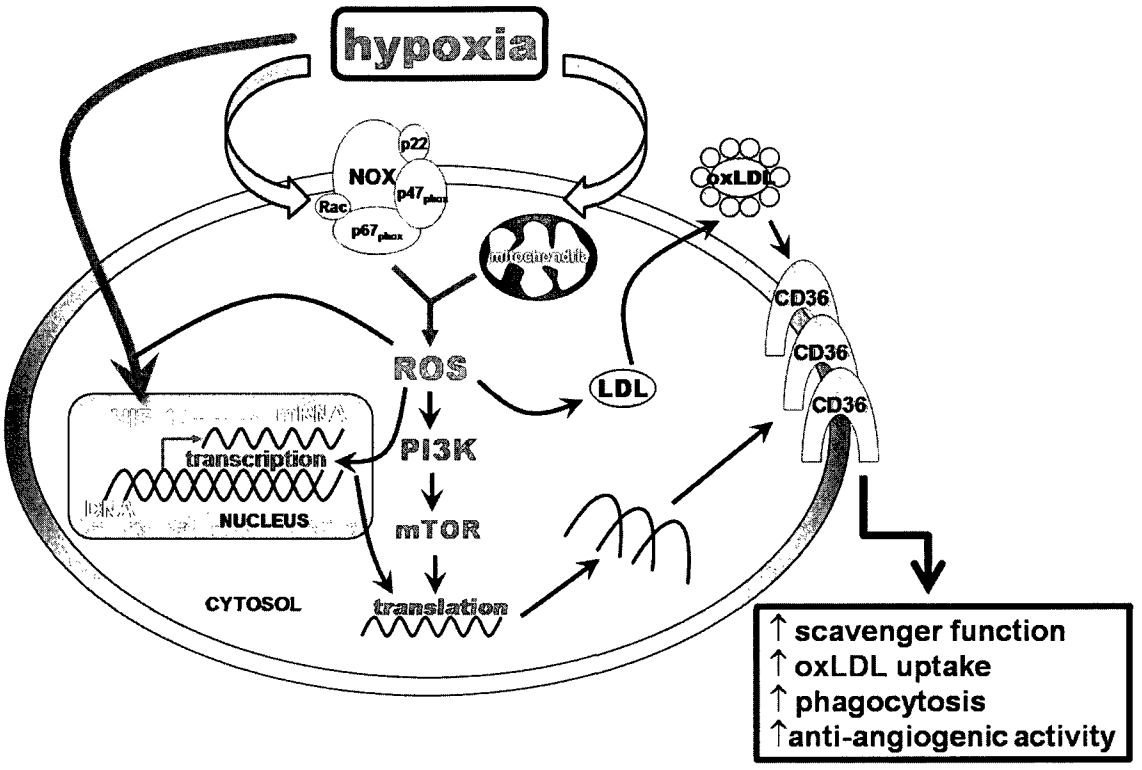

Figure 8. Schematic model depicting the putative mechanisms involved in the hypoxic regulation of CD36. NADPH oxidase and electron leak from mitochondria represent processes by which hypoxia augments the production of ROS leading to transcriptional activation of CD36 in addition to stimulation of the PI3K/mTOR translational cascade with ensued increases in CD36 surface expression and scavenging activity. Hypoxia-related lipid peroxidation further generates CD36 ligands such as oxLDLs which serve to enhance receptor activation and function. Putative involvement of HIF-1 is proposed via direct and indirect activation of HIF-1 by hypoxia and ROS.

CHAPTER 6: GENERAL DISCUSSION, CONCLUSIONS, AND FUTURE PERSPECTIVES

6.1 General discussion

Aberrant neovascularization of the cornea and other parts of the eye is a prevalent cause of visual impairment in all age groups and represents a major public health problem. While numerous molecules are known to contribute to ocular NV, blockade of VEGF has had tremendous beneficial effects in animal models that have now translated to human diseases in the clinic. Accordingly, VEGF inhibitors have emerged as current therapies for neovascular AMD with two new agents having gained clinical approval; pegaptanib (Macugen), an oligonucleotide aptamer that binds and neutralizes VEGF-165, and ranibizumab (Lucentis), a humanized antibody fragment that binds all VEGF-A isoforms (Brown et al., 2006; Rosenfeld et al., 2006; Takeda et al., 2007). Unfortunately, these drugs carry important limitations stemming from their large molecular size, the invasive nature of their intravitreal mode of administration, and their inability to completely repress neovascularization thus entailing repeated and costly injections (Afzal et al., 2007). In this regard, a low cost alternative, still requiring intravitreal injection, is bevacizumab, which is a humanized full-length anti-VEGF antibody already licensed for management of colorectal cancer, but now widely accepted for treatment of choroidal neovascularization and diabetic retinopathy (Cunningham et al., 2005; Rich et al., 2006; Spaide et al., 2006). Based on this precedence, significant efforts in elucidating the underlying molecular mechanisms of ocular NV may lead to new treatments following the lead of VEGF antagonists.

The CD36 scavenger receptor was an appealing target of investigation due to its established roles in normal retinal function and in regulation of angiogenesis. Using the cornea as a model in which to study ocular NV, we explored the involvement of CD36 during postnatal and pathological corneal NV; our data extend CD36's impressive functional repertoire to include maintenance of corneal avascularity and inhibition of corneal NV. Because hypoxia is a powerful stimulus for ocular NV, a third aim of this thesis was to mechanistically decipher the effect of hypoxia on CD36 expression and function. Overall, resultant data support our objectives and demonstrate the following original contributions:

1. Absence of CD36 elicits neovascularization and consequent opacification in the corneas of CD36 knock-out mice (relative to their wild-type counterparts) that manifests at 12 months of age and becomes more pervasive with age (up to 18 months in our study).
2. The age-dependent induction of corneal NV in CD36 null mice is associated with transcriptional downregulation of TSP-1 and upregulation of VEGFA, cJun, and JNK-1.
3. CD36 is constitutively expressed in the normal cornea (epithelium, stromal macrophages) and limbal microvessels, and its expression is persistently increased, particularly in the invading microvessels, following corneal injury.
4. Pharmacological modulation of CD36 using functionally blocking antibodies and oxidized phospholipid ligands correspondingly exacerbates and suppresses the development of corneal NV.
5. Activation of CD36 induces regression of established microvasculature during corneal NV.

6. The mechanisms of CD36 mediated angiostasis involve direct effects on endothelial cells and in part via negative regulation of macrophage-derived VEGFA.
7. Blockade of CD36 during corneal NV induces expression of the pro-angiogenic mediators VEGFA, JNK-1, and cJun.
8. CD36 expression is elevated in the hypoxic cornea and retina, an effect that is non cell-type specific being reproducible in RPE cells, microvascular endothelial cells, and smooth muscle cells.
9. Hypoxia time-dependently augments CD36 mRNA, protein, and surface expression in RPEs.
10. The hypoxic induction of CD36 appears to be reliant on translational mechanisms and not on increases in CD36 mRNA stability.
11. Elevations in CD36 expression and ROS production are positively correlated during hypoxia; mitochondria and NADPH oxidase represent important sources of ROS.
12. Knock-down of HIF-1 by inhibitors and siRNA attenuates the hypoxia-evoked increase in CD36 expression.
13. The functional relevance of hypoxia-induced CD36 expression is demonstrated by increased oxLDL uptake, apoptotic cell clearance, and anti-angiogenic activity.

6.1.1 The cornea as a model for investigating ocular NV

The cornea provides a valuable model system for studying neovascularization primarily because it is an avascular site and is readily

accessible for manipulation. The clarity of the cornea allows for direct identification and quantitation of new vessels in addition to observation of any grossly visible changes in the cornea following treatment. Insights into the neovascular process can also be acquired by evaluating actively growing as well as established blood vessels. An added advantage is that any capillaries arising within the cornea represent true neovascularization, in contrast to a rearrangement of preexisting vessels (Phillips and Knighton, 1995).

For the purposes of our research we utilized an inflammation-induced model of corneal NV as opposed to the frequently employed corneal micropocket assay; the latter is dependent on exogenous stimulation with growth factors thus limiting its pathophysiological relevance and translational capacity. Inflammatory models on the other hand are associated with a pronounced inflammatory response often triggered by trauma, infection or chemical injury. Of note, inflammation-induced corneal neovascular disease remains the most common reason for graft rejection following corneal transplantation (Chang et al., 2001). Finally, by gaining insight regarding the molecular signals involved in corneal NV, general concepts can emerge that may apply to other settings, including tumor angiogenesis and other diseases characterized by inflammation-induced angiogenesis e.g. psoriasis, rheumatoid arthritis.

6.1.2 Therapeutic potential of CD36 in ocular NV

Data generated from the present body of work together with that reported on TSP-1 (Cursiefen et al., 2004a) provide conclusive evidence that CD36 plays a crucial role in attempting to maintain corneal avascularity, a prominent feature of

the normal cornea. Moreover, given the potent angiostatic outcome of CD36 activation, our data support the efficacy of CD36 as a major target in corneal NV, and propose the use of simple oxidized phospholipids as agonists that could facilitate research on treatments for corneal NV or ocular NV in general. Benefits of such a strategy include the twin facts that oxidized phospholipids are inexpensive and can be administered topically, which is far less invasive, but still as effective as intraocular injections (Hosseini et al., 2007; Manzano et al., 2007).

Choroidal NV occurs in diseases of the RPE/Bruch's membrane complex such as AMD, the most prevalent cause of severe vision loss in elderly patients from developed countries. Accordingly, our findings demonstrating a functional role for CD36 in the retina and RPE following hypoxic injury, may extend the therapeutic potential of this receptor to other forms of ocular NV.

6.1.3 CD36 ligands and mimetics as therapeutic agents

With respect to available CD36 agonists, a small peptide mimetic based on the CD36-binding sequence on TSP-1 (ABT-510) is currently in clinical trials for treatment of various malignancies (Hoekstra et al., 2005) including advanced renal cell carcinoma (Ebbinghaus et al., 2007) and metastatic carcinoma (Markovic et al., 2007). Binding of ABT-510 to CD36 is inferred based on the activity of related TSP1-derived peptides to induce CD36-dependent activation of JNK, leading to endothelial cell apoptosis (Jimenez et al., 2001). Studies have also demonstrated that ABT-510 may be effective in treating neovascular diseases such as inflammatory bowel disease (Punekar et al., 2008).

Studies by Ong and colleagues have also identified growth hormone releasing peptides (GHRP) as CD36 ligands (Bodart et al., 2002), leading to the development of a synthetic GHRP-derived CD36 ligand, EP 80317 (Marleau et al., 2005). Incidentally, prolonged treatment with EP 80317 in apolipoprotein E-deficient mice fed a high fat, high cholesterol diet elicited a significant reduction in aortic lesion areas and was associated with a favorable plasma lipid profile (Marleau et al., 2005). As such, EP 80317 is considered a prototype for a novel class of anti-atherosclerotic agents.

6.1.4 Implication of the VEGFA/JNK-1/cJun pathway

In the current thesis, we examined the signal transduction cascade enacted by CD36 that contributed to its angio-inhibitory effects during postnatal and inflammatory corneal NV. We document that both genetic ablation and pharmacological inhibition of CD36 are associated with pronounced induction of VEGFA, the stress-activated kinase JNK-1, and its phosphorylation substrate cJun. This concurs with reports that VEGF-induced proliferation is dependent on JNK activation and subsequent phosphorylation of cJun (Pedram et al., 1998; Zhang et al., 2004; Wang et al., 2007). Interestingly, recent studies have demonstrated that JNK-1 is an essential mediator of corneal inflammation wherein JNK-1 deficiency diminishes neutrophil recruitment and development of corneal haze (Adhikary et al., 2008). Thus antagonism of these factors represents another mode by which CD36 inhibits corneal NV, thus enhancing its therapeutic potential.

6.1.5 On vessel regression and limitations of monotherapies

Regression of neovascularization continues to be a valuable therapeutic endpoint. Incidentally, the efficacy of anti-VEGF monotherapies is not sufficient to cause vessel regression, and regrowth of new vessels, often within months, requires multiple applications of these drugs (Afzal et al., 2007; Bradley et al., 2007). Therefore CD36 may represent an interesting target since its activation was able to induce regression of preformed microvasculature. Other established regulators of blood vessel regression are the Tie 2/angiopoietin (Ang) systems and platelet derived growth factor (PDGF)-B/PDGF receptor (PDGFR)-β, the latter representing an important drug target in tumor therapy, (Hoffmann et al., 2005; von Tell et al., 2006). Vessel regression is also achievable with PEDF wherein its intraocular gene transfer has been shown to induce regression of established choroidal neovascularization (Mori et al., 2002).

Inasmuch as topical application of CD36 agonists caused pronounced inhibition of corneal NV, it did not completely prevent neovascular progression. This is consistent with work by others who have shown that local administration of bevacizumab substantially, but not entirely, limits corneal NV (Hosseini et al., 2007; Manzano et al., 2007) and accredit this outcome to the fact that numerous other factors (e.g. FGF, MMPs) are known to induce corneal NV (Lee et al., 1998; Chang et al., 2001).

6.1.6 Many roads lead to angiostasis: benefits of combination therapy

To date, angiostatics have proven to be modestly effective remedies for neovascular eye disease. As discussed above, anti-angiogenic monotherapies have yet to demonstrate complete suppression of neovascular growth in the clinic and thus far have only delayed vision loss (Bradley et al., 2007). Reasons for this limited success may be attributable to compensatory mechanisms because blocking a single anti-angiogenic pathway may trigger compensatory upregulation of other proangiogenic pathways (Bradley et al., 2007) (Afzal et al., 2007; Dorrell et al., 2007b). Then again, Dorrell and colleagues successfully demonstrated dramatic, synergic inhibition of neovascular growth associated with development, ischemic retinopathy, and tumor growth by combining molecules that target multiple distinct aspects of the angiogenic cascade, with minimal consequences to normal vasculature (Dorrell et al., 2007b). Based on our observations that VEGF antagonism was central to the anti-angiogenic effects of CD36, we propose that co-administration of CD36 agonists and anti-VEGF drugs may lead to more potent suppression of corneal NV. This is because activation of CD36 would result in increased scavenging activity and antagonism of the VEGF pathway. Pharmacological intervention with JNK-1 inhibitors or antagonists may also prove beneficial. Overall, combination therapy has important implications for better management of ocular pathologies where complete inhibition of neovascularization is warranted.

6.1.7 Is complete inhibition of neovascularization always desirable?

While complete angiostasis may be desirable under certain conditions (e.g. to inhibit tumor growth), it is yet to be established whether complete inhibition of neovascularization in the hypoxic/ischemic eye is a desired therapeutic endpoint. Moreover, others have questioned whether an already ischemic tissue might be further damaged if the underlying hypoxia is potentiated via destruction of proliferating, albeit abnormal, blood vessels (Dorrell et al., 2007a). While this may be a valid consideration for the diabetic retina, in the cornea, avascularity is indispensable for optimal vision; hence it appears that the need for complete angiostasis depends upon the region of the eye that is affected. Interestingly, Dorrell et al. have proposed that vascular reconstruction to rebuild or stabilize damaged ocular vasculature using stem cell based technologies might be the optimal clinical approach (Dorrell et al., 2007a).

6.1.8 Upregulation of CD36: a protective or injurious phenomenon?

It is well established that hypoxia is a major stimulus of angiogenesis. An intriguing observation garnered from our studies was the significant induction of CD36 by both inflammatory and hypoxic stimuli, wherein we identified CD36 as a HIF-1 regulated gene. Similar yet conflicting effects have been documented for the CD36 ligand, TSP-1; independent groups have reported TSP-1 downregulation (Laderoute et al., 2000; Tenan et al., 2000) or induction (Phelan et al., 1998; Favier et al., 2005; Distler et al., 2007) following hypoxia and/or

ischemia. Nonetheless, we presume that upregulation of CD36 may represent a negative feedback response to prevent excessive angiogenesis in hypoxic environments; whether this can be inferred for other pathological situations is indeterminate.

Hypoxia/ischemia is known to generate several ligands, including oxidized lipids, that can activate CD36 and initiate subsequent signalling and function (Cho et al., 2005). However, the question begs to be asked, does CD36 induction confer protection or cytotoxicity in the affected tissue? On the one hand, CD36-null mice are protected from the tissue damage and functional impairment induced by cerebral ischemia (Cho et al., 2005), whereas in the cornea, CD36 deficiency compromises corneal avascularity (Barcia et al., 2007) and exacerbates the outcome of corneal NV (see Chapter 4). In the retina, upregulation of CD36 following hypoxic stress may protect it from further injury as CD36 participates in the phagocytosis of damaged photoreceptor outersegments or clearance of apoptotic cells. Together these evidences underscore the functional diversity and complexity of this scavenger receptor; depending on the nature of the injury, the cell type or tissue affected, and the ligands involved, modulation of CD36 expression exerts very varied effects. It is then apparent that the outcome of activating or inhibiting CD36 signaling or receptor function becomes difficult to predict in complex environments.

This level of complexity is not unique to CD36 but is characteristic of numerous genes. For example, it remains unclear whether HIF-1 affords a protective host defense during inflammation/hypoxia or whether it is part of a pathogenic response elicited by invading pathogens or excessive inflammation

(Kuhlicke et al., 2007; Ziello et al., 2007). As such, in the context of ischemia, HIF-1α upregulation stimulates angiogenesis and increases blood flow hence preventing tissue and cardiac damage (Date et al., 2005; Kido et al., 2005). Conversely, downregulation of HIF-1 is protective against lipopolysaccharide-induced sepsis (Peyssonnaux et al., 2007; Oh et al., 2008) and metastasis (Ziello et al., 2007).

6.1.9 Establishing a link between CD36, ROS, and HIF-1

The relationship between CD36, ROS, and HIF-1 under low oxygen conditions deserves brief mention. Though the role of ROS in regulating HIF-1 activity during hypoxia is still hotly debated, a growing body of literature supports the essential involvement of mitochondrial ROS (Brunelle et al., 2005; Guzy et al., 2005; Guzy and Schumacker, 2006; Lin et al., 2008). Because ROS are known to stabilize HIF-1 and given that HIF-1 inhibition has been shown to perturb hypoxia-dependent ROS production (Lin et al., 2008), a plausible mechanism by which HIF-1 regulates CD36 may be linked to enhanced ROS production. With respect to modulating CD36, the use of antioxidants, versus inhibition of the ETC may be more feasible for limiting hypoxia-dependent ROS production since antioxidants minimize toxicity concerns and do not interfere with mitochondrial respiration. Mechanistically, antioxidants may suppress CD36 expression by disrupting ROS-dependent CD36 signaling events or by inhibiting lipid peroxidation and apoptosis, hence effectively reducing ligand levels (Cho et al., 2007). Finally, besides indirect regulation of CD36 via the proposed HIF-1 ROS-

dependent pathway, HIF-1 may directly modulate CD36 promoter activity; such a conjecture is yet to be investigated.

6.2 Conclusion

Neovascular diseases of the eye affect a number of distinct tissues, including the cornea and retina, where robust, pathological neovascularization is a common etiological factor. Many treatments for these diseases attempt to prevent the formation of new but typically abnormal blood vessels. Despite the proliferation of anti-VEGF therapies, significant visual loss remains a problem in anti-VEGF treated patients. As such, delineating the underlying molecular mechanisms responsible for these complex yet debilitating disorders is of paramount importance if new therapeutic strategies are to be developed. Data presented in the current thesis establishes CD36 as an important target against ocular diseases hallmarked by neovascularization, inflammation, and hypoxia (refer to schematic overview in Figure 1). This work constitutes the very first references for the involvement of CD36 in modulating corneal avascularity and inflammatory neovascularization via antagonism of the VEGFA/JNK-1/cJun cascade. Novel HIF-1 dependent hypoxic regulation of CD36 is also presented and implicates ROS and translational mechanisms.

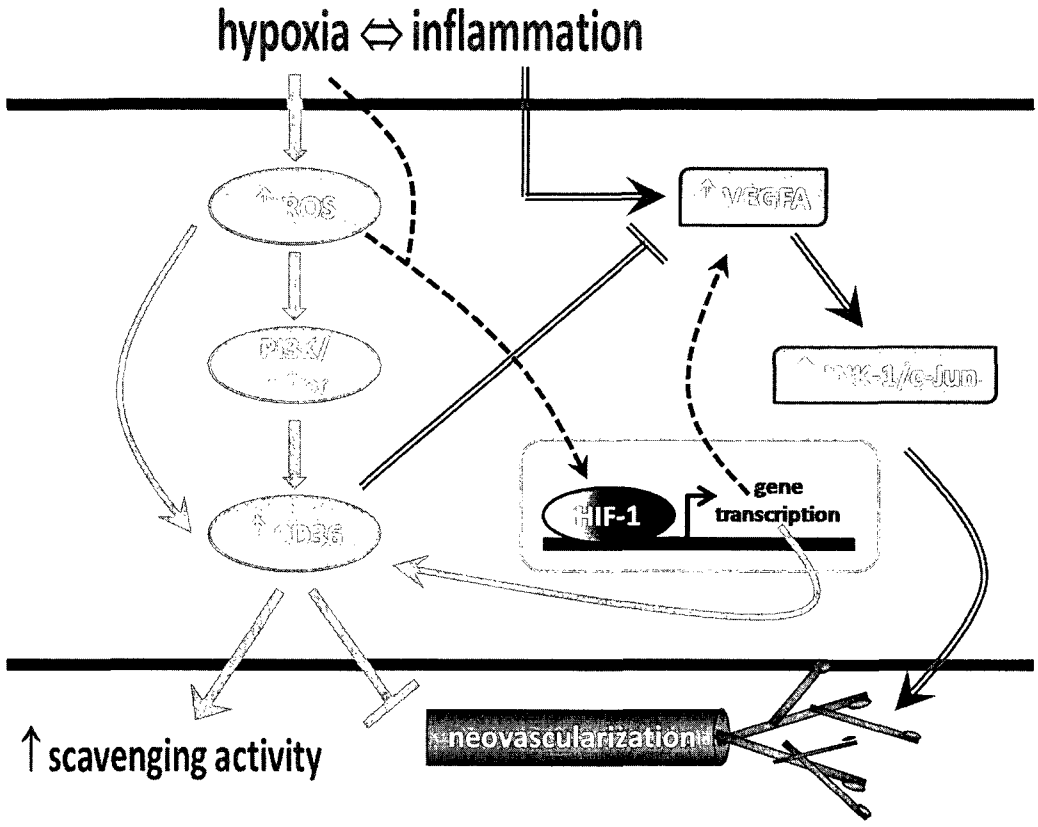

Figure 1. Mechanisms implicated in CD36 angiostatic and scavenging activities during ocular neovascularization. Ocular neovascularization (NV) is often hallmarked by inflammation and hypoxia which are closely integrated events. Via a proposed mechanism (doube-lined red arrows or lines), CD36 antagonizes inflammatory induction of the VEGFA/JNK-1/cJun pathway, which unhindered, would ordinarily culminate in neovascularization. In an alternative mechanism (solid green arrows or lines), hypoxia augments production of reactive oxygen species (ROS) which in turn activate HIF-1 (dashed black arrows) and the PI3K/mTOR translational cascade; together this results in elevated CD36 expression and consequent increases in CD36 scavenging and anti-angiogenic activity.

6.3 Future Perspectives

This work imparts several novel avenues for future research. Regression of established microvasculature remains a desirable outcome of any angiostatic therapy. Having shown that activation of CD36 induces blood vessel regression, a possible direction of research could investigate the mechanisms underlying CD36 mediated vasoregression during developmental and pathological angiogenesis. This could involve determining how CD36 expression correlates with coverage of blood vessels by pericytes, which are key players in vessel maturation and stabilization (Mitchell et al., 2008). Other questions that could be explored are whether CD36 disrupts endothelial pericyte interactions during vessel remodelling, or if CD36 antagonizes vessel stabilizing factors such as the PDGF-B/PDGFR-β or the Tie 2/Ang systems (Hoffmann et al., 2005; von Tell et al., 2006).

Another potentially interesting field of research concerns the role of CD36 in modulating developmental retinal angiogenesis and ischemic retinopathies. Continued work on the CD36 knock-out mouse may shed valuable insight into the precise contribution of CD36 during these processes including any ultrastructural histological features associated with this phenotype. Because VEGF-induced vascular leakage is closely linked with angiogenesis in a host of eye diseases (Erickson et al., 2007), future research could also address whether CD36 alters the blood-retinal barrier or affects expression of tight junction proteins.

A common hallmark of solid cancers is the presence of regions with low vascular perfusion termed "hypoxic regions". In keeping with this idea, it may be

interesting to determine the expression profile of CD36 in ocular cancers such as retinoblastoma, a common intraocular malignancy of childhood that is characterized by a solid well-vascularized tumor (Lee et al., 2008). In this process, the role and regulation of CD36 in the tumor hypoxia response could be investigated.

Several transcription factors are known to participate in hypoxic gene regulation, while novel factors responsible for the hypoxic regulation of CD36 are yet to be elucidated. Future studies could thus perform CD36 promoter analyses in order to identify putative binding motifs for other hypoxia- and/or redox-sensitive transcription factors. Such studies could also unveil whether CD36 is a direct and/or indirect target of HIF-1, by identifying and characterizing the presence of functional HIF-1 binding site(s) in the CD36 promoter. Finally, it may be worthwhile to clarify the contributions of HIF-1α and HIF-2α to the CD36 hypoxic response because others have reported differential or overlapping regulation by these two transcription factors during hypoxia (Anelli et al., 2008).

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APPENDIX A: LYMPHOCYTIC MICROPARTICLES INHIBIT ANGIOGENESIS BY STIMULATING OXIDATIVE STRESS AND NEGATIVELY REGULATING VEGF-INDUCED PATHWAYS

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LYMPHOCYTIC MICROPARTICLES INHIBIT ANGIOGENESIS BY STIMULATING OXIDATIVE STRESS AND NEGATIVELY REGULATING VEGF-INDUCED PATHWAYS

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Running head: LMPs inhibit angiogenesis

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Abstract

Recent studies have demonstrated that lymphocyte-derived microparticles (LMPs) impair endothelial cell function. However, no data currently exist regarding the contribution of LMPs in the regulation of angiogenesis. In the present study, we investigated the effects of LMPs on angiogenesis in vivo and in vitro and demonstrate that LMPs strongly suppressed aortic ring microvessel sprouting and in vivo corneal neovascularization. In vitro, LMPs considerably diminished human umbilical vein endothelial cell survival and proliferation in a concentration dependent manner. Mechanistically, the antioxidants U74389G and U83836E, were partially protective against the anti-proliferative effects of LMPs, while the NOX inhibitors, apocynin and diphenyleneiodonium, significantly abrogated these effects. Moreover, LMPs increased not only the expression of the NADPH oxidase (NOX) subunits $gp91^{phox}$, $p22^{phox}$, and $p47^{phox}$, but also the production of reactive oxygen species (ROS) and NOX-derived superoxide (O_2^-). Of importance, LMPs caused a pronounced augmentation in the protein expression of the CD36 anti-angiogenic receptor, while significantly downregulating the protein levels of VEGFR2 and its downstream signaling mediator phosphorylated ERK 1/2. In summary, LMPs potently suppress neovascularization in vivo and in vitro by augmenting ROS generation via NOX and interfering with the VEGF signaling pathway.

Key words: Microparticles, NADPH oxidase; CD36; VEGFR2, Angiogenesis

Introduction

Microparticles (MPs) are small membrane vesicles (14; 24) released upon activation or during apoptosis from various cell types including lymphocytes, platelets and endothelial cells (23; 31; 38). Microparticles have been implicated in the pathogenesis of cardiovascular and inflammatory diseases that are associated with vascular damage and impaired angiogenesis. Of relevance, lymphocyte derived MPs (LMPs) have been detected at elevated levels in atherosclerotic plaques (32) and in patients with myocardial ischemia or preeclampsia (31; 49). Recent observations have further demonstrated that MPs released from apoptotic lymphocytes or from plasma of diabetic patients induce endothelial dysfunction by modulating nitric oxide pathways (46).

Angiogenesis is involved in physiological events such as embryonic development and wound healing, as well as in pathological conditions such as tumor growth, diabetic retinopathy, and chronic inflammation.(8; 18) This tightly regulated and complex process involves endothelial cell survival, proliferation, migration, differentiation, and tube formation (17). It is widely accepted that angiogenesis is determined by a relative balance between pro- and anti-angiogenic factors.(22) Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors known and exerts its mitogenic effects primarily through the VEGF receptor type 2 (VEGFR2), which is almost exclusively expressed on endothelial cells. Moreover, VEGFR2 possesses instrinsic tyrosine kinase activity and therefore transduces signals leading to stimulation of mitogen activated protein kinases (MAPK). (44) Nonetheless, angiogenesis is also determined by

the presence of angiostatic molecules. CD36 is a potent anti-angiogenic surface receptor that is expressed by microvascular endothelial cells and binds to numerous ligands including thrombospondin (TSP)-1, an endogenous inhibitor of angiogenesis.(15) Interestingly, a previous study demonstrated that activation of CD36 by TSP-1 down-modulated VEGFR2 expression and p38 MAPK phosphorylation.(39) Then again, increased CD36 expression has been associated with pro-oxidative conditions such as atherosclerosis, inflammation, and ischemia.(12; 15; 40)

Reactive oxygen species (ROS) are involved in the development and progression of various cardiovascular diseases and oxidative stress is considered the central mechanism.(10) Furthermore, oxidative stress is thought to contribute to angiogenesis by mediating endothelial cell proliferation and migration.(30; 43) The major source of ROS in endothelial cells is NADPH oxidase (NOX); increasing NOX–driven ROS stimulates VEGF expression and enhances VEGFR2 autophosphorylation.(28; 47) In this context, LMPs could be one of the key factors linking oxidative stress and angiogenesis.

Previously published studies have documented that microparticles released from platelets (PMPs) induce angiogenesis and stimulate post-ischemic revascularization, whereas endothelial cell derived microparticles (EMPs) suppress angiogenesis by altering the redox balance.(25; 35) Nevertheless, the involvement of LMPs in regulating angiogenesis is yet to be established. Herein, we report for the first time that LMPs significantly inhibit blood vessel formation in the ex vivo aortic ring angiogenesis assay and in vivo corneal neovascularization (CNV) model. Moreover, the current findings suggest that

LMPs strongly diminish VEGF induced endothelial cell proliferation and migration by enhancing ROS production primarily from NOX with accompanied increases in CD36 expression and suppression of VEGFR2 signaling.

Materials and Methods

Compounds and reagents

Actinomycin D, 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), Lucigenin (N,N-dimethyl-9,9'biacridinium dinitrate), and angiotensin (Sigma Aldrich); β-actin (Novus Biologicals); Flk-1 (VEGFR2) rabbit polyclonal antibody and horseradish peroxidase linked anti-rabbit IgG, antibodies against $gp91^{phox}$, $p22^{phox}$ (FL-195), $p47^{phox}$, ERK1/2, phospho-ERK1/2, TSP-1, and rabbit polyclonal CD36 antibody (Santa Cruz Biotechnology; Santa Cruz, CA); U83836E and U74389G (Biomol, PA, USA); [^{3}H]-thymidine (Amersham, Mississauga, Ontario, Canada); hrVEGF and apocynin (Calbiochem, La Jolla, California, USA); mitomycin C (Fluka Biochemika); Annexin-V-Cy5 (BD pharmagen, Sandiego, CA); vybrant apoptosis assay kit, propidium iodide (PI) and fluorescent microbeads, 1 μM (Molecular Probes, Eugene, OR, U.S.A); NADPH (Roche Diagnostics, Laval, QC Canada); diphenyliodonium (DPI) (Calbiochem, La. Jolla, Calif., USA).

Cell culture

CEM T cells were purchased from ATCC (Manassas, USA) and cultured with X-VIVO medium (Cambrex, Walkersville, MD). Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex (Walkersville, MD)

and cultured as recommended. The immortalized human microvascular endothelial cell line-1 (HMEC-1) was kindly supplied by Dr Candal FJ (Centers for Disease Control and Prevention, Atlanta, GA). HMEC-1 were grown in Endothelial Basal Medium (Cambrex,Walkersville, MD) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA), 100 μg/mL streptomycin, 100 U/mL penicillin, 10 ng/mL epidermal growth factor (BD, Oakville, Ontario, Canada) and 1 μg/mL hydrocortisone (Sigma).

LMPs production

LMPs were generated as described.(33) Briefly, CEM T cells were treated with 0.5μg/mL of actinomycin D for 24 hours, a supernatant was obtained by centrifugation at 750g for 15 min, then 1500g for 5 min to remove cells and large debris. MPs from the supernatant were washed after three centrifugation steps (50 min at 12,000g) and recovered in saline or basic cell culture mediums. Washing medium from last supernatant was used as control. LMPs were characterized with annexin V staining by FACS analysis, and gated using 1.0 μM beads in which 97% of MPs (≤ 1μM) were annexin-V-Cy5 positive.(41) The concentrations of LMPs were determined by the BioRad protein assay. Using the same protocol, we also generated LMPs from hyperoxia (95% O_2, 36h) or hypoxia (5% O_2, 36h) exposed CEM T cells.

Animals

Six week old male C57BL/6 mice purchased from Charles River (St-Constant, Quebec, Canada) were used according to a protocol approved by the Sainte-Justine Research Center Animal Care Committee.

Aortic ring angiogenesis assay

The aortic ring assay was performed as described previously.(37) In brief, 1mm thoracic aortas were embedded in 3-dimensional growth factor reduced Matrigel (BD Biosciences) and cultured in EGM-2 medium at 37°C. The culture medium was changed on day 3 and the aortic rings were treated with saline or 30μg/mL of LMPs until day 7. Aortic rings were photographed on days 5 and 7 using a Nikon eclipse TE300 inverted microscope. The angiogenic response was determined by measuring the area of neovessel formation using Image Pro Plus software.

Murine model of corneal neovascularization

Angiogenesis was investigated in vivo using a murine model of corneal neovascularization (CNV) as described previously.(37) Briefly, each mouse was anesthetized with isoflurane (Abbott, Canada), and topical proparacaine (Alcon, Canada) and 2 μL of 0.15M NaOH were applied to the central cornea. The corneal and limbal epithelium were removed by scraping with a scalpel. Gentamicin sulfate ophthalmic solution (Sabex Inc., Quebec, Canada) was instilled immediately following epithelial denudation. Buprenorphine (0.05 mg/kg; Schering-Plough Ltd) was administered post-operatively for analgesia. Twenty-four hours after corneal injury, mice were randomly divided into two groups that received either saline or 50 μg/mL LMPs. Treatments were administered topically three times daily for 7 days, after which corneas were harvested, flatmounted and immunostained with FITC-conjugated anti-CD31. Images were captured with a Nikon digital camera DXM 1200 using Nikon ACT

1 version 2.62 software. The CNV was quantified in a masked fashion using Adobe Photoshop 7.0 image analysis software. The total corneal surface area was outlined using the innermost vessel of the limbal arcade as the border and the ratio [(neovascularized area/total cornea area) x 100] was used to provide a measure of the percentage vascularized cornea.

Cell viability assay

Cells at approximately 60% confluence were incubated for 24 hours with vehicle or the indicated concentrations of LMPs. Cell viability was estimated by mitochondrial-dependent reduction of MTT. Essentially, MTT (0.5 mg/mL in PBS [pH 7.4]) was added to the culture medium and incubated at 37°C for 3 hours, media was aspirated, formazan product solubilized with acidified isopropanol, and the optical density was read at 545 nm with reference wavelength at 690 nm.

[^{3}H]-thymidine incorporation assay

$4x10^4$ HUVEC were plated and serum starved for 24 hours. After synchronization, cells were cultured in complete medium with vehicle or 10 ug/mL LMPs for an additional 24 hours. Thereafter, 1μCi/mL [^{3}H]-thymidine was added to each well, and incubated for 24 hours. [^{3}H]-thymidine DNA incorporation was assayed by scintillation counting.

Apoptosis assay

HUVEC were treated with or without 10 μg/mL LMPs for 8, 18 and 24 hours, then treated with reagents from the Vybrant Apoptosis Assay Kit

(Molecular Probes, Invitrogen) followed by flow cytometry analysis according to the manufacturer's protocol. The rate of apoptosis or necrosis was expressed as the percentage of apoptotic cells relative to the total number of cells per condition.

Measurement of ROS generation and the NADPH Oxidase assay

Induction of reactive oxygen species (ROS) was measured using the fluoroprobe DCFDA (Molecular Probes). Endothelial cells were cultured in 24-well plates and treated with LMPs and /or apocynin at indicated concentrations for three hours, or angiotensin (Ang II; 100 nM) for 45 minutes as a positive control. Cells were stained with DCFDA (10 μM) for another 30 minutes. After staining, the extracellular dye was washed twice with 10.0mM HEPES buffer (pH 7.4), and the fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a multi-well fluorescent plate reader (Wallac 1420 VICTOR Multilabel Counter).

NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method as described.(20) Briefly, HUVEC were treated with 10 μg/mL LMPs for different time periods, washed in ice-cold PBS, harvested, and homogenized via sonication (1 second) (Brandson Sonifier 150, USA) in lysis buffer (20 mM KH_2PO4, pH 7.0, 1 mM EGTA, 10mM complete protease inhibitor cocktail). Homogenates were centrifuged at 800 x *g* at 4 °C for 10 min to remove the unbroken cells and debris, and aliquots were used immediately. To initiate the assay, 100-μl aliquots of the homogenates were added to 900 μl of 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH. Photon emission in terms of relative light units

was measured in a luminometer every 2 mins for 30 min. There was no measurable activity in the absence of NADPH. Superoxide anion production was expressed as relative chemiluminescence (light) units (RLU)/μg protein. Protein content was determined by the Bio-Rad protein assay.

Western blot analysis

Cells were plated at a density of 1 x 10^6 cells per 100-mm plate and incubated with 7.5, or 10 and 15 μg/mL LMPs for 24 hours. Soluble proteins were extracted using cell lysis buffer (10mM Tris-HCl, 1.5mM $MgCl_2$, 1mM DTT, 1 μM pepstatin, 0.75mM EDTA, 1% (v/v) SDS, 10mM protease inhibitor cocktail (Roche, pH 7.5). Following centrifugation, the supernatant was collected and total protein concentration was determined (Bio-Rad assay). 25μg of protein was fractionated by SDS-PAGE. The resolved proteins were transferred onto a PVDF membrane on a semi-dry electrophoretic transfer cell (Bio-Rad) for Western blot analysis. Membranes were blocked, and then incubated overnight at 4°C with an anti-VEGFR2 polyclonal antibody (1:500 dilution), anti-$gp91^{phox}$ (1:100), anti-$p22^{phox}$ antibody (1:200), anti- $p47^{phox}$ (1:200), phospho-ERK1/2 antibody (1:200), ERK1/2 (1:200), TSP-1 (1:400), and anti-CD36 polyclonal antibody (1:400). After washing, membranes were incubated with a horseradish peroxidase linked anti-rabbit IgG (1:5000) for 1 h at room temperature. β-actin was used as a loading control (1:10000). Proteins were visualized using the ECL Western blotting detection system (Perkin Elmer).

Cell migration assay

Two cell migration assays were used to facilitate our analysis. Cell migration was first determined using a coverslip border migration assay. Briefly, $0.5x10^6$ HUVEC were seeded onto 12 mm-coverslips in a 24-well plate. Cells were serum starved for 4 hours and proliferation was inhibited by adding 10 μg/mL mitomycin C for 30 minutes. Next, coverslips were carefully removed, washed with fresh media, and transferred into a 12-well plate containing 10 ng/mL VEGF in the presence or absence of 10 μg/mL LMPs. Images were captured between 48 and 72 hours using an Axiovert 200M inverted microscope (Zeiss). At 72 hours, the coverslips were removed and the proportion of migrated cells was quantified by MTT assay.

The Boyden chamber migration assay was also used. A 96-well chemotaxis chamber with five micron polycarbonate filter was purchased from Corning Incoporated, NY. The filter was placed over a bottom chamber containing 10 ng/mL of hrVEGF. 10,000 HUVEC were seeded to each well in the upper chamber. For testing the effects of LMPs and apocynin on the cell migration, HUVEC were incubated with LMPs and/or apocynin in the upper chambers. The assembled chemotaxis chamber was incubated for 24 hours at 37°C with 5% CO_2 to allow cells to migrate through the filter. Non-migrated cells on the upper surface of the filter were removed by scraping with a wiper tool (Neuro Probe, Inc., Gaithersburg, MD) and a cotton swab, and the filter was stained with coomassie blue. The total number of migrated cells per well were counted; the assays were performed in quadruplicate.

Statistical analysis

All experiments were repeated at least three times and values are presented as means ± SEM. Data were analyzed by one-way ANOVA followed by post-hoc Bonferroni tests for comparison among means. Statistical significance was set at $p<0.05$.

Results

LMPs suppress aortic ring angiogenesis and in vivo corneal neovascularization

The first objective was to determine whether LMPs affect vessel development. For this purpose, we utilized the aortic ring angiogenesis assay and a pathophysiologically relevant CNV model that is largely driven by VEGF. Incubation of aortic rings with saline or 30 μg/mL LMPs for 48 and 96 hours significantly reduced neovessel formation by 50% (2.2± 0.2 mm^2 vs. 1.1 ± 0.1 mm^2; $p<0.05$) and 58% (7.7 ± 0.3 mm^2 vs. 3.2 ± 0.5 mm^2; $p<0.001$) (Figures 1 A, B) respectively. Having established that LMPs inhibit ex vivo angiogenesis, we analyzed its significance in vivo by treating mice subjected to CNV with saline or 50 μg/mL LMPs three times daily for 7 days. Compared to saline treatment, LMPs caused a 23% reduction in CNV (80.0 ± 3.6% vs. 61.6 ± 2.3%; $p < 0.001$; Figures 1 C, D).

LMPs inhibit human endothelial cell survival and proliferation

Cell survival and proliferation are critical steps during angiogenesis. To determine the effect of LMPs on vascular cell survival, HUVEC and HMEC-1

were exposed to different concentrations of LMPs and their viability was assessed by MTT assay. LMPs significantly diminished cell viability in both cell types in a concentration dependent manner (Figures 2 A- B). In order to determine whether the effect of LMPs on cell proliferation is stimulus-dependent, LMPs were generated from hyperoxia or hypoxia exposure. LMPs produced under both hyperoxic and hypoxic conditions potently suppressed HUVEC proliferation (45.8 ± 1.4 and 50.8 ± 2.3, respectively; $p<0.001$ vs. control) to a comparable degree as actinomycin D-derived LMPs (49.0 ± 0.8; $p<0.001$ vs. control). This indicates that the effects of LMPs are not stimulus-dependent.

The observed reduction in cell survival could be caused by decreased cell proliferation or increased apoptosis or necrosis. [^{3}H]-thymidine DNA incorporation was applied and LMPs (10 μg/mL) reduced HUVEC proliferation by 60% ($p<0.001$) (Figure 2 C). To ascertain whether LMPs were inducing apoptosis or necrosis, both LMPs treated and control HUVEC were double labelled with FITC-conjugated annexin-V and PI; however, induction of apoptosis or necrosis was not observed under all test conditions ($P>0.05$) (Figure 2 D).

Antioxidants partially block the anti-proliferative effects of LMPs

Previous studies have shown that EMPs increase superoxide production (9) and lead to impairment of angiogenic pattern.(35) Moreover, NOX, a major source of superoxide free radicals, was highly expressed by endothelial cells.(16) We therefore postulated that LMPs were exerting their anti-angiogenic properties via oxidative stress mechanisms. To address this hypothesis, we utilized two well known lipid peroxidation inhibitors, namely U83836E and U74389G (3; 29; 45)

and tested their ability to attenuate the anti-proliferative effects of LMPs. U83836E and U74389G at 5 and 10 μM concentrations respectively, led to a partial but statistically significant increase in cell proliferation compared to LMPs treatment alone ($p<0.05$) (Figure 3A). Additionally, pre-treatment of HUVEC with two specific NOX inhibitors, apocynin (1.5 mM) and diphenyleneiodonium (DPI; 5 μM), significantly abrogated the LMP-induced anti-proliferative effects (*$p<0.05$ and ***$p<0.001$ respectively; Figures 3 B-C).

LMPs increase ROS and NOX activity

Having demonstrated the important role of oxidative stress in LMP-mediated activities, we were next interested in investigating the effects of LMPs on ROS generation. The latter was determined by measurement of intracellular ROS levels using DCF fluorescence following a 3 hour pretreatment with 10 μg/mL LMPs. As shown in Figure 4A, compared to control, LMPs significantly increased ROS production as indicated by a rise in the DCF signal ($p<0.05$). Moreover, LMP-induced ROS generation was significantly attenuated by pretreatment with apocynin (1.5 mM; $p<0.05$).

Because the superoxide-generating NADPH oxidase has been described to largely contribute to ROS formation in endothelial cells, (27) we next investigated the effect of LMPs on superoxide generation from this enzyme. Superoxide anion production was measured in LMP-treated HUVECs as lucigenin-enhanced chemiluminescence using NADPH as the substrate. As indicated in Figure 4B, LMPs increased the rate of superoxide formation after 1 hour incubation and reached a peak after 8 hours ($P<0.001$).

LMPs induce protein levels of $p22^{phox}$, $p47^{phox}$, $gp91^{phox}$, and the CD36 scavenger receptor

Owing to the ability of LMPs to induce NOX activity, we next investigated their effect on the expression of $p22^{phox}$, $p47^{phox}$ and $gp91^{phox}$, which are critical subunits of NADPH oxidase.(48) Indeed, LMPs strongly upregulated $p22^{phox}$, $p47^{phox}$ and $gp91^{phox}$ protein expression in a concentration-dependent fashion ($P<0.05$) (Figures 5 A-F). Although LMPs demonstrated very low level expression of $p22^{phox}$, $p47^{phox}$ and $gp91^{phox}$ were undetected.

The CD36 scavenger receptor and its endogenous ligand TSP-1 are potent inhibitors of in vitro and in vivo angiogenesis(37; 39) whose expression are potentiated in pro-oxidative environments (12; 13; 40) as well as by NADPH oxidase activation.(4) In this context, human microvascular endothelial cells treated with 10 and 15 μg/mL LMPs dose-dependently augmented CD36 protein levels by 1.9 and 2.3 fold respectively (Figures 5G,H), whereas expression of TSP-1 was not significantly changed. Moreover, TSP-1 was not detected in LMPs per se which is in agreement with the published results from the proteomic analysis of LMPs.(36)

LMP mediated anti-migratory effects are reversed by NOX inhibitors

Because cell migration plays a pivotal role in angiogenesis, we sought to elucidate the effect of LMPs on VEGF-induced cell migration. HUVECs were plated onto coverslips and exposed to 10 ng/mL VEGF with or without LMPs. Cell migration was substantially decreased by 58% after 72 hours of LMPs treatment ($p<0.001$; Figures 6A, B).

Cell migration was also evaluated using the modified Boyden chamber assay. LMPs strongly inhibited VEGF-induced cell migration by 40% ($p<0.001$; Figure 6C) and apocynin (1.5mM) was able to partially rescue the LMP mediated anti-migratory effects ($p<0.01$ vs. LMP; Figure 6C).

LMPs reduce VEGFR2 protein and phospho-ERK levels

Having observed that LMPs induced CD36 expression, we surmised that LMPs were further suppressing angiogenesis by antagonizing the VEGF signaling pathway. This hypothesis is corroborated by evidence that activation of CD36 leads to suppression of VEGF-induced VEGFR2 phosphorylation.(39) Accordingly, HUVEC proliferation was assessed following pre-incubation with 1.5 μg/mL anti-VEGFR2 antibody in the presence or absence of LMPs (10 μg/mL). As expected, the anti-VEGFR2 antibody alone strongly decreased cell proliferation ($p<0.01$); however, co-treatment with the anti-VEGFR2 antibody and LMPs did not result in a synergistic reduction of cell proliferation (#$p>0.05$, compared to Ab-VEGFR2 group Figure 7A). Consistent with this data, Western blot analysis of HUVEC treated with 7.5 and 15 μg/mL LMPs, caused a dose-dependent downregulation of VEGFR2 protein expression by 50% and 65% respectively vs. control (**$p<0.01$; Figures 7 B, C). Phospho-ERK1/2 levels were also significantly inhibited by 35% (*$p<0.05$; Figures 7 D, E)

Discussion

Microparticles (MPs) are known to contribute to the pathogenesis of cardiovascular diseases, including inflammation and vascular dysfunction.

Another important action of MPs in the vascular system is their ability to modulate angiogenesis.(34) Nevertheless, despite the escalation in MP research, very little is known regarding the role of T lymphocyte-derived microparticles (LMPs) in regulating angiogenesis.(19; 34) Herein, we report that LMPs inhibited angiogenesis *in vivo* and *in vitro* by suppressing vascular cell survival, proliferation and migration. Of particular interest, our data demonstrate that LMPs induced ROS production via NOX activation while antioxidants and NOX inhibitors attenuated the anti-angiogenic effects of LMPs. Furthermore, through CD36 induction and VEGFR2 and phospho-ERK1/2 downregulation, we provide evidence that LMPs interfered with the VEGF signalling pathway. Taken together, these findings strongly support a role for LMPs in regulating angiogenesis during pathological conditions.

MPs are released from the plasma membrane during cell activation by apoptosis, shear stress, or agonists. In our study, MPs were obtained by apoptosis from T lymphocytes treated with actinomycin D. Moreover, the characteristics of MPs seem to depend on the mechanism of stimulation and the activation status of the cell from which they originate.(25; 35) This is clearly highlighted by the reported effects of MPs on angiogenesis. For example, although we show that LMPs possess anti-angiogenic properties, others have shown that MPs from endothelial cells inhibit, whereas platelet-derived MPs promote angiogenesis. (7; 25; 35) In our study, the anti-angiogenic effects of LMPs seem to occur as a result of decreased cell proliferation and not increased cell apoptosis or necrosis (Figure 2). This is in agreement with observations by Andriantsitohaina's group who showed that pathophysiological levels of LMPs failed to induce endothelial cell

apoptosis. (33) Nonetheless, the pathophysiological relevance of our study is limited given that there is presently no standardized method for measuring MPs and one cannot rely upon the numbers provided by other studies since they highly depend upon the method of measurement and the sensitivity of detection.(1; 6; 26)

It has been documented that oxidative stress is one of the central mechanisms responsible for endothelial cell dysfunction.(16; 21) The major sources of ROS in endothelial cells are endothelial nitric oxide synthase (eNOS) and NOX. In line with this, there is a general consensus that nitric oxide (NO) inhibits both vascular smooth muscle and endothelial cell proliferation. (11; 50; 51) In our study, nitrite levels were unchanged by LMP treatment and eNOS blockers did not prevent the anti-proliferative effects of LMPs (data not shown). Conversely, LMPs increased ROS levels and NOX activity (Figure 4) as well as upregulated expression of the $gp91^{phox}$, $p22^{phox}$ and $p47^{phox}$ NOX subunits (Figure 5). Consistent with this, inhibition of NOX partly abrogated the inhibitory effects of LMPs on both cell proliferation and migration (Figures 3B, C; 6C). Collectively, these results support that the NOX, and not the eNOS-NO signaling pathway, is involved in generating ROS that mediate the angiostatic effects of LMPs.

One of the detrimental consequences of oxidative stress is peroxidation of membrane lipids. Lipid peroxidation induces site-specific changes in the organization of the phospholipid bilayer thus leading to cellular dysfunction. The lipid peroxidation inhibitors, U74389G and U83836E, are lipophilic steroid compounds that intercalate into biological membranes thus enhancing their stability in the event of oxidative stress. (5; 42) In this study, both compounds

partially attenuated the anti-proliferative effects of LMPs (Figure 3 A), thus suggesting that LMPs' angiostatic activities also involve increased lipid peroxidation.

Ample studies have demonstrated that oxidative stress stimulates CD36 expression and that antioxidants attenuate its expression and function. (12; 15; 40) It was therefore intriguing to observe that LMPs upregulated CD36 expression, which is consistent with the pro-oxidant actions of LMPs (Figures 3, 4, 6). However, we presume that the LMP-mediated upregulation of CD36 is TSP-1 independent since LMPs had no significant effect on TSP-1 expression. Moreover, because CD36 is a well established anti-angiogenic receptor, (15; 37) it is tempting to speculate that the generation of ROS by LMPs occurs upstream of the induction of CD36 with subsequent suppression of the VEGF/VEGFR2 signaling pathway, as has been proposed by us and others. (37; 39)

It is well known that VEGF plays a pivotal role in developmental and pathological angiogenesis. VEGF stimulates angiogenesis through VEGFR2 (KDR/Flk-1), which is expressed mainly on endothelial cells. (2; 44) In the present study, several lines of evidence supported our hypothesis that LMPs antagonized the VEGF/VEGFR2 pathway. First, we demonstrate that LMPs potently inhibited VEGF-induced inflammatory corneal neovascularization (Figures 1C, D). Secondly, VEGF-induced endothelial cell migration was dramatically reduced by LMPs (Figure 6). Thirdly, inhibition of VEGFR2 activity had no synergistic effect on the anti-proliferative effects of LMPs, suggesting that both VEGFR2 and LMPs signal via the same pathway (Figure 7A). Finally, we show that LMPs significantly downregulated VEGFR2 and phosphorylated

ERK1/2 expression (the main downstream effector of the VEGF signaling pathway) (Figure 7), while increasing CD36 protein levels (Figures 5 G, H), a known negative regulator of this pathway. (37; 39)

In conclusion, we provide evidence for the first time that MPs from T cells inhibit angiogenesis *in vivo* and *in vitro*. We demonstrate that LMPs impair vascular cell survival, proliferation, and migration. The present data also suggest that LMPs regulate angiogenesis by acting through the NADPH oxidase and VEGFR2 pathways. Given the pivotal role of the VEGF/VEGFR2 signaling in angiogenesis, understanding the mechanisms of how LMPs interrupt VEGFR2 signaling could provide attractive therapeutic strategies aimed at reducing the deleterious effects of MPs on the vascular system.

Perspectives and significance

Having long been considered as cellular debris, microparticles constitute reliable markers of vascular damage. Released into biological fluids, microparticles are involved in the modulation of key functions including immunity, inflammation, vascular remodeling and angiogenesis. LMPs can be considered a hallmark of stress-injured or dying lymphocytic cells and may be recognized in the future as a marker of lymphocytic dysfunction. Our data demonstrate that LMPs have considerable impact on angiogenesis *in vitro* and *in vivo*. In view of this, LMPs may be important contributors to the pathogenesis of diseases that are accompanied by impaired angiogenesis and could thus influence vascular function (microvascular angiogenesis and vasopermeability) of ischemic tissue, alerting the body for special attention and the need for emergency repair

procedures. Pharmacological modulation of circulating LMP concentrations could become a major future therapeutic target.

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Figures

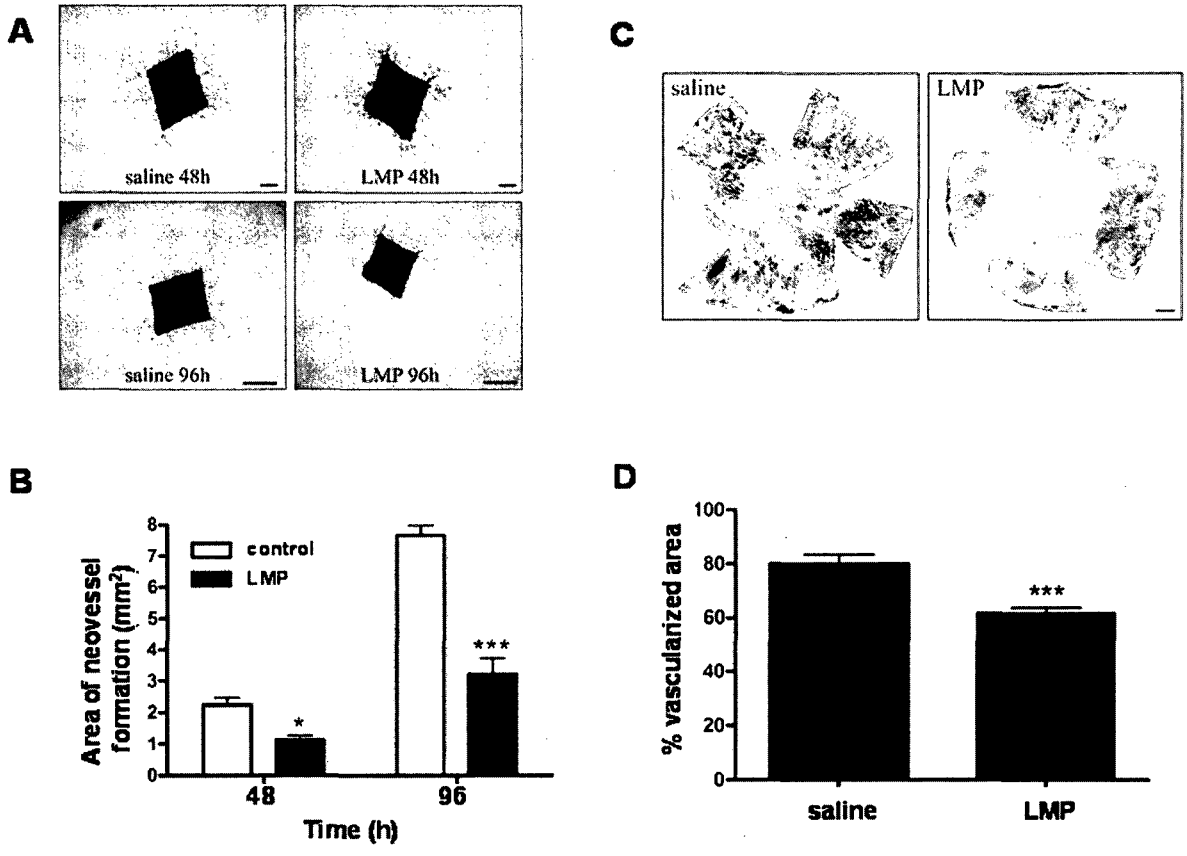

Figure 1

Figure 1. LMPs inhibit angiogenesis in the aortic ring assay and in vivo corneal neovascularization model. **(A)** Representative illustrations of neovessels arising from aortic rings after 2 and 4 days treatment with saline or 30 μg/mL LMPs. **(B)** Quantitative analysis of the area of neovessel formation in aortic rings (n=8 per group). Scale bar: 200 μm. **(C)** Mice subjected to inflammation-induced CNV were treated three times daily for 7 days with vehicle or 50 μg/mL LMPs. **(D)** Quantification of the vascularized corneal area (n=7 per group). Values are means ± SE . *$p < 0.05$; ***$p < 0.001$ vs. CTRL.

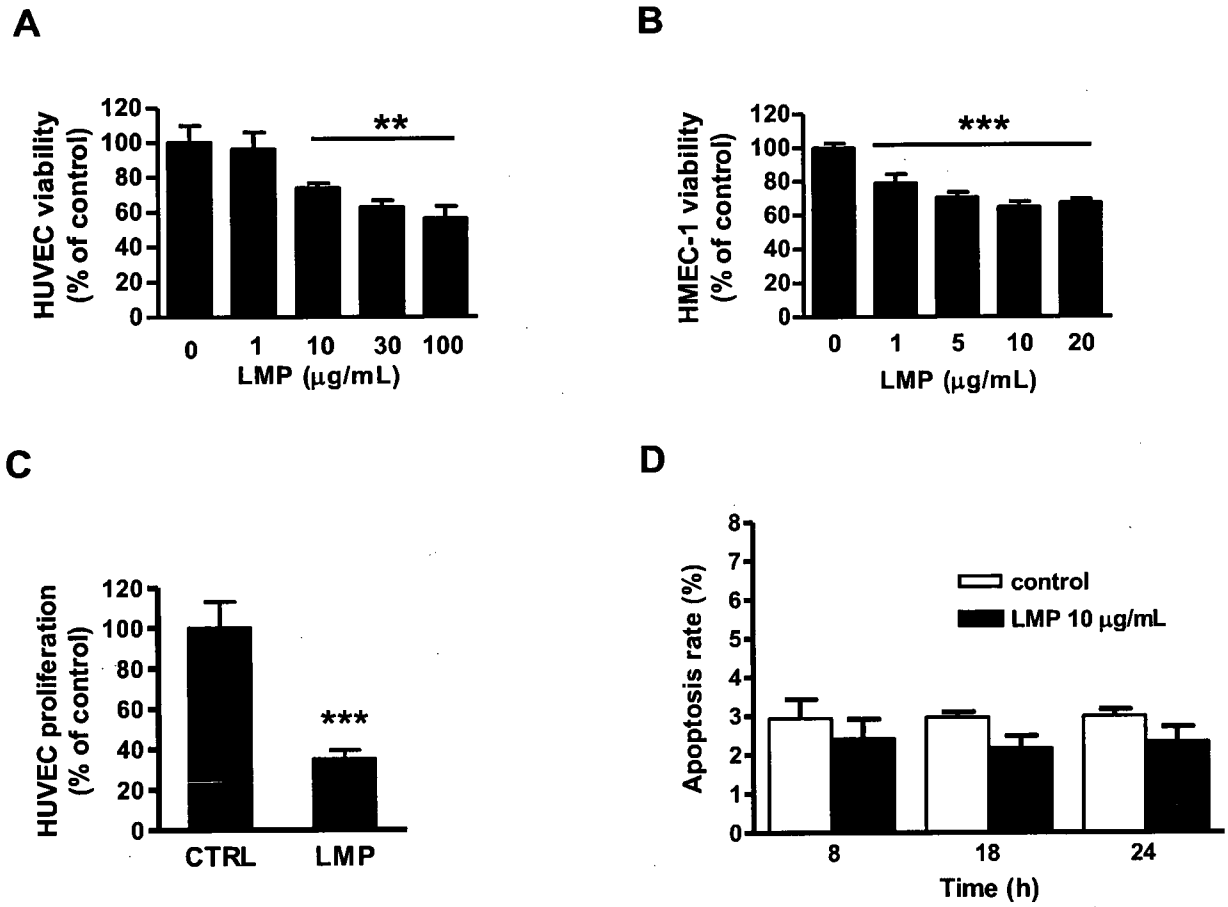
A
HUVEC viability (% of control)
120
100
80
60
40
20
0
**
0 1 10 30 100
LMP (μg/mL)
B
HMEC-1 viability (% of control)
120
100
80
60
40
20
0
***
0 1 5 10 20
LMP (μg/mL)
C
HUVEC proliferation (% of control)
120
100
80
60
40
20
0
***
CTRL LMP
D
Apoptosis rate (%)
8
7
6
5
4
3
2
1
0
control
LMP 10 μg/mL
8 18 24
Time (h)

Figure 2

Figure 2. LMPs reduce endothelial cell survival and proliferation. (A) HUVEC or (B) HMEC-1 were incubated with the indicated concentrations of LMPs for 24 hours and cell viability was evaluated by MTT assay. (C) HUVEC were treated with or without 10 μg/mL LMPs for 24 hours. Cell proliferation was assessed by [3H]-thymidine DNA incorporation and normalized to control. (D) HUVEC were treated with 10 μg/mL LMPs for different time periods, then apoptotic and necrotic cells were determined by flow cytometry and expressed as the percentage of apoptotic cells relative to the total number of cells per condition. Values are means ± SE of 3-5 individual experiments, each performed in triplicate. **$p<0.01$, ***$p<0.001$ vs.CTRL.

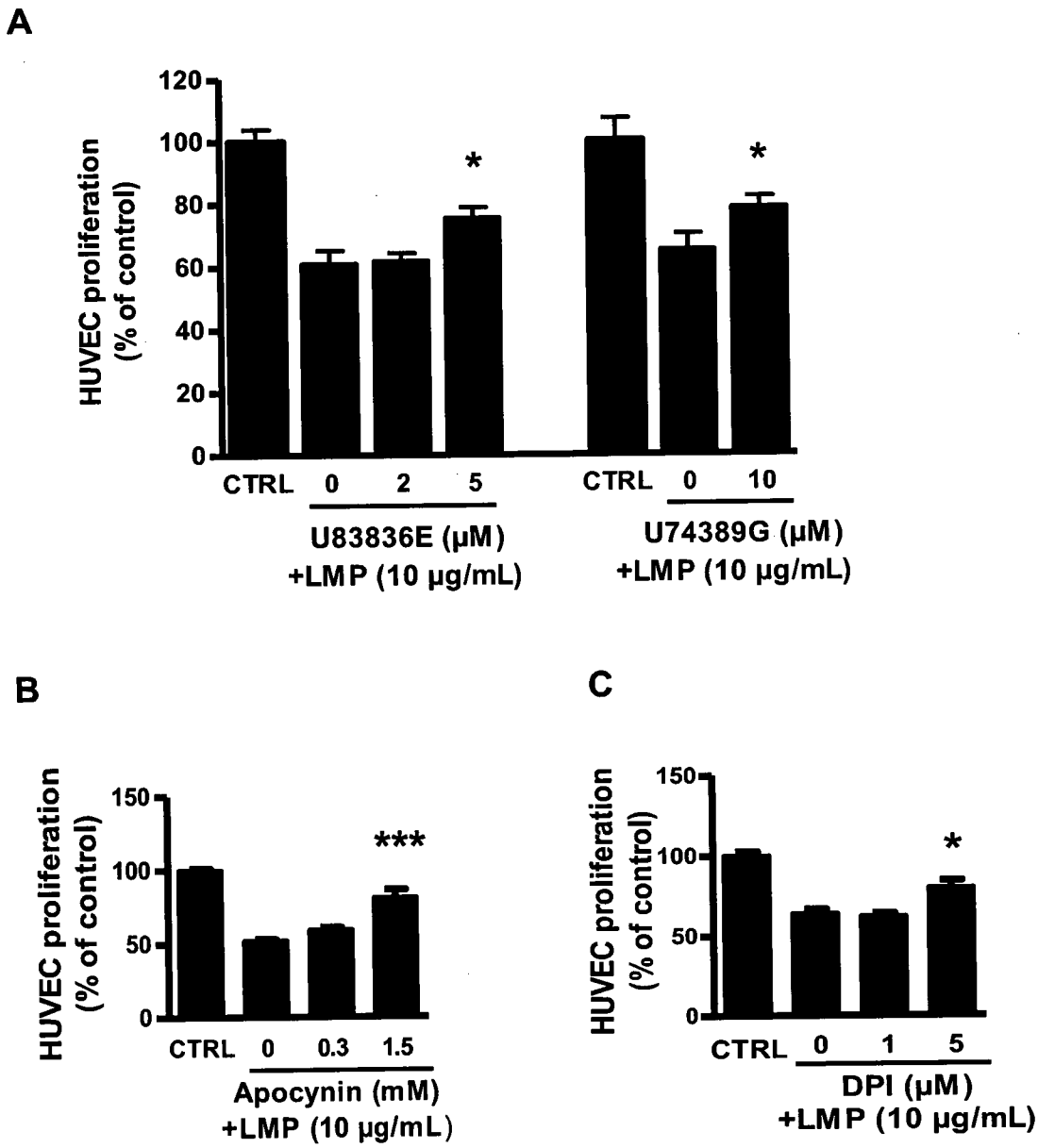
A
HUVEC proliferation (% of control)
120
100
80
60
40
20
0
*
*
CTRL 0 2 5
U83836E (μM)
+LMP (10 μg/mL)
CTRL 0 10
U74389G (μM)
+LMP (10 μg/mL)
B
HUVEC proliferation (% of control)
150
100
50
0
***
CTRL 0 0.3 1.5
Apocynin (mM)
+LMP (10 μg/mL)
C
HUVEC proliferation (% of control)
150
100
50
0
*
CTRL 0 1 5
DPI (μM)
+LMP (10 μg/mL)

Figure 3

Figure 3. Antioxidants partially restore LMP-mediated anti-proliferative effects. HUVEC were pretreated for 3 hours with the indicated concentrations of (**A**) U83836E, U74389G (**B**) apocynin or (**C**) diphenyleneiodonium (DPI), after which 10 μg/mL LMPs were added and incubated for an additional 24 hours followed by cell proliferation measurements. Values are means ± SE of 3 individual experiments performed in triplicate.*$p<0.05$, ***$p<0.001$ vs. LMP treatment.

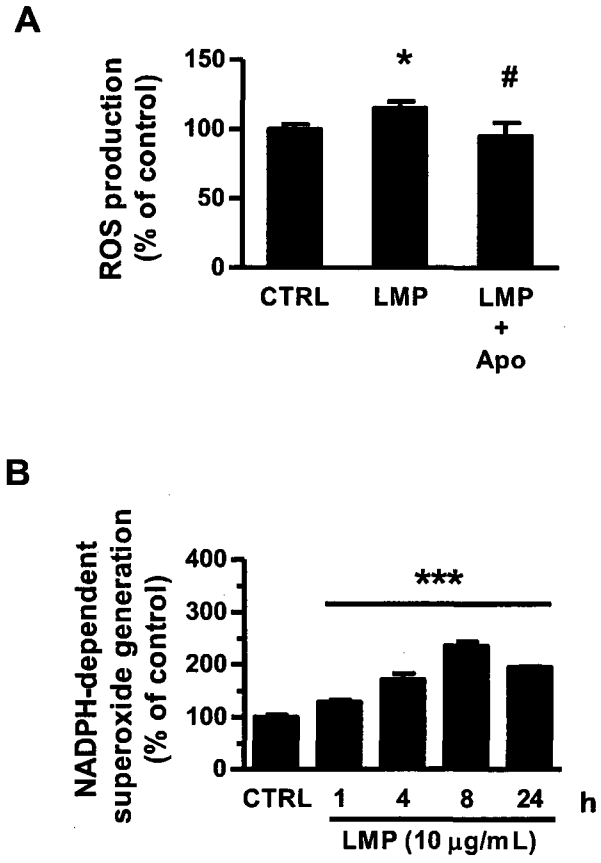

Figure 4. LMPs induce ROS production and NADPH-dependent superoxide generation. **(A)** HUVEC were incubated with 10 µg/mL LMPs in the absence or presence of apocynin (1 mM). Intracellular ROS generation was measured by DCF fluorescence. Data are expressed as relative to control. **(B)** HUVEC were incubated with 10 µg/mL LMPs for different time points, superoxide anion (O_2^-) production was measured as lucigenin-enhanced chemiluminescence using NADPH as substrate. Data are expressed as percentage of control. Values are means ± SE of 3 individual experiments, each performed in triplicate. *$p<0.05$ vs. CTRL, #$p<0.05$ vs. LMPs, ***$p<0.001$ vs. CTRL.

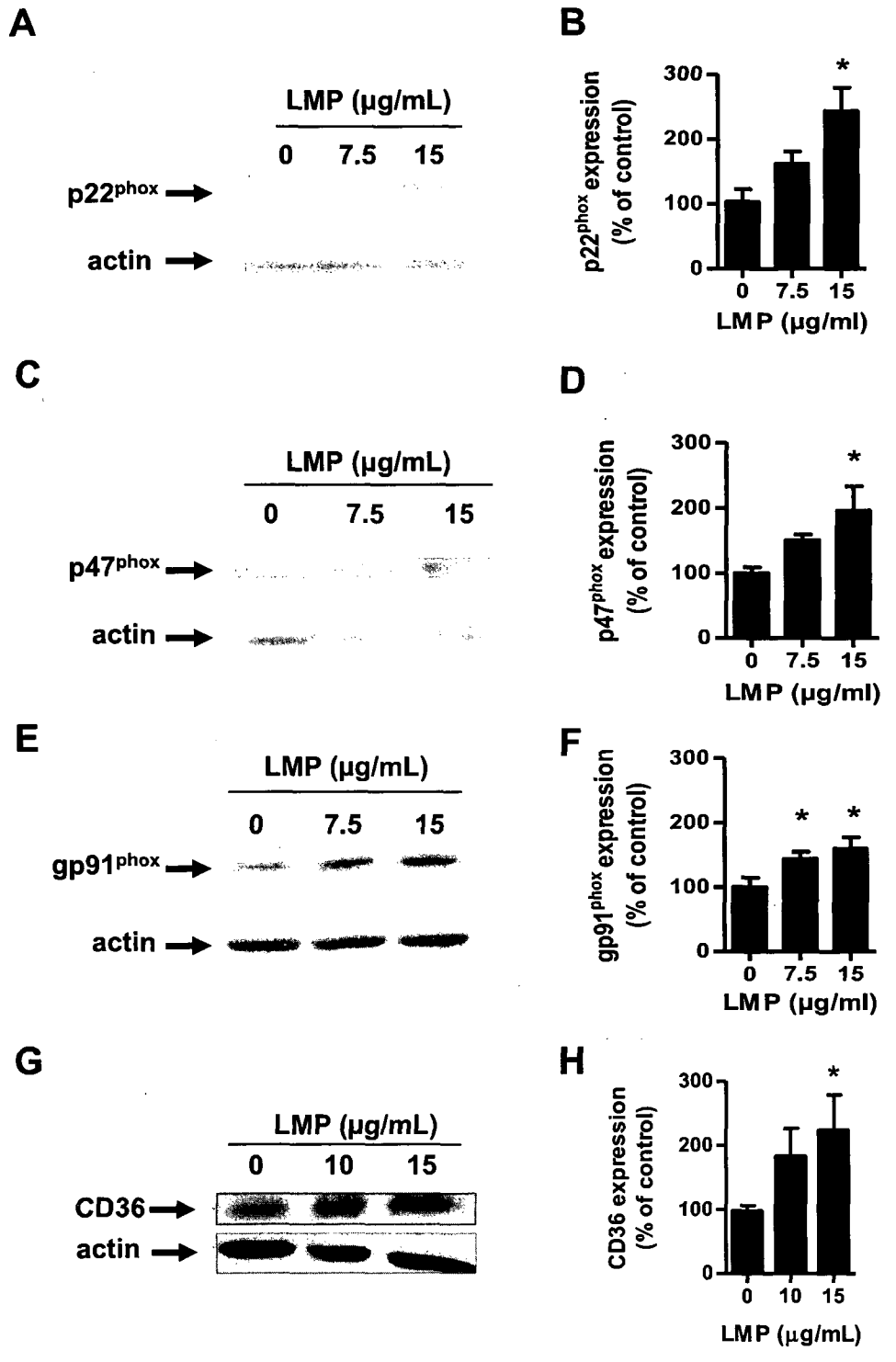
A
LMP (μg/mL)
0 7.5 15
p22phox
actin
B
p22phox expression (% of control)
300
200
100
0
0 7.5 15
LMP (μg/ml)
C
LMP (μg/mL)
0 7.5 15
p47phox
actin
D
p47phox expression (% of control)
300
200
100
0
0 7.5 15
LMP (μg/ml)
E
LMP (μg/mL)
0 7.5 15
gp91phox
actin
F
gp91phox expression (% of control)
300
200
100
0
0 7.5 15
LMP (μg/ml)
G
LMP (μg/mL)
0 10 15
CD36
actin
H
CD36 expression (% of control)
300
200
100
0
0 10 15
LMP (μg/mL)

Figure 5

Figure 5. LMPs increase p22phox, p47phox, gp91phox, and CD36 expression. (**A, C, E,**) HUVEC were treated with 7.5 and 15 μg/mL LMPs for 24 hours and p22phox, p47phox, gp91phox expression were detected by Western blot. (**B, D, F**) The p22phox, p47phox and gp91phox protein levels were normalized to β-actin and the untreated condition was set to equal 100%. (**G, H**) CD36 protein levels were determined by Western blot in human microvascular endothelial cells treated with 10 and 15 μg/mL LMPs and data was normalized to β-actin. Values are means ± SE of 3 experiments. *$p<0.05$ vs. control.

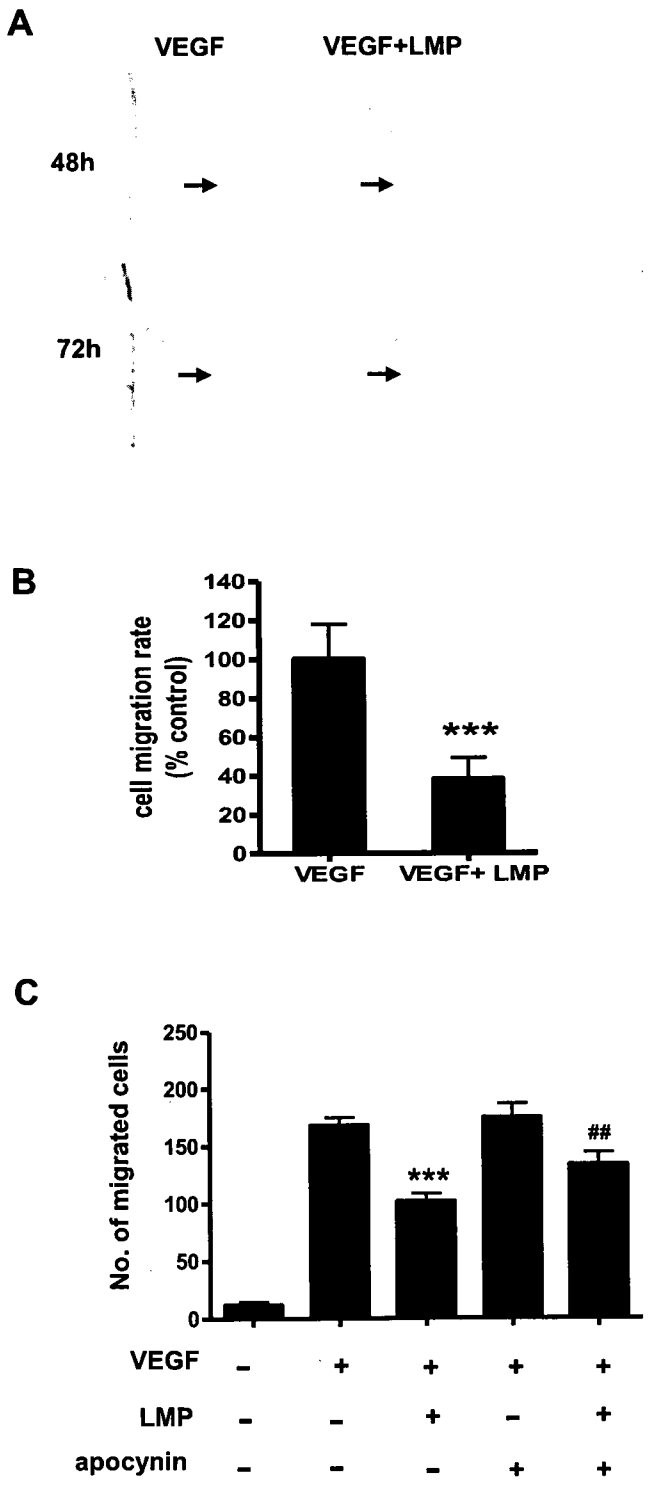
A
VEGF
VEGF+LMP
48h
72h
B
cell migration rate (% control)
140
120
100
80
60
40
20
0
***
VEGF
VEGF+ LMP
C
No. of migrated cells
250
200
150
100
50
0
***
##
VEGF - + + + +
LMP - - + - +
apocynin - - - + +

Figure 6

Figure 6. LMPs inhibit VEGF-induced cell migration. (**A**) Migration of HUVEC was induced by 10 ng/mL VEGF in the absence or presence of 10 μg/mL LMPs. Photographs were taken at 48h and 72h. Black arrows indicate direction of cell migration. Representative images (4X) are shown from three independent experiments performed in duplicate. (**B**) Cell migration (72 h) was quantified by MTT assay and is represented as the relative cell migration rate compared to VEGF alone ***$p<0.001$. (**C**) LMPs inhibit VEGF-induced cell migration in Boyden chamber assay. HUVEC were incubated with 10 μg/mL LMPs in the absence or presence of 1.5 mM apocynin for 24 hours under induction of VEGF (10 ng/mL). The total number of migrated cells per well were counted and presented as means ± SE. ***$p<0.001$ vs. VEGF and ## $p<0.01$ vs. VEGF + LMP.

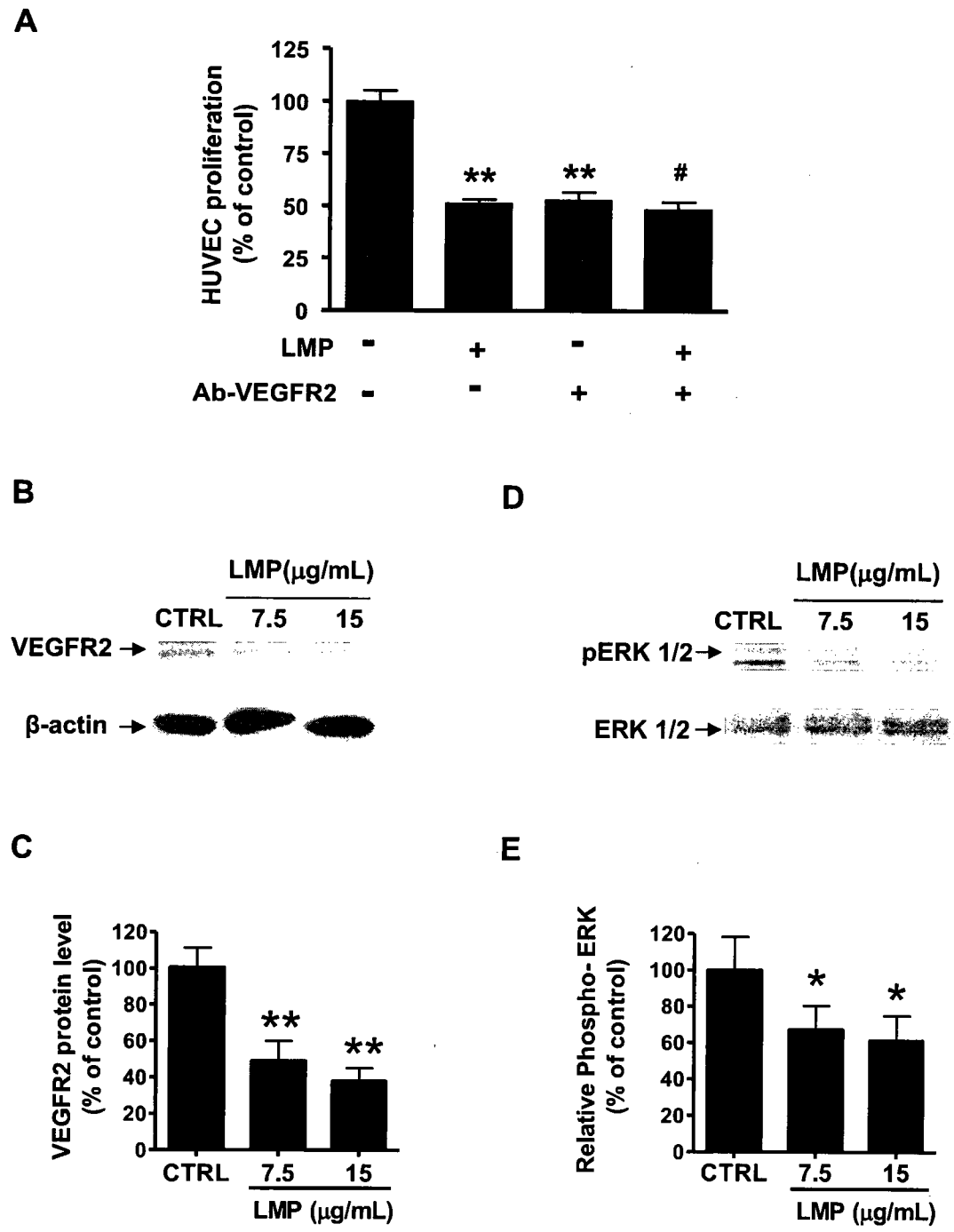
A
HUVEC proliferation (% of control)
125
100
75
50
25
0
**
**
#
LMP - + - +
Ab-VEGFR2 - - + +
B
LMP(μg/mL)
CTRL 7.5 15
VEGFR2
β-actin
D
LMP(μg/mL)
CTRL 7.5 15
pERK 1/2
ERK 1/2
C
VEGFR2 protein level (% of control)
120
100
80
60
40
20
0
**
**
CTRL 7.5 15
LMP (μg/mL)
E
Relative Phospho- ERK (% of control)
120
100
80
60
40
20
0
*
*
CTRL 7.5 15
LMP (μg/mL)

Figure 7

Figure 7. LMPs downregulate VEGFR2 protein expression and ERK 1/2 phosphorylation. (**A**) HUVEC were treated with a VEGFR2 polyclonal antibody (1.5 μg/mL) in the absence or presence of LMPs (10 μg/mL) for 24 hours followed by cell proliferation measurements. Relative proliferation rates are presented as means ± SE. **p<0.01 vs. CTRL; #p>0.05 vs. LMP or Ab-VEGFR2 group. HUVEC were pre-exposed to 7.5 and 15 μg/mL LMPs for 24 hours, VEGFR2 protein level (**B**) and phosphorylated, total ERK1/2 (**D**) were determined by Western blot. (**C**) VEGFR2 protein levels were normalized to β-actin and presented as relative to control, **p<0.01 vs. CTRL.(**E**) The level of phosphorylated ERK1/2 are normalized to total ERK1/2 and depicted as relative to control, *p<0.05 vs CTRL.