Novel Regulators of Microvascular Function

by

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CERTIFICATE OF APPROVAL

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This dissertation is dedicated to my parents,
Agustina and Omar Zamora,
and to my wife, Melody Maani-Zamora

Through their sacrifice, encouragement and
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Abstract

Over the past two decades, vascular biologists have changed our perception on the importance of the endothelial cell in physiologic and pathologic processes in the body. No longer is the endothelium considered a non-thrombogenic lining of blood vessels, which acts as a passive, selective filter between the blood and its surrounding tissue. The endothelium is now recognized to be a significant player in such biological processes as inflammation, blood coagulation, angiogenesis, tumor growth, arthritis, diabetes, atherosclerosis and ischemia/reperfusion injuries. Furthermore, it is now understood that endothelial cell morphology, function, and protein expression can vary drastically between large/small vessels and arteries/veins, all of which are varied even further in a tissue-specific manner.

The central theme of this body of work is ocular endothelial cells and their response to inflammatory/angiogenic mediators at both *in vitro* and *in vivo* levels. The initial focus of this work was to test the hypothesis that the basic inflammatory properties of human iris endothelial cells (HIEC) can contribute to leukocyte trafficking during inflammation. To test this premise, we first developed a method to routinely isolate purified HIEC which could be propagated and studied *in vitro*. Using these cells, in conjunction with a variety of gene, protein, and functional analytical methods, knowledge was furthered on the expression profiles of various biological mediators during inflammation (e.g., adhesion molecules, cytokines and chemokines). We next hypothesized that EphrinB2 could be expressed by human peripheral blood leukocytes in response to inflammatory mediators and that EphrinB2 could regulate the intracellular signaling activities of HIEC. Data generated from this study indicate that EphrinB2, a

ligand up regulated by stimulated lymphocytes, can activate HIEC. These observations suggest a novel mechanism by which HIEC regulate lymphocyte trafficking during inflammation. Lastly, the potential role of EphrinB2 and EphB4 molecules in retinopathy of prematurity (ROP) was investigated. EphrinB2 ligands and their EphB4 receptors are key regulators of endothelial cell proliferation and migration. We hypothesized that these molecules play a role in pathological neovascularization (NV) in the mouse model of oxygen-induced retinopathy and that soluble versions of these molecules can alter this NV. Results generated from this study support the hypothesis that endogenous EphrinB2 and EphB4 are regulators of retinal NV during oxygen-induced retinopathy and may serve as novel targets for therapeutic intervention.

If I have seen further than others, it is by standing upon the shoulders of giants.

-Newton, Isaac (1642-1727)

Chapter 1: Introduction

1A: The Endothelial Cell: A Historical Perspective

In 1661, a new era in vascular biology began when Marcello Malpighi presented the final missing piece of evidence for the theory of blood circulation set forth by William Harvey (reviewed in Thilo-Korner, 1983)[1]. He described the first piece of evidence for the existence of capillaries in blood circulation[1]. The contributions of Harvey, and those of Antony von Leuwenhoek (better microscopes) and Jan Swammerdam / Friedrich Ruysch (blood vessel dye injection techniques), provided essential stepping-stones that brought vascular biologists closer to discovering the endothelial cell (EC)[1]. Between 1840 and 1860, Henle, von Koelliker and Frey provided the first report on cells lining the walls of capillaries and described as "structure-less skin with nuclei"[1]. Little did they know they had just described the ECs that line the walls of capillaries. It was not until 1860 when von Recklinghausen developed the silver precipitation injection technique that the "structure-less skin" was further described as a single sheet of cells. It took another five years before the term "endothelium" was coined specifically referring to the cells lining the luminal side of a blood vessel wall.

At the turn of the century, Harrison (1907) first reported on the analysis of cells derived from pieces of tissue, thus coining the term "tissue culture"[2]. In 1910 Carrel and Burrows adopted Harrison's technique in an attempt to culture arteries derived from dogs and cats[3]. They were able to obtain a variety of cells growing out of the vessels, but the cells were of mixed population and only viable for a few days. In the 1920's Lewis observed the outgrowth of ECs from cultured fragments of chick embryo liver[4], and in 1931 Lewis[5] and Shabuya[6] published their first results on the cultivation of ECs.

However, their EC isolation technique was plagued by two issues: 1) limited EC proliferation and 2) overgrowth of EC by "spindle shaped" cells, known today as fibroblasts. It was not until 1959 that Lazzarini-Robertson reported the cloning of two cell types derived from arterial explants, one clone was described as ECs and the other as fibroblasts[7]. Although Lazzarini-Robertson is typically given credit with developing the first purified culture of ECs, the technique was still plagued by limited proliferation of their cells. An alternative approach for the isolation and culture of ECs was reported in 1963 by Maruyama[8], and by Fryer[9] in 1966, by demonstrating that they could obtain ECs after perfusing umbilical cord vessels with trypsin solutions. However, the ECs isolated still demonstrated limited proliferation and stromal cell contamination.

In the 1970's, another chapter in EC biology was opened when Eric Jaffe drastically modified Maruyama's and Fryer's techniques and isolated ECs from umbilical cord vein. These cells could be passed repeatedly and remained pure[10-12]. One of the many modifications implemented was the use of collagenase as the digesting enzyme, instead of trypsin. The collagenase only released the ECs lining the vessel. This technique reduced the possibility of digesting the internal elastic tissue (Figure 1) of the umbilical vein, eliminating the possibility of obtaining smooth muscle cells and fibroblasts (collagenase selectively digests subendothelial basement membrane leaving the internal elastic lamina intact). By this method, Jaffe significantly increased his initial yield of ECs and provided the cells with an optimized culture technique, which supported their proliferation over longer periods. Over the past 30 years, Jaffe's technique has provided a significant cornerstone for vascular biologists. Numerous variations and modifications of Jaffe's technique exist, which has allowed EC to be isolated from

various parts of the vascular tree. Over the past decade, EC-isolation techniques allowed for vascular cells from the human eye to be isolated and cultured. The eye's first tissues to have their ECs isolated and studied were the choroid and retinal tissues[13,14].

- In this dissertation, I report on the isolation and purification of human iris ECs, the first and only report of its kind to date[15].

1B: The Endothelial Cell: A Contemporary View

A monolayer of EC lines the entire vascular tree. As reported by Jaffe, this monolayer covers a surface of greater than $1000m^2$ in the human adult, and adds up to approximately 10^{12} cells with a total weight greater than 100g [16]. ECs are polarized with their apical surface being in direct contact with blood, and their basal surface in contact with the extracellular matrix, which is in proximity to smooth muscle cells, pericytes and tissue-specific stromal cells (Figure 2A). ECs are a highly specialized form of simple squamous epithelial cells.The junctions between ECs are similar to those of other epithelial cells and consist of adherens junctions, tight junctions, gap junctions, and complexus adherents junctions. These junctions must be highly regulated in order to maintain the integrity of the endothelium and protect the vessels from uncontrolled increases in permeability, inflammation or thrombotic reactions. Furthermore, these junctions not only function as sites of EC-EC attachment, but have emerged as significant signaling structures in the regulation of vascular hemostasis (Figure 2B).

Although the adult endothelium is relatively quiescent in normal situations, it is highly metabolically active. Over the past two decades, old perceptions have evolved from the endothelium acting as a non-thrombogenic lining of blood vessels to acting as a

passive selective filter between blood and tissue. The endothelium is now recognized to be a significant player in various physiological and pathological processes such as inflammation, blood coagulation, angiogenesis, tumor growth, arthritis, diabetes, atherosclerosis and ischemia/reperfusion injuries (Reviewed in Boeynaems and Pirotton, 1994)[17]. The pleiotropic role of ECs involves several basic functions including:

- Secretion of various biologically active factors (i.e., nitric oxide, prostacyclin, and von Willebrand factor).
- Expression of binding proteins on their luminal surface such as thrombomodulin which inhibits thrombin's procoagulant activity, thus contributing to the non-thrombogenic surface of the endothelium.
- Expression of adhesion molecules for leukocytes, tumor cells and metabolic enzymes (i.e. angiotensin-converting enzyme and lipoprotein lipase).
- Regulation of the vessel permeability and transport between the blood and tissue.
- Regulation of contractility of vascular smooth muscle cells.
- Control of smooth muscle cell proliferation and angiogenic processes.

1C: The Heterogeneity of Endothelial Cells

Although all vascular ECs perform certain functions in common, there is a high diversity of specialized functions that depend on the type of the blood vessel and the requirements of the surrounding tissue [18]. In many cases, our understanding of these diverse functions remain on the level of "phenomenon" with little to no molecular explanation. We are at the advent of a new era in EC biology with the discovery of EC heterogeneity, which furthers our understanding from the morphological level to the

functional and transcriptional levels as well. EC heterogeneity is most evident from the morphology of the ECs that line the vessel of various tissues (continuous, fenestrated, or discontinuous; Figure 1B). Furthermore, ECs that line microvessels are generally flattened, elongated, and often fenestrated, while ECs that line large vessels are generally polygonal, nonfenestrated and thicker[19]. Even between capillary beds of different tissues, organ-specific differences exist. For example, capillaries of skeletal muscle, heart, lung and brain have continuous endothelium, while capillaries of endocrine and exocrine glands, choroid plexus and intestinal villi will typically possess fenestrations[20]. It is suggested that there are as many types of varieties of capillaries as there are organs and tissues[21].

ECs will also vary in the types of function they perform based on the tissue in which they reside. For example, brain endothelium interacts with astrocytes to comprise the highly regulated blood-brain barrier by forming a continuous endothelial barrier with complex tight junctions[22]. In contrast, liver ECs are highly fenestrated and discontinuous, and line hepatic sinusoids in the mediation and exchange of metabolites, and process toxins between the portal blood, Kupffer cells and hepatocytes[23].

A new concept in EC heterogeneity has emerged over the past decade. It is now understood that ECs exhibit a high level of diversity in the types of proteins they express at their luminal surface. It was reported over a decade ago that ECs in the vasculature of different organs express organ-specific proteins, suggesting the presence of vascular bed "addresses."[24]. More recently, organ-specific differences in vascular EC protein expression have been discovered using a biopanning technique termed "phage display." This technique utilizes small peptides expressed on the surface of bacteriophages that are

capable of differentially binding to ECs of different vascular beds[20]. Using this technique, investigators have reported on the identification of lung-specific, breast-specific, and prostate gland-specific EC surface proteins through the binding of phage-presented peptides to membrane dipeptidase, aminopeptidase P, and interleukin-11, respectively[20,25,26]. Phage display technologies have revealed a map of "vascular addresses" which can potentially be used to selectively target therapeutic agents to specific tissue vascular beds, while sparing others. Furthermore, genetic studies have revealed differences between the endothelium of arteries and veins. For example, Wang et al. (1999) reported on the expression of EphrinB2 on arterial ECs, while EphB4, the receptor tyrosine kinase that binds EphrinB2, is expressed at much higher levels in veins than in arteries[27]. Although it is clear that the endothelium between different vascular beds can be highly heterogeneous, many questions remain as to how these differences have arisen and how much plasticity the ECs have retained. Equally as important, it remains to be determined how the heterogeneity of ECs between vascular beds affects their susceptibility to inflammation.

1D: Inflammation and the Multistep Process of Leukocyte Adhesion-Extravasation

Cornelius Celsus is credited with the phrase "rubor et tumor cum calore et dolore," or "redness and swelling with heat and pain," which dates back to his first century AD writings[28]. However, Julius Conheim, a student of Virchow, is generally credited with the observation that inflammation also includes plasma exudation and local leukocyte extravasation into the affected tissue[28].

During inflammation, the EC plays the critical role of "gatekeeper" and selectively regulates which leukocytes pass through its barrier to enter the tissue. Leukocyte recruitment into inflamed tissues is a well-characterized process and occurs in a multistep fashion (Figure 3)[29-32]. Initially, activated ECs reduce the velocity of the flowing leukocyte by presenting a variety of adhesion molecules on their luminal surface. These molecules interact with the adhesion molecules expressed on the surfaces of leukocytes and cause the leukocyte to roll on the endothelium. This process is a prerequisite for the latter steps of leukocyte "firm adhesion" and "extravasation"[33,34]. Rolling is mediated by L-selectin on leukocytes, and E- and P-selectins on ECs[35] to reduce the speed of the leukocytes entering the venular portion of the microcirculation[33,35-41]. Studies *in vivo* suggest that initial leukocyte rolling is mainly P-selectin dependent [39,40,42], whereas E- and L-selectin appear to be responsible for rolling only after inflammatory stimulation for an hour or more[41-44].

After rolling, leukocytes become activated, which in turn activates integrins expressed on ECs and causes the arrest and firm adhesion of the leukocyte[45]. This process is mediated by leukocyte-expressed integrins (e.g., LFA-1, Mac-1, VLA-4, LPAM-1) interacting with members of the immunoglobulin superfamily of adhesion molecules (ICAM-1, ICAM-2, VCAM-1, MAdCAM-1) expressed on the activated endothelium. Leukocytes also firmly adhere via interaction with platelet-EC adhesion molecule (PECAM; also a member of the immunoglobulin superfamily; Figure 2A and 2B) and is expressed on leukocytes and at endothelial junctions. Chemokines also play a vital role in firm adhesion by mediating the rapid clustering of intergrins[46,47]. The chemokine superfamily of low molecular weight chemotactic cytokines bind to and signal through

seven-transmembrane, G-protein coupled receptors expressed on the leukocyte[48-51]. The chemokines are expressed by ECs and stromal cells in the local microenvironment and are presented on the endothelial surface to activate leukocytes[52,53]. Each of these processes set the stage for the leukocyte's final act, the compromising of the endothelial barrier, a process generally referred to as "extravasation."

Extravasation, the least characterized step in leukocyte trafficking during inflammation, begins when the leukocyte squeezes through an inter-endothelial cleft to enter the inflamed tissue. Vasoactive agents induce the formation of gaps between adjacent ECs. *In vitro* studies have demonstrated that histamine can increase the permeability of ECs, and this process requires the release of cytoplasmic calcium[54]. The increases in cytoplasmic calcium cause a calcium-dependent activation of myosin light chain kinase, an enzyme that phosphorylates myosin light chains to induce actin polymerization, and results in EC retraction[54-58]. During this process, the leukocyte encounters different junctional proteins (Figure 2A and 2B). Vascular endothelial cadherin, generally located between ECs, redistributes to the EC surface (Figure 2B), while PECAM and junctional adhesion molecule (JAM) remain concentrated along the EC margins (Figure 2B)[59]. CD99, a membrane protein expressed by both leukocytes and ECs functions to guide the migrating leukocyte through the EC-EC barrier. Blockage of PECAM or CD99 during this process can prevent the leukocyte from extravasating[59]. Upon crossing the endothelial barrier, the leukocyte encounters the extracellular matrix (ECM) and will then secrete degradative enzymes to help clear a path to enter the inflamed tissue[60]. This extravasation route described here is generally referred to as the "paracellular" route, but it should be noted that this is not an exclusive pathway for

crossing a monolayer of endothelium. Early structural studies suggest that lymphocytes can pass through the body of an EC[61,62]. Feng et al., (1998) recently demonstrated that under certain conditions neutrophils can migrate "transcytotically" (directly through an EC) within inflamed venules[63]. However, it is generally thought that the former route mentioned occurs the majority of the time.

1E: Inflammation and the Eye

Although inflammatory processes throughout the body share the same basic mechanisms, it is becoming increasingly evident that the vascular beds of the body do not recruit leukocytes equally[64], nor are they equally susceptible to inflammation[65]. Site-specific differences such as EC heterogeneity, molecular microenvironment, and hemodynamics are thought to contribute in tissue-specific inflammation.

My research has focused on elucidating the pathogenic mechanism(s) involved in a collection of inflammatory diseases involving the anterior uveal tract. This inflammatory process is generally referred to as anterior uveitis (AU)[66]. The uveal tract is a highly vascularized region of the eye and consists of the iris, ciliary body, and choroid tissues (Figure 4A). At the onset of AU, ECs and leukocytes in the iris become activated. This results in the transmigration of leukocytes from the blood into the iris stroma. This phenomenon is orchestrated by chemokines and adhesion molecules on the surfaces of the iris endothelium and leukocytes[67-70]. In extreme conditions, leukocytes can transmigrate through the body of the iris, into the anterior chamber where they can affect vision (Figure 4B). Interestingly, the exact cause of this tissue-specific inflammation remains unknown, but is it thought to stem from an autoimmune reaction. The question

still remains as to why the vasculature of the anterior uveal tract (iris) becomes inflamed, while sparing the choroid and/or retinal vascular beds. Similarly, other ocular diseases are characterized by the selective inflammation of choroid and retinal tissues[71]. Various animal models[71] were developed in an attempt to answer this question, and many of the molecular players and underlying biology were elucidated in these systems[67]. However, species differences make it difficult to carry these findings into the human.

One approach taken by our lab to understand iris-specific inflammation better is to isolate, culture and experimentally test the inflammatory properties of human iris ECs (HIEC). Ultimately, procurement of these EC has allowed the inflammatory properties of iris endothelium to be studied[15] and has allowed for the side by side comparison of inflammatory properties between iris, choroid and retinal ECs (manuscript in preparation; Silverman *et al.*; Rosenbaum Laboratory).

1F: Vasculogenesis vs. Angiogenesis

Two distinct mechanisms exist in the formation of the vascular network in the embryo, 1) vasculogenesis and 2) angiogenesis. While vasculogenesis refers to differentiation and development of blood vessels from mesodermal-derived angioblasts *in situ* (Figure 5B)[72], angiogenesis refers to the remodeling and expansion of this network.

Vasculogenesis

The initial steps in the formation of the extra-embryonic vasculature begins in the embryonic yolk sac by the induction of the ventral-lateral mesoderm via members of the fibroblast growth factor (FGF) and transforming growth factor beta (TGF-β) families[73]. *In situ* differentiation of hemangioblasts from the mesoderm begins the process of

vasculogenesis by forming blood islands which then interconnect to each other (Figure 5A)[74]. The peripheral cells of the blood island will then differentiate into pre-ECs, while the cells centrally located become hematopoietic cells (Figure 5A and 5B)[75]. Vasculogenesis then proceeds by fusion of the pre-ECs into a primitive capillary plexus (Figure 5A and 5B), which is then expanded by angiogenic processes[76].

Intra-embryonic vascularization follows similar steps to those of the yolk sac. However, a major difference exists between extra-embryonic and intra-embryonic vascular development. Extra-embryonically, EC differentiation occurs simultaneously and in close association with hemopoietic precursor cells in the blood island[77,78]. Intra-embryonically, ECs differentiate from the mesoderm as solitary angioblasts, without the presence of hemopoietic precursor cells (with the exception of a small region of the aorta)[79,80]. Embryonic angioblasts then migrate and fuse with other angioblasts and other forming capillaries to form a vessel *in situ*[78,81]. Once extra- and intra-embryonic vascular networks connect, the intra-embryonic vessels are then populated by hemopoietic cells derived from the yolk sac or aorta[78]. Vasculogenesis, which is mainly restricted to embryonic development, proposedly also occurs in adults. This notion is supported by the finding that circulating angioblasts exist in human peripheral blood and are capable of differentiating *in vitro* into ECs[82].

Angiogenesis

Angiogenesis and vasculogenesis are distinct from one another on the cellular level, however the molecular mechanisms controlling both processes overlap since both processes share several required molecules[76,83]. Angiogenesis is a dynamic, multistep process that requires the retraction of pericytes from the abluminal surface of the

capillary, and the release of proteinases from the endothelium to degrade the ECM surrounding the vessel[84]. This process allows the EC to be released from its vessel, migrate towards the angiogenic stimulus, proliferate and form tube-like structures[85]. The tubes will then fuse with other pre-formed vessels, which leads to their filling of blood to initiate flow[85]. Angiogenesis is the major form of vascularization in adults and occurs throughout adult life (i.e., female reproductive system, wound healing, and various pathological conditions).

In general, capillaries can be formed by sprouting and non-sprouting angiogenesis[76]. Sprouting angiogenesis occurs extra-embryonally in the yolk sac and intra-embryonally during brain development. Non-sprouting angiogenesis occurs in the lung and accounts for splitting of pre-existing vessels by transcapillary pillars. Non-sprouting angiogenesis can also occur by the intercalated growth of blood vessels, a process by which pre-existing capillary merge, or additional ECs fuse into existing vessels to increase their diameter and length[74,86]. The yolk sac, lung and heart are sites where both sprouting and non-sprouting angiogenesis can occur[76]. After the primary capillary plexus has expanded by angiogenesis, it becomes remodeled into a mature system of arteries, capillaries, and veins. During maturation, the vessels are then surrounded by pericytes and smooth muscle cells. These cells generally sprout in concert with ECs, and this process is regulated by platelet derived growth factor[87], Tie-2 and angiopoietin-1[74]. Once positioned in proximity of the EC-tube structures, the pericytes and smooth muscle cells then deposit an ECM which provides structural strength to the developing vasculature[74].

Major Regulators of Angiogenesis

VEGF and its receptors are key players of angiogenesis[88-90]. VEGF is a potent EC mitogen and inducer of angiogenesis that is devoid of consistent and appreciable mitogenic activity for other cell types[91,92]. Furthermore, disruption of VEGF receptors leads to defective large vessel formation and subsequent cardiovascular failure[90]. TGF-β and its receptor are also major players in angiogenesis[93,94]. EphrinB2, a member of the Ephrin family of transmembrane ligands, marks arterial ECs, where as its cognate receptor EphB4 is expressed on veins. Inactivation of EphrinB2 or EphB4 prevents the remodeling of arteries and veins into properly branched structures and results in lethality[27], underscoring their importance in angiogenesis. Angiogenesis is also dependent on αvβ3 adhesion receptor which allows ECs to interact with a variety of extracellular matrix components and allows their migration[95-97]. Furthermore, αvβ3 plays a significant role in allowing the activation of VEGF receptor-2[98]. Matrix metalloproteinase-2 co-localizes with αvβ3 on the cell surface of invasive cells, and is proteolytically active[99]. VE-cadherins are shown to play a vital role in angiogenesis by mediating EC-EC interactions to regulate their organization into vessel-like structures[100]. In fact, VE-cadherin negative EC demonstrate vascular defects during expansion of the primitive vascular network by angiogenesis, but not during vasculogenesis[100]. Truncation of VE-cadherin is capable of blocking the capacity of EC to respond to survival signals induced by VEGF[101].

1G: Retinopathy of Prematurity

Retinopathy of prematurity (ROP) is generally characterized by extensive

angiogenesis in the retina which can lead to blindness[102]. ROP is a condition of growing concern in the United States. Incidence of blindness associated with this condition is estimated at nearly 700 infants per year, and some permanent vision loss is expected to occur in nearly 4000 infants annually due to this disease[103,104]. The increasing incidence of ROP is linked to the implementation of new neonatal care technologies which enhance the survival of very low birth weight infants and the continued need for supplemental oxygen[104]. The pathological conditions of ROP are unique in that normal and abnormal vessel growth occurs simultaneously in the retina and in close proximity.

In general, astrocytes emigrating from the optic nerve[105] enter the avascular retina. As astrocytes become hypoxic, they express high levels of vascular endothelial growth factor (VEGF)[106]. Secretion of VEGF by the astrocytes induces the growth of blood vessels from the optic nerve into the superficial layer of the retina and alleviates hypoxia in this region (Figure 6A). This phenomenon is immediately followed by the downregulation of VEGF in the superficial region, but a relative hypoxia of the deeper, avascular layers (intermediate and deep) still exists. This condition induces the sprouting of superficial vessels into the intermediate/deep layers of the retina and fully vascularizes the tissue. During these critical periods of vascular development, any alteration in VEGF expression can alter retinal vascular formation. Infants whose lungs are not fully developed are placed in a high-oxygen environment, which alters VEGF expression in the retina. Upon return to normal room air, the retina experiences a relative hypoxia, high VEGF expression, retinal neovascularization, and associated vitreal bleeding and retinal detachment[107].

Animal models of this disease process have been generated and closely mimic the human condition[108]. One of these models experimentally places neonatal mice in high oxygen for several days. This results in the downregulation of retinal VEGF, thus stopping further vascular development and causing some vascular regression (Figure 6B). This treatment results in large avascular regions in the retina, and a severe ischemic condition when these mice are placed back in normal room air. This process induces high levels of retinal VEGF expression and an overgrowth of its vasculature. This constitutes the murine model of ischemic retinopathy and closely relates to human forms of ROP[109].

1H: Ephrins and Ephs

Eph receptors and their Ephrin ligands comprise the largest family of receptor tyrosine kinases[110-112]. Eph receptors are classified into two groups, A or B, depending on whether they interact with an Ephrin of the A class (no cytoplasmic tail) or of the B class (possess cytoplasmic tail; Figure 7). Resultantly, engagement of EphB receptors with ephrinB ligands can induce a "bi-directional" signal into both the EphB and EphrinB expressing cells. EphrinB2 ligands and EphB4 receptors are key regulators of vascular development and are expressed by ECs of developing arteries and veins, respectively. Specifically, endothelium of developing arteries express EphrinB2 ligands, while EphB4 receptors are expressed by venous endothelium[27,113-115]. During vessel formation, arteriole and venule ECs undergoing proliferation and migration come into proximity of each another. Engagements of EphB4 on venule ECs by EphrinB2 on arteriole ECs cause the ECs to stop proliferation and migration, and this phenomenon is thought to prevent these cell types from intermingling (Figure 7)[116]. In general, adhesive/repulsive signaling

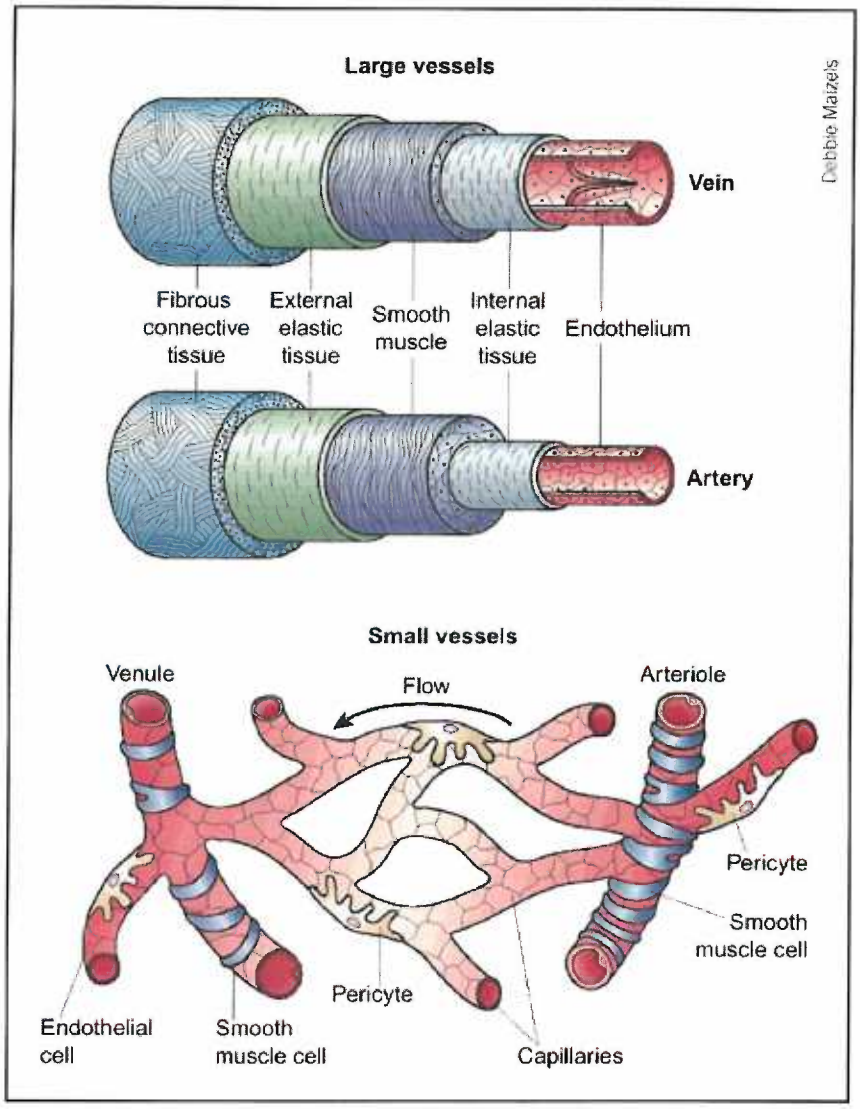
events induced by Eph/Ephrin interactions occur via modulation of integrin activity and by actin cytoskeletal rearrangement. I hypothesize that the Ephrin/Eph signaling system also serves in the regulation of leukocyte trafficking during iris inflammation.

1I: Goals of This Study

The impetus for the proceeding studies stemmed from the need to better understand the role of HIEC in AU. To this end, I detail in this thesis a protocol devised to purify HIEC cultures and analyze their basic inflammatory properties in response to biologic stimuli. Secondly, the observation that the Ephrin/Eph family of signaling molecules can regulate cell migration led to the hypothesis that they may also perform similar functions during HIEC-leukocyte interactions. In this section, I set forth to determine if human peripheral blood leukocyte express EphrinB2, and if their expression profiles could be altered in response to inflammatory mediators. Additionally, I report on the functional relevance that EphrinB2 has on the intracellular signaling properties of EphB4-expressing HIEC. Lastly, since EphrinB2 and EphB4 are known regulators of angiogenesis, I hypothesized that these molecules could play a vital role in pathological neovascularization (NV) during ROP. To test the functional role that EphrinB2 and EphB4 may have in retinal neovascularization, I implemented a mouse model of ROP in which soluble versions of EphrinB2 or EphB4 were intravitreally injected. The results generated from this study suggest that the endogenous Ephrin/Eph signaling mechanism is a critical functional regulator of retinal NV during oxygen-induced retinopathy, and should be considered as therapeutic targets in the treatment of this disease.

Figure 1: Composition of Blood Vessels and Types of Capillary EC. (A) Heterogeneity of vessel wall composition between artery and vein, arteriole and venule, and capillaries. **(B)** Types of EC that line various capillary walls. **(B-a)** Continuous capillaries have no openings in their walls. **(B-b)** Fenestrated capillaries have small openings (80-100nm in diameter). Fenestrae are covered by a nonmembranous, permeable diaphragm, and allow for the passage of macromolecules. **(B-c)** Discontinous capillaries (sinusoids) have a large lumen with many fenestrations. The fenestrations do not have a diaphragm and the basal lamina is either absent or discontinous. *Adapted from Cleaver and Melton (2003) Nat. Med. 9(6):661-668*

A


Large vessels
Vein
Fibrous connective tissue
External elastic tissue
Smooth muscle
Internal elastic tissue
Endothelium
Artery
Small vessels
Venule
Flow
Arteriole
Pericyte
Smooth muscle cell
Endothelial cell
Smooth muscle cell
Pericyte
Capillaries
Debbie Maizels

B

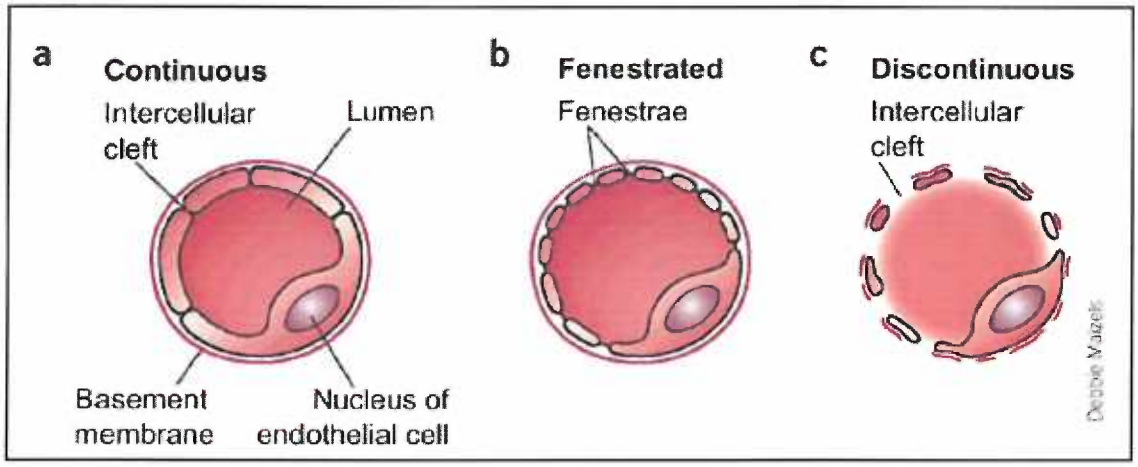
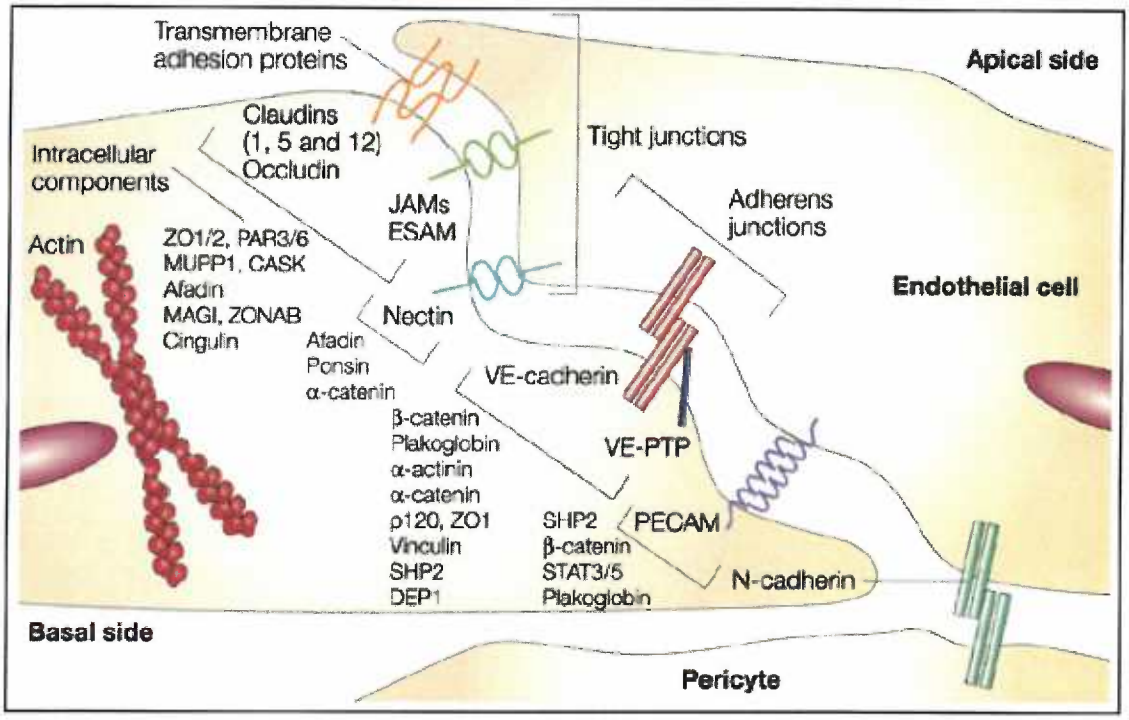

a
Continuous
Intercellular cleft
Lumen
Basement membrane
Nucleus of endothelial cell
b
Fenestrated
Fenestrae
c
Discontinuous
Intercellular cleft
Debbie Maizels

Figure 2: Molecular Components of Endothelial Junctions and Leukocyte Extravasation. **(A)** Depicted are two main types of EC junctions (tight and adherens junctions), and the molecules that comprise these junctions (i.e., cadherins, PECAMs, Nectins, JAMs, Claudins). Also depicted are the various cytoskeletal and signaling proteins that can associate with junction-forming proteins. **(B)** During diapedesis, leukocytes express many of the same junctional proteins already implicated between EC. However, some proteins such as VE-cadherin re-distribute to the apical surface of the EC. *Adapted from Dejana (2004) Nat. Rev. Mol. Cell Biol., 5(4):261-70.*

A


Transmembrane adhesion proteins
Apical side
Claudins (1, 5 and 12) Occludin
Tight junctions
Intracellular components
JAMs ESAM
Adherens junctions
Actin
ZO1/2, PAR3/6 MUPP1, CASK Afadin MAGI, ZONAB Cingulin
Endothelial cell
Nectin
Afadin Ponsin α-catenin
VE-cadherin
β-catenin Plakoglobin α-actinin α-catenin p120, ZO1 Vinculin SHP2 DEP1
VE-PTP
SHP2 β-catenin STAT3/5 Plakoglobin
PECAM
N-cadherin
Basal side
Pericyte

B

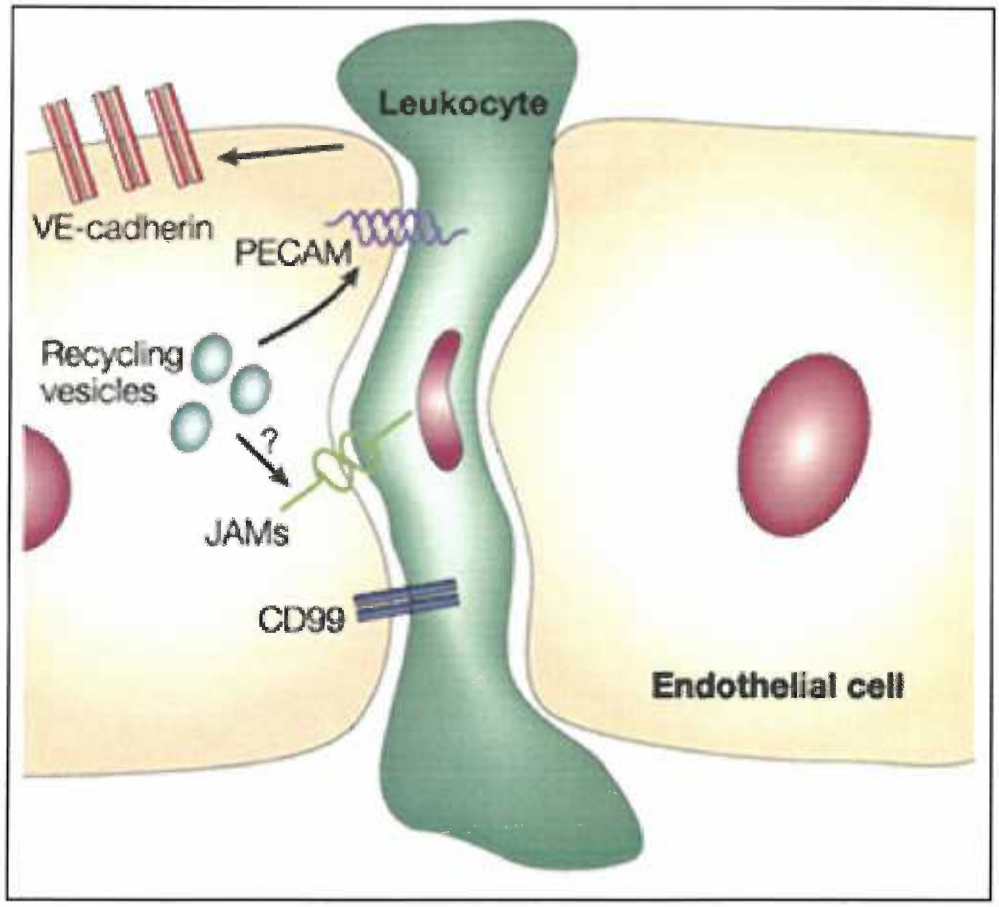

Leukocyte
VE-cadherin
PECAM
Recycling vesicles
?
JAMs
CD99
Endothelial cell

Figure 3: Molecular Players Involved in the Multistep Adhesion Process. There are four distinct steps in leukocyte adhesion to endothelium (1. tethering, 2. rolling, 3. activation, 4. arrest). Depicted under each of these steps is a "magnified" view of the molecular players involved in leukocyte-EC interactions. The double-headed arrows between leukocyte- and EC-expressed molecules depicts their possible interaction. *Adapted from: von Andrian and Mackay (2000) N. Eng. J. Med., 343(14):1020-1034.*

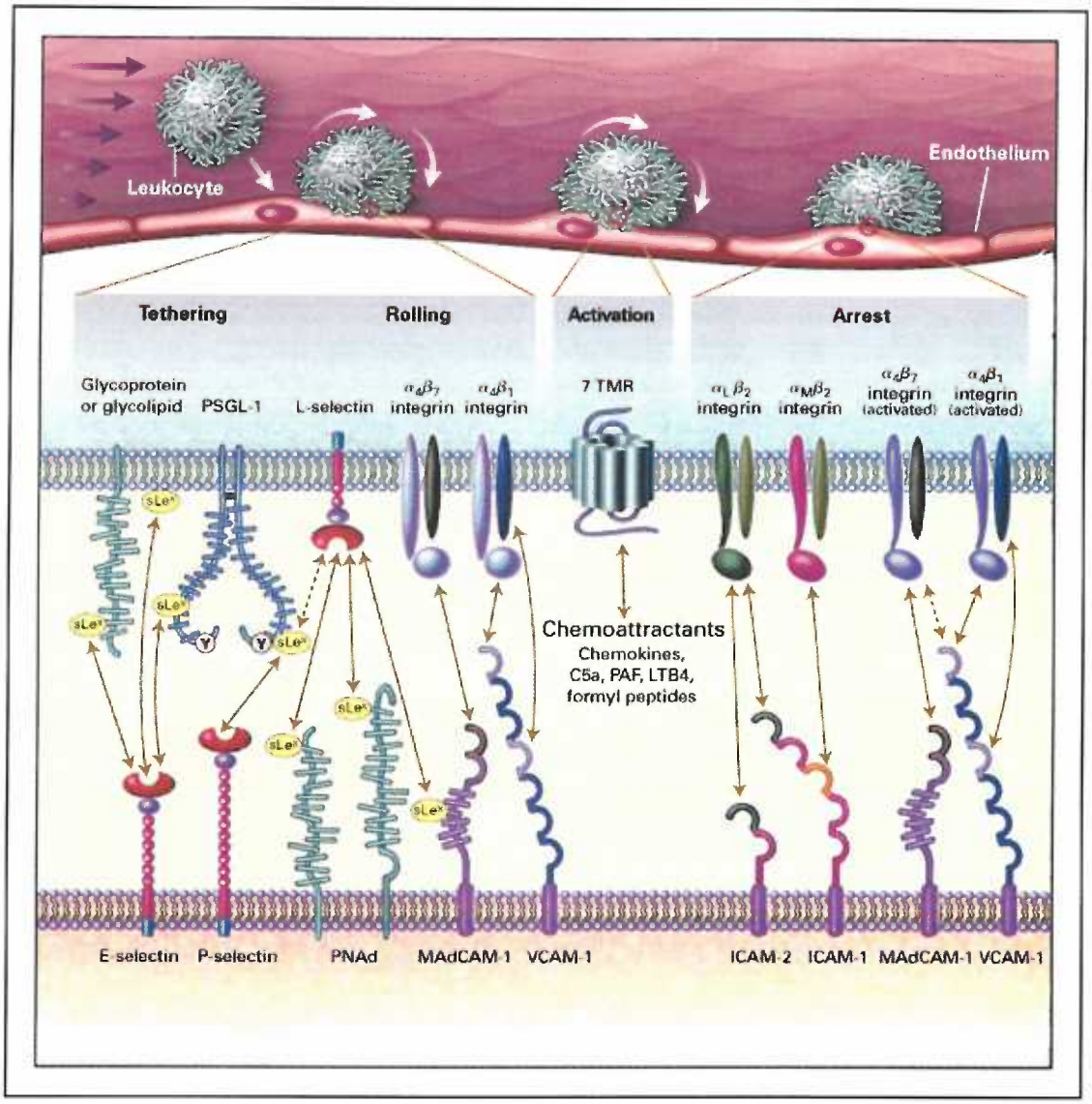
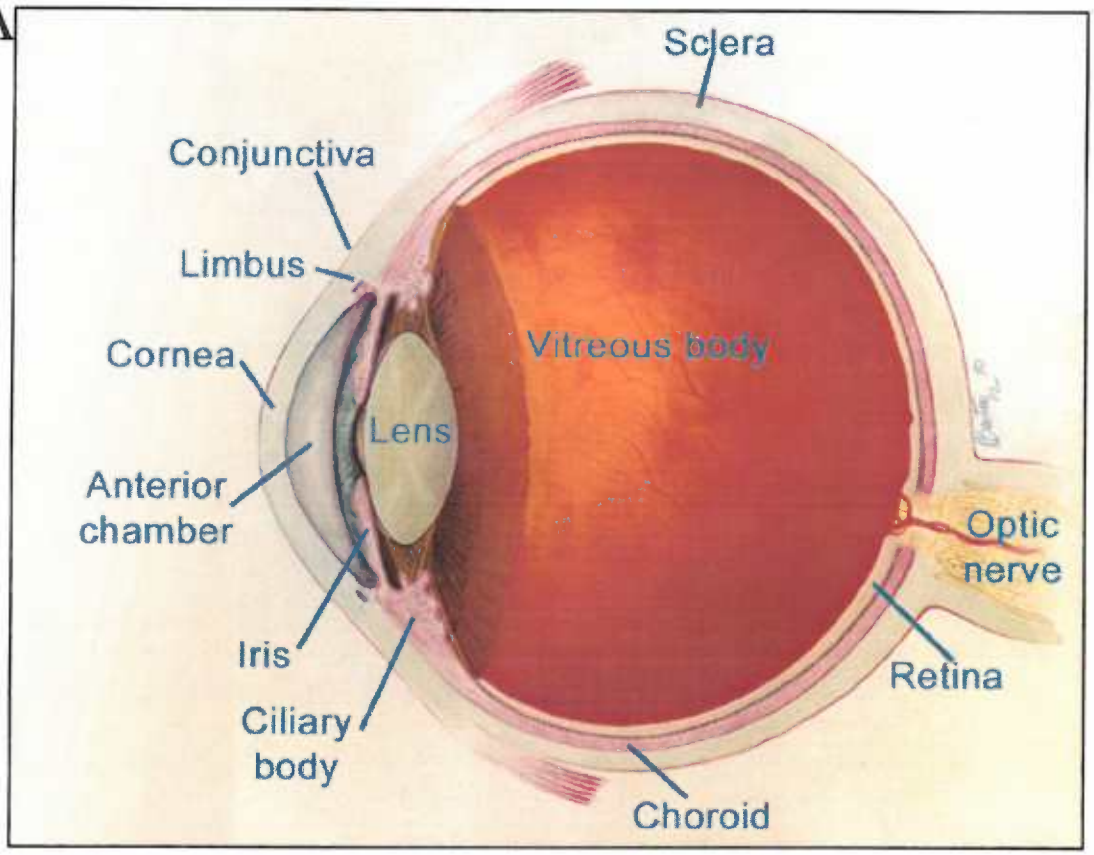

Leukocyte
Endothelium
Tethering
Rolling
Activation
Arrest
Glycoprotein or glycolipid
PSGL-1
L-selectin
$\alpha_4\beta_7$ integrin
$\alpha_4\beta_1$ integrin
7 TMR
$\alpha_L\beta_2$ integrin
$\alpha_M\beta_2$ integrin
$\alpha_4\beta_7$ integrin (activated)
$\alpha_4\beta_1$ integrin (activated)
sLe^x
Chemoattractants
Chemokines, C5a, PAF, LTB4, formyl peptides
E-selectin
P-selectin
PNAd
MAdCAM-1
VCAM-1
ICAM-2
ICAM-1
MAdCAM-1
VCAM-1

Figure 4: The Human Eye and Anterior Uveitis. **(A)** The uveal tract of the human eye includes the iris, ciliary body, and choroid tissues. The term uvea is derived from Latin terminology meaning "grape" since the uveal tissue resembles the skin of a grape. *Adapted from the NEI website, with modifications made by Dr. Stephen R. Planck.* **(B)** During extreme bouts of anterior uveitis (iritis), leukocytes can extravasate out of the iris vasculature, through the iris stroma, and into the anterior chamber. Once in the anterior chamber, leukocytes can "float" in the aqueous humor and disrupt vision. Gravity can act on floating leukocytes and cause them to accumulate within the anterior chamber (hypopyon; arrow). *Obtained from Dr. James T. Rosenbaum.*

A


Sclera
Conjunctiva
Limbus
Cornea
Lens
Vitreous body
Anterior chamber
Optic nerve
Iris
Retina
Ciliary body
Choroid

B

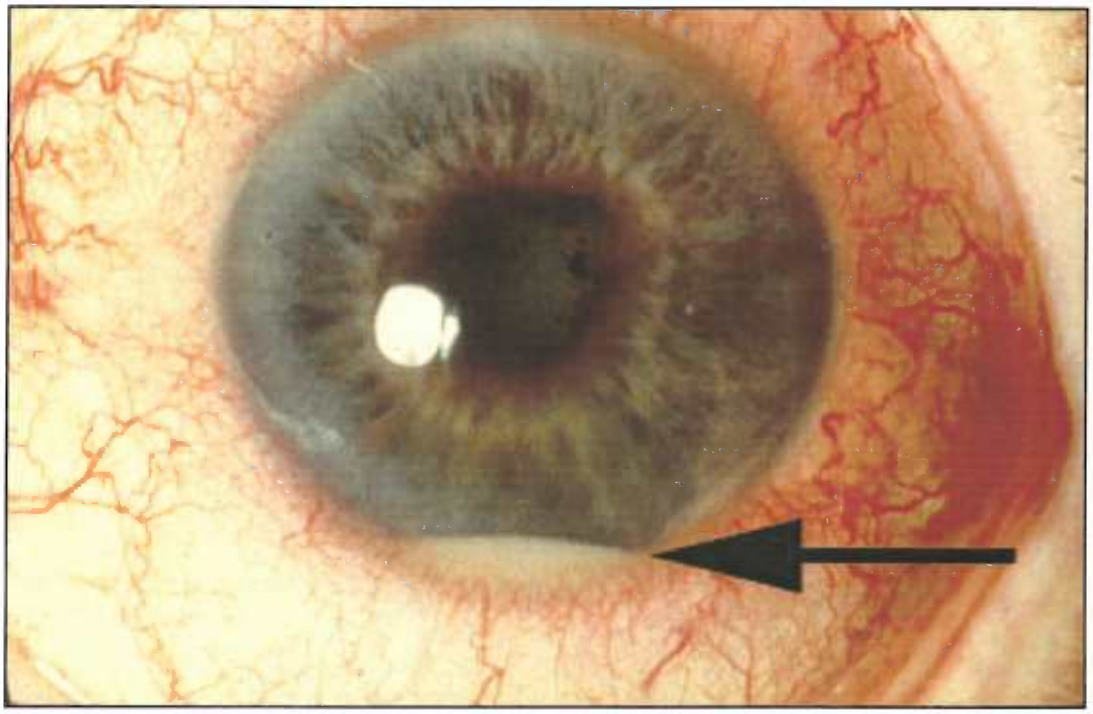
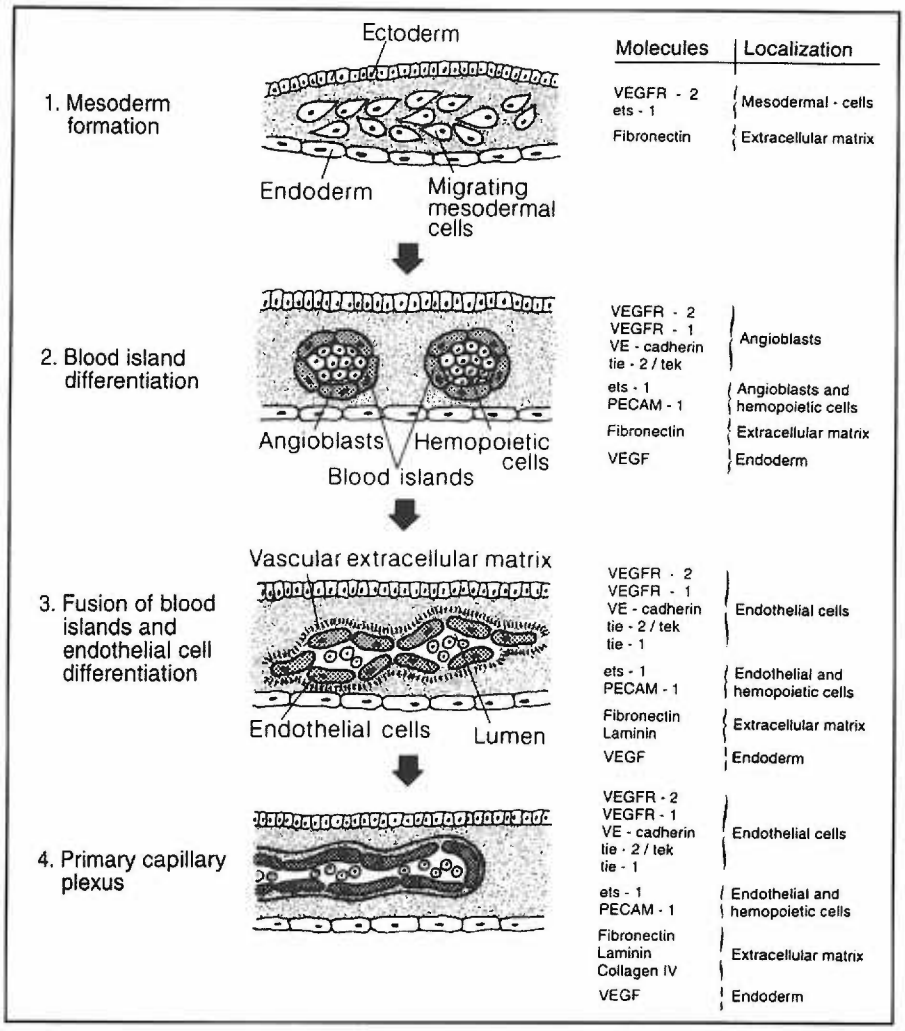


Figure 5: Vasculogenesis and Hematopoiesis. **(A)** Schematic representation of the processes and major molecular players of vasculogenesis. 1) The process begins by the formation of the mesoderm which arises from epiblast pre-cursor cells. 2) Mesodermal derived hemangioblasts form cell aggregates (blood islands), and the cells in the center differentiate into hematopoietic stem cells, while peripheral cells differentiate into angioblasts (endothelial cell precursors). 3) Proximal blood islands fuse to form an outer EC barrier with a lumen that contains hemopoietic cells, thus forming the primary capillary plexus (4). *Adapted from: Risau and Flamme (1995) Annu Rev Cell Dev Biol, **11**:73-91.* **(B)** Diagram depicting the hemangioblast as the precursor of both vascular EC and hematopoietic cells. *Adapted from: Carmeliet (2003) Nat. Med. 9(6):653-660.*

A


Molecules
Localization
1. Mesoderm formation
Ectoderm
Endoderm
Migrating mesodermal cells
VEGFR - 2
ets - 1
Mesodermal - cells
Fibronectin
Extracellular matrix
2. Blood island differentiation
Angioblasts
Hemopoietic cells
Blood islands
VEGFR - 2
VEGFR - 1
VE - cadherin
tie - 2 / tek
Angioblasts
ets - 1
PECAM - 1
Angioblasts and hemopoietic cells
Fibronectin
Extracellular matrix
VEGF
Endoderm
3. Fusion of blood islands and endothelial cell differentiation
Vascular extracellular matrix
Endothelial cells
Lumen
VEGFR - 2
VEGFR - 1
VE - cadherin
tie - 2 / tek
tie - 1
Endothelial cells
ets - 1
PECAM - 1
Endothelial and hemopoietic cells
Fibronectin
Laminin
Extracellular matrix
VEGF
Endoderm
4. Primary capillary plexus
VEGFR - 2
VEGFR - 1
VE - cadherin
tie - 2 / tek
tie - 1
Endothelial cells
ets - 1
PECAM - 1
Endothelial and hemopoietic cells
Fibronectin
Laminin
Collagen IV
Extracellular matrix
VEGF
Endoderm

B

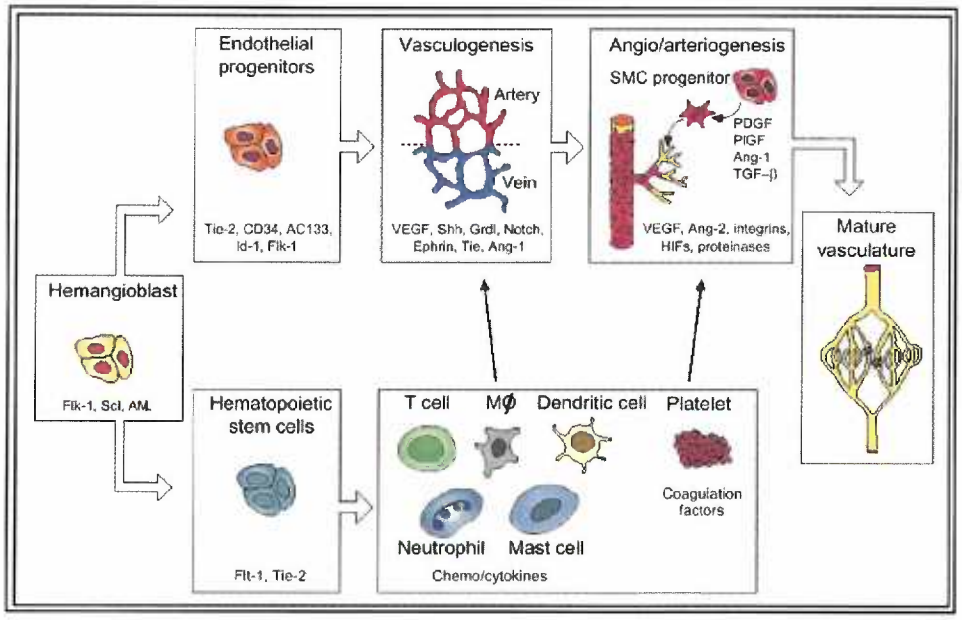
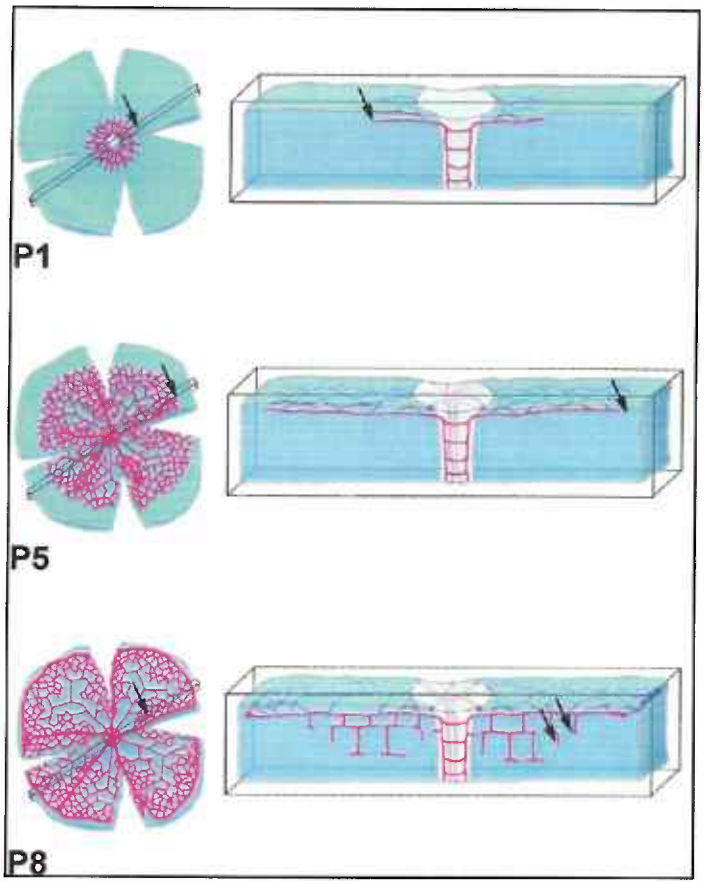

Endothelial progenitors
Tie-2, CD34, AC133, Id-1, Flk-1
Vasculogenesis
Artery
Vein
VEGF, Shh, Grdl, Notch, Ephrin, Tie, Ang-1
Angio/arteriogenesis
SMC progenitor
PDGF
PlGF
Ang-1
TGF-β
VEGF, Ang-2, integrins, HIFs, proteinases
Mature vasculature
Hemangioblast
Flk-1, Scl, AML
Hematopoietic stem cells
Flt-1, Tie-2
T cell
MØ
Dendritic cell
Platelet
Coagulation factors
Neutrophil
Mast cell
Chemo/cytokines

Figure 6: Vascularization of the Retina and the Role of VEGF in the mouse model of ROP. **(A)** Vessels sprout from the optic disk into the periphery of the superficial retina (arrows; postnatal day (P) 1). By P5, a primary plexus is formed, which subsequently penetrates into deeper layers of the retina (arrows; P8). Vessel branching and fusion form a fully function plexus. *Adapted from: Gerhardt et al. (2003) J. Cell Biol. 161(6):1163-1177.* **(B-1)** Normal retinal vessel development is stimulated by production of VEGF (red) anterior to the developing vasculature. **(B-2)** In the first phase of ROP, exposure to relative hyperoxia after birth interrupts the gradient of physiologic hypoxia in the immature retina, leading to down-regulation of VEGF production and a cessation of vessel growth. **(B-3)** Upon return to room air, the metabolic demand of the developing retina increases and the nonperfused portions of the retina become hypoxic, overproducing VEGF. **(B-4)** Neovascularization occurs in response to the overproduction of VEGF, producing ROP. *Adapted from: Pierce et al. (1996) Arch Ophthalmol. 114(10):1219-28.*

A


P1
P5
P8

B

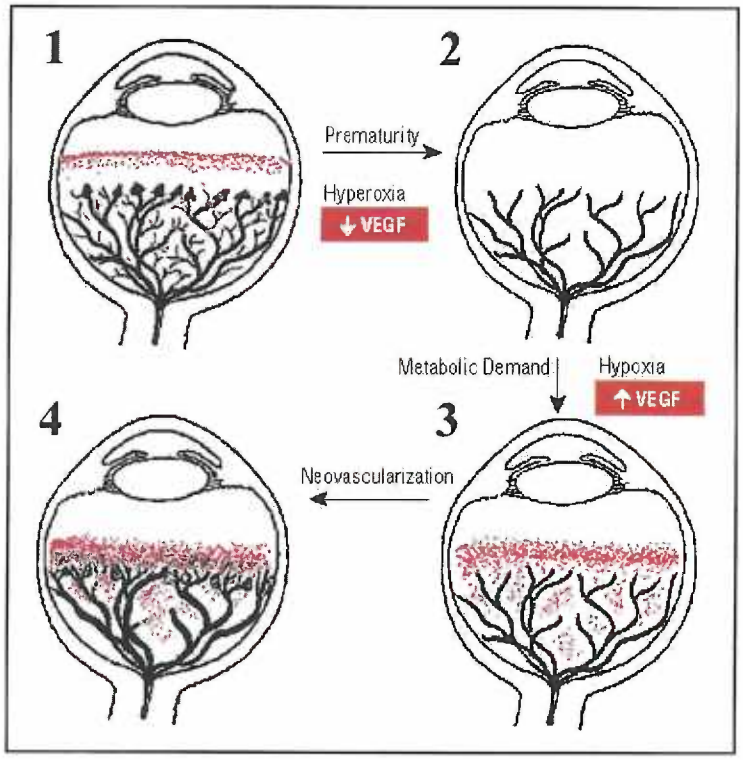
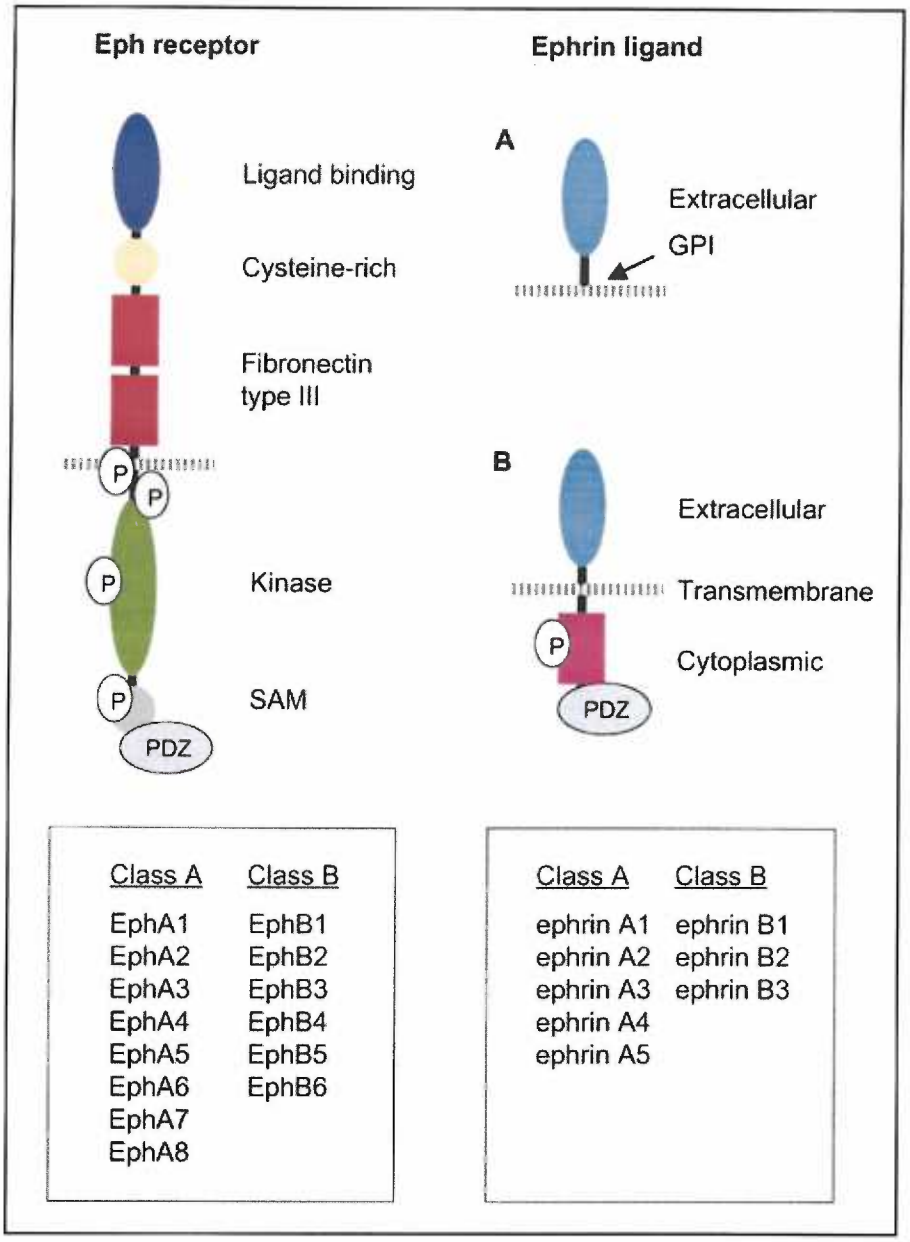
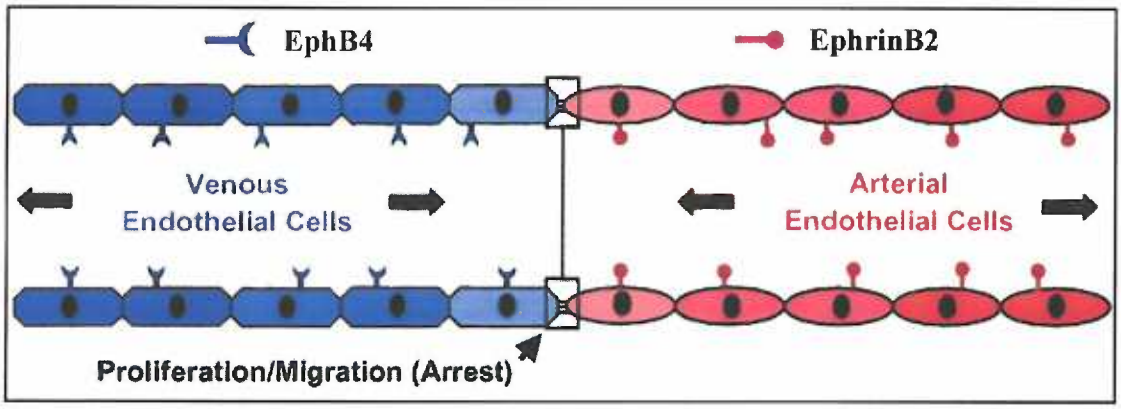

1
2
Prematurity
Hyperoxia
VEGF
Metabolic Demand
Hypoxia
VEGF
4
3
Neovascularization

Figure 7: Ephrin Ligands and Eph Receptors. **(A)** Eph receptors are classified into 2 subgroups (class A or class B) depending on whether they bind to a class A Ephrin or class B Ephrin. *Adapted from: Cheng et. al., Cytokine Growth Factor Rev. (2002), 13(1):75-85.* (B) During capillary formation, arterial EC EphrinB2 interacts with venule EC EphB4. This interaction suppresses the proliferation and migration of these cells which prevents these cells from co-mingling and allows proper vessel formation. *Adapted from: Kim et. al., (2002) FASEB J. 16(9): p. 1126-8.*

A


Eph receptor
Ephrin ligand
Ligand binding
Cysteine-rich
Fibronectin type III
Kinase
SAM
PDZ
P
A
Extracellular
GPI
B
Extracellular
Transmembrane
Cytoplasmic
PDZ
Class A
Class B
EphA1
EphA2
EphA3
EphA4
EphA5
EphA6
EphA7
EphA8
EphB1
EphB2
EphB3
EphB4
EphB5
EphB6
Class A
Class B
ephrin A1
ephrin A2
ephrin A3
ephrin A4
ephrin A5
ephrin B1
ephrin B2
ephrin B3

B


EphB4
EphrinB2
Venous Endothelial Cells
Arterial Endothelial Cells
Proliferation/Migration (Arrest)

To me it seems that all sciences are vain and full of errors that are not born of Experience, mother of all certainty, and that are not tested by Experience; that is to say, which do not at their beginning, middle, or end, pass through any of the five senses.

-Leonardo da Vinci (1452-1519)

Chapter 2: Materials and Methods (M&M)

2A: M&M for Chapter 3

Iris EC Cultures

All usage of human tissues and cells in this study was in accordance with IRB approved protocols. Irises from anonymously donated human eyes (Lions Eye Bank, Portland, OR; donor age range 16-42 in this study; no known history of ocular or cardiovascular disease), were digested in 0.2% type II collagenase (Sigma Chem. Co., St. Louis, MO) in medium MCDB-131, for 20-30 min at 37°C, after having the iris pigment epithelial layer mechanically removed with a cotton swab. Following digestion, EC were purified away from iris stromal cells using monoclonal anti-human platelet-EC adhesion molecule-1 (PECAM-1) antibody-coated magnetic beads (Dynal, Inc., Lake Success, NY), and were cultured in MCDB-131 supplemented with 10% fetal bovine serum (FBS, Invitrogen Corp., Carlsbad, CA), EC growth factors (EGM-MV2 BulletKit™, with hydrocortisone omitted; Clonetics/Biowhittaker, Walkersville, MD; complete medium), gentamycin (10 μg/ml), and amphoteracin-B (Fungizone®, 250 ng/ml, Invitrogen Corp./Gibco). Cultures were trypsin passaged at a 1:3 split ratio, and used in subsequent experiments between passages 3 and 6.

Confirmation of EC Nature of Cultured Cells

Following one or two rounds of magnetic separation, cultures were ≥99.5% pure on the basis of PECAM-1 and von Willebrand factor (vWF) expression[18,117], and uptake of dil-acetylated-LDL[118]. In specialized culture conditions with a provisional extracellular matrix, monolayers of EC are capable of reorganizing into capillary-like

networks[119]. Seventy-five thousand HIEC were plated on polymerized Matrigel® (14.1 mg/ml, 200 μl/well, BD Biosciences, Bedford, MA) within 24-well tissue culture plates, and were allowed to attach overnight. Cells were then refed, with some wells receiving 10 ng/ml phorbol myristate acetate (PMA; Sigma) to induce tube formation[120].

RT-PCR Analysis of Adhesion Molecule mRNA Expression

Confluent HIEC monolayers were stimulated for up to 21 hrs with either lipopolysaccharide (LPS, 10 μg/ml, from *E. coli* 055:B5, List Biological Laboratories, Inc., Campbell, CA) or recombinant human tumor necrosis factor-alpha (TNFα, 10 ng/ml, R&D Systems, Minneapolis, MN). Total RNA was extracted using a commercial kit (RNApure™, GenHunter Corp., Nashville, TN). Touchdown RT-PCR detection of gene expression was done as previously described in detail[121]. Positive-control total RNA was obtained from synovial samples of rheumatoid arthritis patients. Diethyl pyrocarbonate-treated water (DEPC-H_2O, Ambion, Inc., Austin, TX) was used as a negative-control. Human ICAM-1 primer sets (Sense, 5'–CCGGAAGGTGTATGAACTG–3'; Anti-sense, 5'–TCCATGGTGATCTCTCCTC–3'), ICAM-2 primer sets (Sense, 5'-CCGTGGCAATGAGACTCTGCACTA-3'; Anti-sense, 5'-ATGGTTGCTATGGCCGGAAGG-3'), and VCAM-1 primer sets (Sense, 5'-CTCCGTCTCATTGACTTGC-3'; Anti-sense, 5'GAACAGGTCATGGTCACAG-3', all from Operon Technologies, Alameda, CA), were used to probe cDNAs reverse-transcribed from the experimental, positive, and negative control RNA samples. A primer pair for cyclophilin was included in each assay as an internal control (Sense, 5'–TGTTCTTCGACATTGCCGTCGAC–3'; Antisense, 5'–

GCATTTGCCATGGACAAGATGCCAGGA– 3'; Operon). PCR reaction products were electrophoresed in 3% agarose gels in Tris-acetate buffer containing ethidium bromide, and UV-induced fluorescent bands were photographed and digitized.

Protein Analysis

Cell adhesion molecule (CAM) protein expression on intact monolayers of HIEC was evaluated using a fluorescent enzyme-linked immunocellular assay (ELICA) in a 96-well microtiter plate format, as previously described in detail[122]. Mouse monoclonal anti-human ICAM-1 antibody (Clone W-CAM-1, Neomarkers, Inc., Fremont, CA) rabbit polyclonal anti-ICAM-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-human VCAM-1 antibody (clone BBIG-V1-4B2, R & D Systems), and monoclonal anti-human E-selectin antibody (clone BBIG-E4-5D11, R & D Systems) were used as primary detection antibodies (all diluted to 1 μg/ml in 1% bovine serum albumin/bicarbonate buffered saline). Affinity-purified, alkaline phosphatase-conjugated, goat anti-mouse or anti-rabbit polyclonal antibodies (1:1000, Sigma) were used for secondary detection. Methylumbelliferyl phosphate (100 μg/ml, Sigma) was used as the fluorescent detection substrate. In some cases, ELICA results were confirmed by western blotting in which HIEC lysates (3×10^4 cells/lane) were electrophoresed on 4-15% linear gradient SDS-PAGE gels, followed by transfer to nitrocellulose and protein detection with the same antibodies used in the ELICA. Bands were visualized using an NBT/BCIP detection kit (Vector Labs, Burlingame, CA), and images were digitized.

Leukocyte Adhesion Studies

To explore the utility of our cell culture as an *in vitro* model of ocular inflammation, we tested the ability of activated HIEC cultures to adhere U937 monocytes (ATCC). HIEC were grown to confluence in 48-well plates and were then stimulated with either LPS (10 μg/ml), TNFα (10 ng/ml), or vascular endothelial growth factor (VEGF; 100 ng/ml, R&D Systems) for 12 hrs. In some cases, LPS-stimulated HIEC were incubated with either anti-ICAM-1 mAb (20 μg/ml), anti-ICAM-2 pAb (10 μg/ml), or anti-VCAM-1 mAb (10 μg/ml, Clone 1G11.B1, Neomarkers Inc.), for the last 30 minutes prior to monocyte addition. All stimulations and antibody incubations were done in complete medium at 37°C. Following EC treatments, the wells were aspirated, quickly washed with MCDB-131/10% FBS, and 500 μl of the same medium containing 2.5×10^5 monocytes was added to each well and incubated for 15 minutes at 37°C. After gently washing off unbound monocytes, the EC monolayers and adherent monocytes were briefly fixed with 0.5% glutaraldehyde, and attached monocytes were visually counted (four 1-mm^2 regions per well).

Statistical Analysis

Data presented for ELICA and monocyte adhesion assays represent mean fluorescence/well and monocytes bound/mm^2 HIEC monolayer surface area, respectively, +/- S.D, for the indicated number of replicates. Asterisk (*) in figures indicates a significant difference ($p < 0.05$) between the means of experimental and respective control groups, by ANOVA (SigmaStat™ 2.0 software, SPSS Science, Chicago, IL).

2B: M&M for Chapter 4:

Cell and Tissue Culture

Human blood was obtained from normal healthy volunteers who had given informed consent according to IRB protocols. The blood was immediately diluted with phosphate-buffered saline (PBS) and placed over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). The cells were then centrifuged for 20 minutes at 500g and mononuclear cells were collected from the interface. Contaminating red blood cells were removed with Red Cell Lysis Buffer (Sigma; St. Louis, MO). Cells were then cultured for approximately 4 hours in RPMI-10% FCS (heat-inactivated; Gibco BRL/Invitrogen; Carlsbad, CA) at 37°C in a 5% CO_2/95% air atmosphere for monocyte depletion. Peripheral blood lymphocytes (PBLs) were then collected from the culture supernatant, washed once with PBS and plated in RPMI-2% FCS (heat-inactivated) for experimentation. Some PBLs were stimulated between 0-24 hours with TNFα (10 ng/ml; R&D Systems, Minneapolis, MN) or SDF1α (40 ng/ml; Peprotech, Rocky Hill, NJ).

To isolate $CD4^+$, $CD8^+$ and $CD19^+$ cells, PBLs were isolated and monocytes depleted as mentioned above. Before labeling, Fc receptors on cells were blocked with 2% bovine serum albumin (BSA), 10 μg/ml of mouse IgG in PBS (labeling buffer) for 30 minutes on ice. Antibodies were then added to cells according to manufacturers recommendations. Briefly, PBLs were incubated with the following antibodies at 4°C for 30 minutes: 1) Simultest two-color anti-CD3-fluorescein isothiocyanate (FITC)/-CD4-phycoerythrin (PE) conjugated antibodies, 2) Simultest two color anti-CD3-FITC/-CD8-PE conjugated antibodies (both from Becton Dickinson, San Jose, CA), and 3) anti-CD19 TRI-COLOR conjugated antibodies (Caltag Labs; Burlingame, CA). Labeled cells were

then washed with PBS, re-suspended in labeling buffer and subsets isolated using a FACS Turbo Vantage® cell sorter (Becton Dickinson; Frankin Lakes, NJ). A purity of greater than 90% was obtained for $CD3^+/CD4^+$ and $CD3^+/CD8^+$ cells, while $CD19^+$ cells averaged ~88% purity. $CD4^+$, $CD8^+$, and $CD19^+$ cells were then cultured overnight in RPMI-2% FCS (heat-inactivated) before experimentation. $CD4^+$ and $CD8^+$ cells were stimulated between 0-6 hours with TNFα (10 ng/ml; R&D Systems), while $CD19^+$ cells were activated for 0-6 hours by crosslinking their B cell receptor with 1μg/ml of anti-human IgM-specific F(ab') framents (Southern Biotechnology Assoc., Birmingham, AL). Total RNA was then extracted (described below) upon termination of experiment.

Polymorphonuclear cells (PMNs=neutrophils) were isolated with Polymorphprep (Axis-Shield; Oslo, Norway) according to the manufacturers protocol. Briefly, human blood was obtained from normal healthy donors, and layered over Polymorphprep to a final volume of 1:2. Samples were then centrifuged at 500g for 30 minutes at 22°C. The PMN fraction was then carefully removed from the appropriate interface and diluted with an equal volume of RPMI to restore normal osmolality. The cells were then spun down at 400g for 10 min at 22°C, and then resuspended in medium. The wash step was repeated once more before suspending cells in RPMI-2% FCS (heat-inactivated) and plating for experimentation. Some PMNs were stimulated between 0-6 hours with TNFα (10 ng/ml) or IL-8 (10 ng/ml; R&D Systems).

HIEC cultures were established as previously described by our lab[15]. Briefly, human eyes were obtained from anonymous donors (Lion's Eye Bank, Portland, OR), within 24 hrs postmortem. Iris tissue was aseptically dissected from donor eyes, collagenase digested, and ECs isolated from contaminating cells using mouse monoclonal

anti-human CD31 antibody-coated magnetic beads (Dynal Biotech Inc., Lake Success, NY). EC were then cultured in MCDB-131 medium (Sigma)-10% FCS containing EGM-2™ and antibiotic supplements (hydrocortisone omitted; Clonetics Inc., Walkersville, MD; complete medium), and grown to confluency. EC were typically used between passages 2 to 5. Twenty-four hours prior to experimentation, medium was changed to MCDB-131-10% FCS, with supplemental growth factors omitted. HIEC were then stimulated with either TNFα (10 ng/ml), IL-1α (1 ng/ml; R&D Systems), LPS (10 μg/ml; E. coli 055:B5, List Biological Laboratories; Campbell, CA), or EphrinB2/Fc (2 μg/ml; R&D Systems) for periods that ranged between 0 to 24 hours. Upon termination of experiment, total RNA or protein was immediately extracted and stored at -80°C. Human mesenteric venule EC (MesVEC) were cultured under identical conditions as HIEC and were generously provided by Dr. Jena Steinle (Southern Illinois University; Carbondale, IL). For explant studies, whole irises were aseptically dissected and cultured in MCDB-131 complete medium. After acclimating explants for 6 hours in culture, some tissues were exposed to LPS (10 μg/ml) for 6 hours. Upon termination of experiment, explants were washed in PBS and total RNA extracted.

RT-PCR

Total mRNA from whole PBLs, PBL subsets, HIEC, MesVEC cells and iris tissue was isolated and DNase treated using Qiagen's RNeasy Mini kit (Valencia, CA). The RNA was then quantitated by UV-spectroscopy and reverse transcribed to obtain cDNA. Touchdown RT-PCR was then performed as previously described[123]. Briefly, multiplex PCR reactions were conducted to simultaneously detect the expression of human

EphrinB2 or EphB4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript expression. Human specific EphrinB2 ligand primer sets (sense, 5'-GCCAGACAAGAGCCATCAAGA-3', antisense, 5'-TCTCCTCCGGTACTTCAGCAAGA-3'; 308 base pair amplicon); and EphB4 receptor primer sets (sense, 5'-ACCTTGCCGAGATGAGCTACG-3', antisense, 5'-CTGCCAACAGTCCAGCATGAG-3'; 386 bp amplicon), all from Integrated DNA Technologies Inc. (IDT, Coralville, IA), were used to amplify specific cDNAs. A primer pair for the constitutively expressed GAPDH gene was included in each assay as an internal control (sense, 5'-AGCTGAACGGGAAGCTCACTGG-3', antisense, 5'-GGAGTGGGTGTCGCTGTTGAAGTC-3'; 209 base pair amplicon; IDT). The PCR products were then electrophoresed in 3% agarose gels in Tris-acetate buffer, containing ethidium bromide, and subsequently photographed under UV light. Although the amplicons detected in our gels matched their predicted base pair size, we sequenced the PCR products and confirmed that the amplicons generated were indeed those of human EphrinB2 and EphB4 (data not shown).

Immunoblotting

For EphrinB2/EphB4 western blots, cells were washed 3 times with PBS and suspended in RIPA lysis buffer (50mM Tris pH 8.0, 137mM NaCl, 10% glycerol, 1% Triton-X100, 100 μM Na-Orthovanadate, 100 μg/ml PMSF, 1 μg/ml Aprotinin, 2 μg/ml Leupeptin). Lysates from approximately 7.5 x 10^4 EC/lane and 1 x 10^5 PBLs/lane were electrophoresed on 4-20% linear gradient SDS-PAGE mini-gel (Biorad; Hercules, CA) and then transferred onto PVDF membranes. Following blocking with 1% BSA in PBS,

membranes were incubated with goat anti-human EphrinB2 IgG antibodies or goat anti-human EphB4 IgG antibodies (each at 5 μg/ml; R&D Systems), and then anti-goat alkaline phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology; Santa Cruz, CA) were used to detect primary antibodies. A chromogenic alkaline phosphatase substrate kit (BCIP/NBT; Vector Laboratories, Burlingame, CA) was used to visualize bands. For phospho-Erk1/2 analysis, EphrinB2/Fc treated HIEC were washed once with PBS, lysed with RIPA buffer, and snap frozen with liquid nitrogen. Lysates from approximately 2.5 x 10^5 HIEC/lane were electrophoresed on an 8% SDS-PAGE large format gel and then transferred onto PVDF membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline, and then incubated overnight at 4°C with rabbit anti-phospho-p44/42 (Erk1/2) MAP kinase antibodies (1:3000, Cell Signaling, Beverly, MA). Primary antibodies were then labeled with anti-rabbit horseradish-peroxidase conjugated antibodies (1:10,000; Santa Cruz) and detected by SuperSignal chemiluminescence substrate (Pierce Rockford, IL) according to the manufacturer's instructions. Images of blots were captured by exposure of membranes to film (Biomax Film; Kodak, Rochester, NY) and followed by film development. Images of the film were then scanned to produce a digital image.

Immunohistochemistry

Donor eyes were obtained from the Lions Eye Bank (Portland, OR) and irises dissected. The iris tissue was then washed once with sterile PBS and fixed overnight in 10% neutral-buffered formalin. The tissues were then paraffin-embedded and sectioned at 5 μm. After the sections were deparaffinized, antigen retrieval was performed by

incubation with trypsin (20 μg/ml; Sigma) for 10 min at 20°C. Routine immunohistochemistry was then performed with rabbit anti-human EphB4 IgG antibodies (0.5 μg/ml; H-200, Santa Cruz Biotechnology), or rabbit anti-human von Willebrand Factor (vWF; 1 μg/ml; A082, Dako, Carpinteria, CA) IgG antibodies. Pre-immune rabbit IgG antibodies (Vector Labs) were diluted to similar concentrations as experimental antibodies and used as a negative control for immunostaining. Anti-rabbit alkaline phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used to detect primary antibodies, and the antibody complexes were visualized using Fast Red substrate kit (BioGenex, San Ramon, CA).

2C: M&M for Chapter 5:

Animals

C57BL/6 mice used in this study were originally obtained from Simonsen Laboratories (Gilroy, CA). The mice were housed and bred in the Oregon Health & Science University animal care facility and treated in accordance with NIH guidelines and the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animals were provided food and water *ad libitum* and were kept on a 12-hour light-dark schedule. To induce retinopathy, postnatal day (P) 7 mice, along with nursing females, were exposed to 75% oxygen for 5 days and then allowed to recover in room air on P12, according to the protocol of Smith *et al.* [109]. Room air litters were maintained under otherwise identical conditions as the hyperoxia-exposed mice.

RT-PCR Analysis

Retinas were dissected at the selected time points (P7-P24) and 4 retinas per condition were pooled for RNA extraction. Total RNA was isolated and DNase treated using RNeasy Mini kit (Qiagen, Valencia, CA), and then reverse transcribed to obtain cDNA. Touchdown RT-PCR detection of gene expression was performed as previously described [123]. Mouse specific EphrinB2 ligand primer sets (sense, 5'-TGTCCAGACAAGAGCCATGA-3', antisense, 5'-TGGTCGTGTGCTGTGGAGAGT-3'; 342 base pair size); and EphB4 receptor primer sets (sense, 5'-ACCTGGCTGCTCGGAACATC-3', antisense, 5'-GCTGCCGTTGGTCCAAGAGT-3'; 492 base pair size), all from Integrated DNA Technologies Inc. (IDT, Coralville, IA) were used to amplify specific cDNAs. A primer pair for the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was included in each assay as an internal control (sense, 5'-GCATGGCCTTCCGTGTTCCTA-3', antisense, 5'-CGGCATCGAAGGTGGAAGAGT-3'; IDT; 204 base pair size). The PCR products were electrophoresed in 3% agarose gels in Tris-acetate buffer containing ethidium bromide and subsequently photographed under UV light. Although the amplicons detected in our gels matched their predicted base pair size, we sequenced the PCR products and confirmed that the amplicons generated were indeed those of mouse EphrinB2 and EphB4 (data not shown).

Intravitreal Injections

Administration of anesthesia and injections were performed as previously described [124]. Pups were deeply anaesthetized by isoflurane inhalation (0.5 L/min in

oxygen). Approximately 1.5 µl of EphrinB2/Fc or EphB4/Fc (100 ng/µl; R&D Systems, Minneapolis, MN) was delivered intravitreally into the right eyes of oxygen-injured mice. Since the chimeric forms of EphrinB2 and EphB4 are dimerized in their active form by the Fc portion of human IgG, human whole IgG was used as the control protein injection. Similar to experimental injections, control human IgG (100 ng/µl; Sigma; St. Louis, MO) was delivered into the left eyes of oxygen-injured mice. Intravitreal injections of were performed with a Hamilton syringe connected to an ultrathin pulled borosilicate glass needle (outer diameter, ~50 μm) to deliver the proteins. EphrinB2/Fc, EphB4/Fc, and human IgG proteins were diluted in sterile Dulbecco's PBS (minus Ca^{++} and Mg^{++}, pH ~7.4; Gibco/Invitrogen; Carlsbad, CA) prior to injection. Control and experimental injections were administered within 5 minutes of each other and were given during the transition from hyperoxia to room air on P12, and repeated on P14. The mice were allowed to recover until P17 and were sacrificed by CO_2 euthanasia. Both eyes were carefully enucleated from each mouse, placed in 10% neutral buffered formalin overnight, and then routinely processed for paraffin embedding. The eyes were then sectioned at 5µm intervals, mounted on slides (SuperFrost Plus; Fisher Scientific, Pittsburgh, PA), and stored at room temperature until used for immunohistologic analysis.

Neovascular Nuclei Quantification

To quantitate the retinal neovascularization, tissue sections were stained with hematoxylin and eosin (H&E). Retinal vascular cell nuclei anterior to the inner limiting membrane of the oxygen-injured and room air control retinas were counted at P17 in a

masked fashion. Care was taken to avoid counting hyaloid vessel nuclei near the optic disk and lens, which are easily distinguishable from neovascularization extending into the vitreous. The average neovascular nuclei per section is calculated for each eye as the mean number counted in 15 sections 40 μm apart. Statistical analysis was based on the median of the averages using Kruskal-Wallis' one-way analysis of variance with a Dunn's method of multiple comparison procedure.

Immunohistochemistry

A rabbit polyclonal anti-mouse type-IV collagen antibody (Collaborative Biomedical Products, Bedford, MA) was used to immunolocalize the basement membrane of blood vessels in the hyperoxia-exposed retinas as well as the age-matched room air control retinas. Before incubation with the type-IV collagen antibody, the sections were digested with 0.1% pepsin (Sigma-Aldrich) for 20 minutes at room temperature. The sections were rinsed with deionized water, and then washed with Tris-buffered saline (50 mM Tris, 0.15 M NaCl (pH 7.5); TBS). Nonspecific binding sites were blocked with 2% normal goat serum (Vector Laboratories, Burlingame, CA), 0.1% BSA, and 0.3% Triton X-100 in TBS for 60 minutes at room temperature. The sections were then washed in TBS, and the antibody-antigen complexes visualized using Fast Red as the substrate (Biogenex Laboratories, San Ramon, CA). Retinal vessels were quantitated in the following categories: (1) superficial vessels, defined as those located between the inner limiting membrane and the ganglion cell layer; (2) deep vessels, defined as those located in the outerplexiform layer. Data gathered in this study were analyzed using a one way ANOVA test with a Tukey's multiple comparison test.

To suppose that the eye with all its inimitable contrivances for adjusting the focus to different distances, for admitting different amounts of light, and for the correction of spherical and chromatic aberration, could have been formed by natural selection, seems, I confess, absurd in the highest degree.

-Charles Darwin (1809-1882)

Chapter 3

Cell Adhesion Molecule Expression in Cultured Human Iris Endothelial Cells

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The manuscript that comprises this chapter appeared in published form in Investigative Ophthalmology & Visual Science 42(12): 2861-2866 (2001) and was co-written with Matthew Silverman. The experiments for Figure 1 were a joint effort of DOZ, MDS, and YP. The experiments for Figure 2 were a joint effort of DOZ, PVT, and MDS. The experiments for Figure 3 and 4 were performed by MDS and YP, with the technical assistance of Leslie O'Rourke and Xiaona Huang.

Purpose: To develop a method to isolate human iris microvascular ECs (HIEC) for exploring their constitutive and inflammatory agent-modulated expression of intercellular adhesion molecules-1 and –2 (ICAM-1 and –2), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin.

Methods: ECs from collagenase-digested irises were isolated on the basis of their expression of platelet-EC adhesion molecule-1 (PECAM-1), using antibody-coupled magnetic beads. Cells were characterized as endothelial based on morphological criteria, their expression of PECAM-1 and von Willebrand factor, their uptake of acetylated low-density lipoprotein, and their ability to form capillary-like networks on Matrigel®. Constitutive and inflammatory agent-modulated expression of ICAM-1 and –2, VCAM-1, and E-selectin was evaluated by the reverse transcription-polymerase chain reaction, enzyme-linked immunocellular assays, western blotting, and by functional studies of leukocyte adhesion to HIEC monolayers.

Results: HIEC constitutively express mRNA and protein for ICAM-1 and ICAM-2, but only low to nondetectable levels of VCAM-1 or E-selectin. Upon endotoxin- or tumor necrosis factor-α-stimulation, ICAM-1, VCAM-1, and E-selectin are potently and time- and dose-dependently upregulated at both message and protein levels. By contrast, ICAM-2 message and protein are slowly down-regulated by inflammatory agents over time, but nonetheless remain present and functional. Overall, cytokine- or endotoxin-activation of HIEC results in enhanced adhesiveness for leukocytes.

Conclusion: ICAM-1, VCAM-1, and E-selectin have been previously implicated in mediating anterior ocular inflammation. Here we report the selective isolation of HIEC and demonstrate differential expression and regulation of these adhesion molecules in them. Additionally, this is the first demonstration of the regulated expression of ICAM-2 in any ocular microvascular cells.

Introduction:

The molecular mechanisms responsible for the tissue-specific inflammation in anterior uveitis (AU) are incompletely understood. AU is often associated with systemic illnesses such as ankylosing spondylitis, inflammatory bowel disease, Behcet's disease, juvenile rheumatoid arthritis, reactive arthritis or sarcoidosis [66,71]. However, AU may also arise in an idiopathic fashion without an associated systemic disease, or it may be secondary to a localized infection such as herpes simplex virus[125]. Regardless of its etiology, the mechanism for triggering an inflammatory response specifically in the iris, while often sparing other ocular tissues, remains unknown. Recently, much emphasis has been placed on understanding how cells in the bloodstream preferentially penetrate specific tissues, e.g., in leukocyte and stem cell migration, and in cancer cell metastasis[126]. In AU, transmigration of leukocytes into the anterior chamber requires that endothelial cells (EC) and the leukocytes within the iris microcirculation become "activated", and then physically approximated. These leukocytes can then leave the bloodstream and migrate through the iris stroma, often into the anterior chamber. Although leukocytes are vital cells in the pathogenesis of inflammation, the essential role of the iris microvascular EC, the gatekeepers in AU inflammatory processes, has received little attention.

Given the limitations of investigating the molecular mechanisms of AU directly in human subjects, a variety of animal models of ocular inflammation have been developed, including endotoxin-induced uveitis (EIU), experimental-autoimmune uveoretinitis (EAU), experimental melanin-induced uveitis, and experimental-autoimmune encephalomyelitis[66,71]. These models attempt to mimic human disease and have provided

important insights into the etiology of AU. Nonetheless, species differences often make it difficult to translate the findings directly from these animal studies over to the human condition. Tissue culture systems provide another means to investigate the biology of ocular inflammation, and others have previously isolated and cultured human choroid and retinal EC for this purpose[13,14]. Functional heterogeneity is known to exist amongst cultured EC derived from the microvasculature of different, often proximally situated, tissues[18,19,127,128]. Thus, in order to evaluate microvascular involvement in AU in an *in vitro* system, we developed a methodology to selectively culture human iris endothelium (HIEC).

In this report we provide the initial characterization of cultured HIEC and describe the constitutive and inflammatory agent-regulated expression of several cell adhesion molecules (CAMs) in HIEC. These molecules are likely key mediators of the leukocyte infiltration observed in AU. Leukocyte adhesion assays suggest that in addition to intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), both of which have been previously implicated in ocular inflammation, ICAM-2 also has the potential to play an important role in leukocyte extravasation through the iris microvascular wall in uveitis.

Results:

HIEC Culture Establishment

Initial digestion of iris tissue yielded a mixed cell population that included cells with morphologies characteristic of EC, fibroblasts, smooth muscle cells, pigment epithelial cells, and pericytes. After a single PECAM-1-based magnetic bead separation, we were able to significantly reduce the number of contaminating cells. We have consistently been able to generate, within two weeks, essentially pure experiment-ready HIEC cultures. To date, we have been successful with over 30 donors. The monolayers thus generated are ≥99.5% pure, as assessed by their cobblestone morphology (Fig. 1A), and by biochemical criteria (detailed below). The average number of HIEC initially isolated from an iris pair is ≈50,000, and these can typically be expanded in culture to ≈3.0×10^6 cells (i.e., 6-7 population doublings), without any detectable changes in morphology or physiology (i.e., CAM expression and stimulability). Additionally, these HIEC cultures are amenable to trypsin passaging and display expected survival rates (≈70-80%) when recovered from cryopreservation.

Physiological Characterization of HIEC

We characterized iris PECAM-1^+ cells as EC by several biological criteria. The presence of functional LDL scavenger receptors on HIEC was demonstrated by the uptake of DiI-Ac-LDL (Fig. 1C). After 4 hrs incubation with fluorescently-labeled Ac-LDL, punctate staining was observed intracytoplasmically throughout the entire EC monolayer. vWF is considered to be a classical marker of EC since it has only been found in EC, megakaryocytes and platelets, the latter two not posing a culture contamination

problem. Immunocytochemistry of HIEC monolayers demonstrated punctate vWF expression within the cytoplasm (Fig. 1D). Although vWF immunostaining intensity varied from cell to cell, greater than 99% of cells stained positively. Lastly, *in vitro*, EC have the ability to form capillary-like networks when grown on three-dimensional matrices under specialized conditions[119]. This characteristic has been used to differentiate between cells that morphologically and biochemically appear to be EC[129]. HIEC isolated by our methods indeed form capillary-like structures *in vitro* when grown on a three-dimensional matrix of Matrigel® and stimulated with PMA. Confluent monolayers of HIEC atop Matrigel® quickly reorganized themselves to form tube-like structures (Fig. 1B), with reorganization apparent as early as 4 hrs after PMA addition.

CAM mRNA Expression in HIEC

Cultured HIEC were tested for by RT-PCR their steady-state mRNA expression of various CAMs in response to the inflammatory mediators LPS and/or TNF-α. Unstimulated HIEC constitutively express low but detectable levels of ICAM-1 message (Fig. 2). Stimulation with 10 μg/ml LPS caused a time-dependent upregulation of ICAM-1 mRNA; an effect obvious at 1.5 hrs post-exposure, maximal around 3-5 hrs, and maintained through at least 21 hrs of continuous stimulation. Similar kinetics of ICAM-1 upregulation were observed when HIEC were stimulated with 10 ng/ml TNFα or with 10 ng/ml IL-1α (not shown). We also evaluated ICAM-2 mRNA expression in these cells by (Fig. 2). We detected significant constitutively expressed ICAM-2 mRNA in HIEC, which was not measurably altered by inflammatory agent stimulation. Only barely detectable VCAM-1 signals (Fig. 2) were seen in unstimulated HIEC. Following

activation with LPS or TNFα, however, we saw a rapid upregulation of VCAM-1 message that was declining after 21 hrs of stimulation.

CAM Protein Expression in HIEC

In line with the mRNA data, using an ELICA we measured a low but detectable constitutive expression of ICAM-1 protein on HIEC cell surfaces, which was markedly elevated by LPS and TNFα stimulation (Fig. 3A). Upregulated ICAM-1 reached maximal levels after 8-12 hours of LPS or TNFα stimulation and remained maximally elevated through at least 2 days of continued stimulation. ICAM-1 upregulation by LPS was confirmed by western blotting of HIEC lysates (Fig. 3A, inset). We also detected significant constitutive expression of ICAM-2 protein in HIEC (Fig. 3B), which, conversely, was decreased in TNFα- and LPS-stimulated HIEC. This trend appeared as early as 6 hrs post-stimulation and by 24 hrs had culminated in a significant 50% decrease in immunodetectable ICAM-2 on HIEC surfaces. Unlike the clear constitutive expression of ICAM-1 and -2, only very low levels of VCAM-1 and no E-selectin protein was detected on unstimulated HIEC. In response to either LPS or TNFα stimulation, however, VCAM-1 protein was markedly elevated by 6 hrs (Fig 3C). After 24 hrs of stimulation, VCAM-1 in TNF α-stimulated EC had begun declining towards baseline but was still significantly elevated versus unstimulated controls. After 24 hrs of stimulation with LPS, VCAM-1 protein had already decayed to control levels (Fig 3C), in good temporal concordance with the declining VCAM mRNA signal observed in these cells. In the case of E-selectin, upregulation by LPS was rapid, with maximal protein levels

detected on HIEC surfaces after only 4 hrs stimulation (Fig 3D). This was followed by a similarly rapid decline to control levels after only 12 hrs.

Leukocyte Adhesion to HIEC

We next investigated whether cultured HIEC monolayers could become more adherent for leukocytes when activated by inflammatory stimuli. When HIEC monolayers were stimulated with LPS (10 μg/ml, 12 hrs), subsequent monocyte adhesion increased almost 10-fold (Fig. 4). Similarly, HIEC stimulation with vascular endothelial growth factor (VEGF, 100 ng/ml, 12 hrs; R&D Systems) resulted in approximately 3-fold increases in monocyte adhesiveness. Pre-incubation of LPS-stimulated HIEC with blocking antibodies against the cell adhesion molecules ICAM-1, ICAM-2, and VCAM-1 resulted in respective 40%, 40%, and 20% decreases in monocyte binding, demonstrating the *in vitro* functioning of multiple adhesion molecules in these activated HIEC.

Discussion:

Microvascular EC play key regulatory roles in the initiation and progression of inflammation[118,126]. The pathological sequelae of AU are often limited to specific tissues (i.e., iris and ciliary body) while neighboring tissues (e.g., retina, choroid, and sclera) are spared, supporting the likely existence of functional heterogeneity amongst the vasculatures of these disparate tissues. That EC from diverse anatomical sites can differ markedly in form and function is now well appreciated; such endothelial heterogeneity has been firmly established both by *in vivo* work and in studies employing cultured EC[18,19,122,127,128]. In ocular pathologies, for example, cytomegalovirus preferentially infects the posterior eye causing a retinitis, while anterior structures are typically unaffected[130]. Additionally, intraocular lymphomas also appear to selectively target the retina and not anterior eye tissues[131]. In appreciation of the likely existence of tissue-specific gene expression amongst the microvasculature of different ocular tissues, we endeavored to establish pure HIEC cultures to investigate some of the molecular mechanisms employed in AU pathogenesis. Although other researchers have previously cultured and experimented with ocular EC from human retina and choroid, [13,14]this is the first report testing pure microvascular EC isolated from human irises.

We evaluated in HIEC the expression of three adhesion molecules of the immunoglobulin superfamily (i.e., ICAM-1, -2, and VCAM-1), and one member of the selectin family (E-selectin), all of which are known to support leukocyte adhesion. Consistent with previous demonstrations in other EC types,[122] these cells display a low but measurable constitutive expression of ICAM-1 that is markedly upregulated at both the mRNA and the protein levels by stimulation with either LPS or, more potently, by

TNFα. By contrast, constitutively expressed ICAM-2 protein is markedly downregulated by the same inflammatory stimuli over the same time period. That we did not detect a coordinate decrease in ICAM-2 mRNA levels using a multiplex RT-PCR assay is likely due to the semi-quantitative nature of this method, since in preliminary experiments using gene array hybridization assays we see an approximate 50% decline in ICAM-2 signal strength after 24 hrs of LPS stimulation[132]. Although this level of change approaches the sensitivity limits of this assay (Clontech Atlas™ Arrays), it does suggest that ICAM-2 mRNA is declining after EC activation. By contrast to the clear constitutive expression of ICAM-1 and –2, negligible VCAM-1 and no E-selectin expression is detectable in unstimulated HIEC, at both mRNA and protein levels. As with ICAM-1, both VCAM-1 and E-selectin are potently upregulated in iris EC upon activation with inflammatory stimuli, in line with their previously suspected roles in uveitis[69,70,133-136]. Unlike the persistent ICAM-1 elevation, VCAM-1 and E-selectin upregulation is transient.

The activated phenotype of HIEC is reflected at the functional level, whereby LPS-stimulated HIEC show a significantly increased adhesiveness for U937 monocytes. This effect is in part blocked by the pre-incubation HIEC with neutralizing antibodies against various adhesion molecules (i.e., ICAM-1 and -2, and VCAM-1). This is consistent with a demonstrated role for EC-expressed ICAM-1 in mediating ocular inflammation in both animal models and clinical investigations, [69,70,121,133-135] and suggests that these cultured HIEC can qualitatively mimic the *in vivo* scenario. Previously, immunohistology performed on biopsied iris specimens from patients with either acute or chronic AU has revealed upregulated expression of ICAM-1 and VCAM-1

on human iris microvascular EC, implicating both adhesion molecules in the increased leukocyte flux through the iris in AU[69,70,136]. Our current data support this likelihood.

To date, this is the first report investigating a role for ICAM-2 in ocular inflammation. Although ICAM-2 is appreciably downregulated in activated HIEC, as has been reported in TNFα- and IL-1β-stimulated umbilical vein EC,[137] we observed that it nonetheless remains present in sufficient amounts to significantly mediate the binding of leukocytes to these vascular cells. Endothelial ICAM-2 is known to support the adhesion a variety of leukocyte subsets to EC, by acting as a ligand for the leukocyte-specific β_2 integrins, LFA-1 and Mac-1,[138,139] and appears to be essential for T-cell transendothelial migration. Interestingly, peptide fragments of ICAM-2 and soluble ICAM-2/Fc chimeras can bind to and rapidly enhance the affinity of these leukocyte integrins for both ICAM-1 and ICAM-2, resulting in a feed-forward enhancement of adhesion[140]. Soluble ICAM-1 exists in normal human plasma in ng/ml quantities, and circulating levels are significantly increased in a variety of inflammatory disorders, including uveitis [135,141]. Additionally, soluble adhesion molecules, including ICAM-1, are elevated in the aqueous humor of uveitis patients,[135] and in the vitreous of patients with proliferative diabetic retinopathy and proliferative vitreoretinopathy[142]. Very recently, soluble ICAM-2 has been detected in the bloodstream of normal humans and leukemia patients, which is significantly lowered after chemotherapeutic reduction of circulating leukemic cells (CG Gahmberg's research team, personal communication). Although currently speculative, if cleavage or shedding of membrane ICAM-2 accompanies its downregulation in activated HIEC, the resulting increased local levels of soluble ICAM-2 might perpetuate inflammation in the iris by activating integrins on nearby leukocytes[140]. Taken together with prior

experimental observations, our current data suggest a potentially important role for ICAM-2 in mediating leukocyte infiltration in uveitis, and warrant further experimentation to this end.

Endothelial expression of various chemokines, cytokines, and cell adhesion molecules are critical determinants in the development of the inflammation in AU. Understanding the EC expression profiles of these molecules and determining their relative importance in uveitis is difficult to test in the clinical scenario, since experimental data gathered from AU patients are limited to procuring small samples of aqueous humor or iris biopsy specimens from patients undergoing ocular surgery. Additionally, anti-inflammatory pharmacologic regimens are often underway in these patients and can complicate interpretation of findings. In order to circumvent these limitations while still working in a relevant human model, cultured HIEC provide a versatile means to expand our understanding of the mechanistic origins of AU. Although cell culture systems are not without their own inherent experimental limitations, this approach complements the existing animal models and clinical investigations in together elucidating the molecular mechanisms of uveitis.

Acknowledgements:

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Figure 1. Phase-contrast and fluorescence micrographs of purified HIEC. **A.)** Phase-contrast micrograph of confluent HIEC monolayer grown on tissue culture plastic. **B.)** Phase-contrast microscopy of HIEC forming capillary-like networks when grown atop a 3-dimensional matrix of Matrigel® and stimulated with PMA (10 ng/ml) for 24 hrs. Initial monolayer reorganization was apparent as early as 4 hrs post-stimulation. Inset shows higher magnification view of apparent lumen formation within capillary-like cords. **C.)** Fluorescent microscopy demonstrates DiI-Ac-LDL uptake by these cells, and **D.)** positive immunoreactivity for von Willebrands Factor. Original magnifications: A & B, 25X; B Inset, 100X; C & D, 100X.

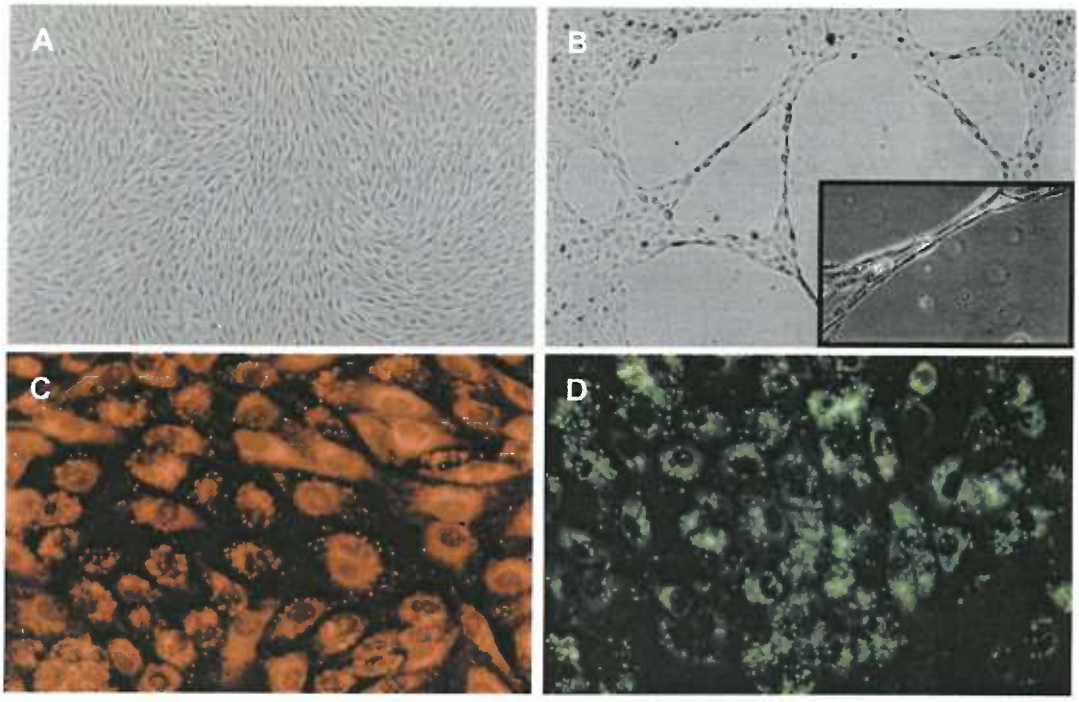

A
B
C
D

Figure 2. RT-PCR Analysis of ICAM-1, ICAM-2, and VCAM-1 mRNA in LPS-stimulated HIEC cultures. HIEC cultures were stimulated for up to 21 hrs with 10 μg/ml LPS. While ICAM-1 and VCAM-1 mRNA levels significantly increased with LPS-stimulation, we did not detect any change in ICAM-2 mRNA expression under the same conditions by RT-PCR. ICAM-1, ICAM-2, VCAM-1, and cyclophilin (CP; internal control) amplicons displayed respective base-pair sizes of 319, 386, 618, and 292. Shown are data from one representative experiment of 3 each for ICAM-1 and ICAM-2, and 2 for VCAM-1.

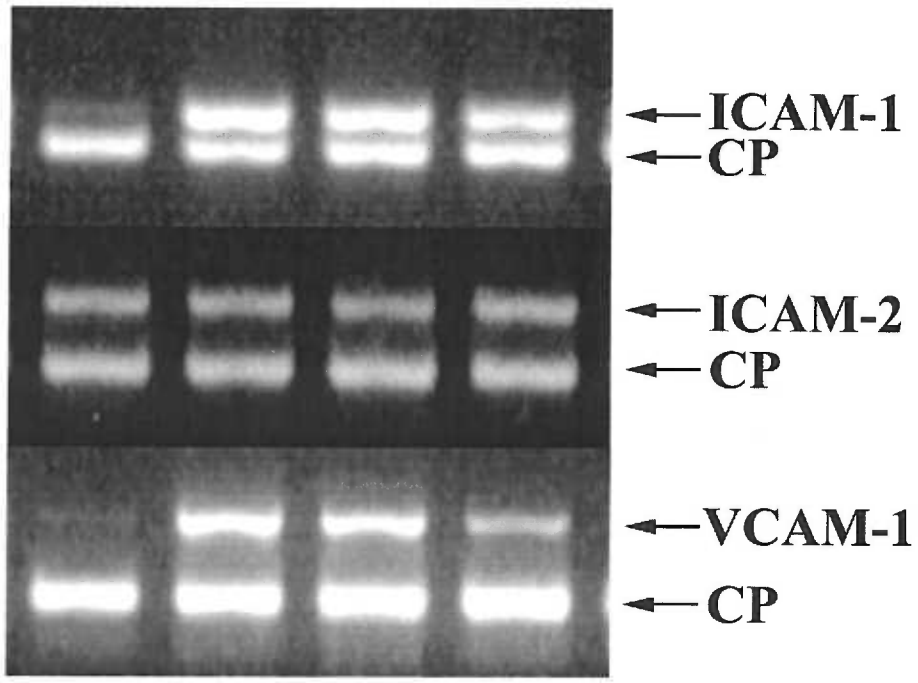

ICAM-1
CP
ICAM-2
CP
VCAM-1
CP
0
3
5
21
LPS Stimulation (hrs)

Figure 3. LPS and TNFα modulate adhesion molecule protein expression in HIEC. Cell surface expression of CAMs was measured in LPS-stimulated (10 μg/ml) and TNFα-stimulated (10 ng/ml) HIEC monolayers using a fluorescent enzyme-linked immunocellular assay (ELICA). **A.** Constitutively detectable ICAM-1 was upregulated as early as 4 hrs post-stimulation, with maximal protein levels attained between 8-12 hrs, and maintained through 48 hrs of exposure. Inset: Western blotting of cell lysates (≈3 x 10^4 cells/lane) from HIEC that had been stimulated with 10 μg/ml LPS for up to 24 hrs confirmed upregulation of ICAM-1 protein; Band molecular weight ≈95 kDa. **B.** Constitutively expressed (CON) ICAM-2 protein in HIEC, unlike the other CAMs measured, is downregulated by LPS- and TNFα-stimulation (10 μg/ml and 10 ng/ml, respectively). **C.** Cultured HIEC expressed very low levels of VCAM-1 protein in the absence of stimulation (CON). VCAM-1 expression was markedly upregulated by LPS and TNFα, whereby levels were maximal after 6 hrs of stimulation and had begun to decline towards baseline by 24 hrs. **D.** E-selectin expression was not detectable in unstimulated HIEC, but was rapidly upregulated in response to LPS and TNFα. Levels were maximal after 4 hrs stimulation and decayed to nearly to baseline by 12 hrs. ELICA data are from one representative experiment of 5 for ICAM-1, 3 for ICAM-2, and 2 for VCAM-1 and E-selectin, each condition having been conducted in 3-6 wells per experiment. Data represent mean fluorescence +/- S.D. Western data are from one representative experiment of 2. Asterisk (*) indicates $p < 0.05$ versus unstimulated controls, by ANOVA.

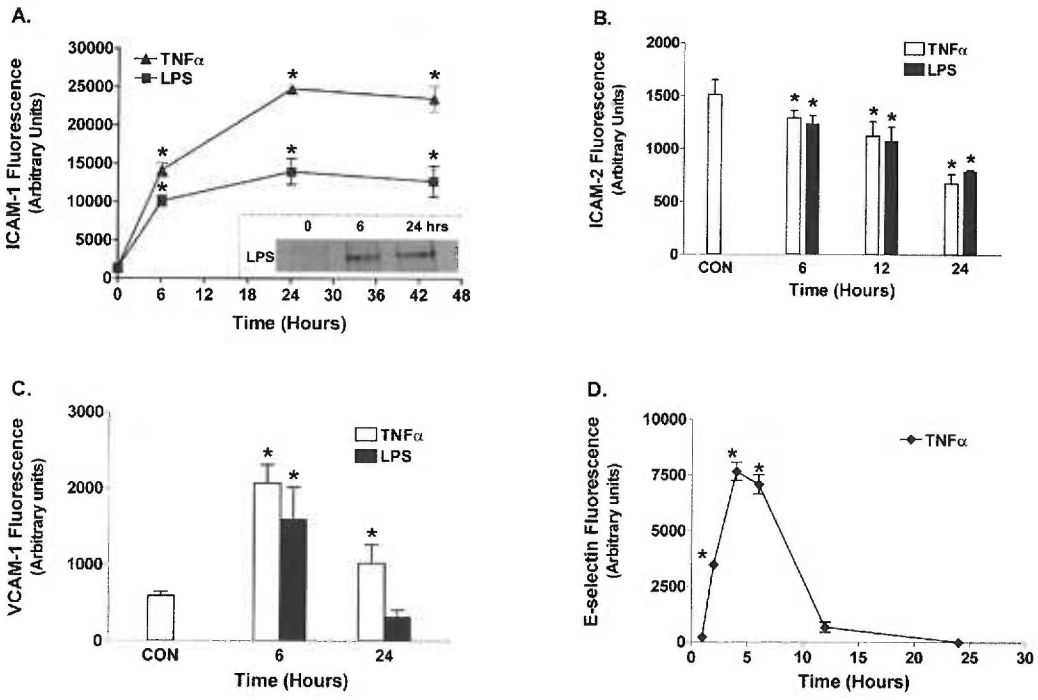
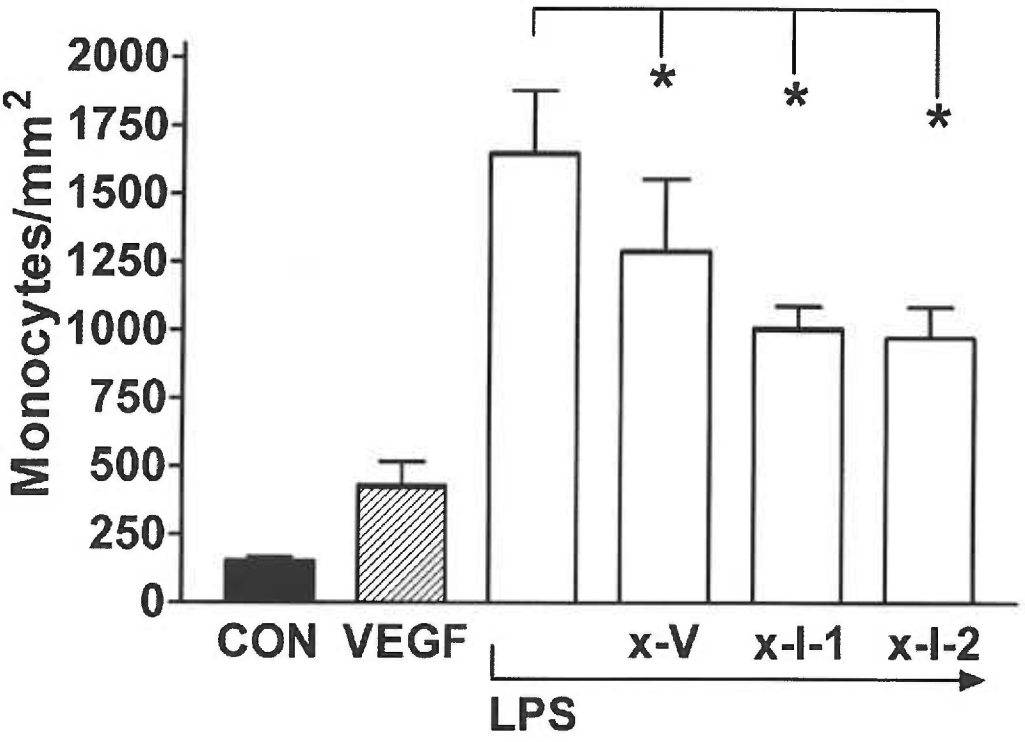

A.
ICAM-1 Fluorescence (Arbitrary Units)
TNFα
LPS
Time (Hours)
0 6 24 hrs
LPS
B.
ICAM-2 Fluorescence (Arbitrary Units)
TNFα
LPS
CON
Time (Hours)
C.
VCAM-1 Fluorescence (Arbitrary units)
TNFα
LPS
CON
Time (Hours)
D.
E-selectin Fluorescence (Arbitrary units)
TNFα
Time (Hours)

Figure 4. Monocyte adhesion to HIEC. Confluent monolayers of HIEC were stimulated with either VEGF (100 ng/ml) or LPS (10 μg/ml) for 12 hrs, followed by the addition of U937 monocytes for 15 minutes; Unstimulated HIEC served as controls (CON). Stimulation with VEGF and LPS resulted in significant 3- and 10-fold increases in monocyte adhesion, respectively ($p<0.01$ by ANOVA, in both cases) Anti-VCAM-1 (x-V), -ICAM-1 (x-I-1), or -ICAM-2 (x-I-2) neutralizing antibodies were added to some LPS-stimulated wells prior to monocyte incubations. All experimental variables were conducted in triplicate or quadruplicate wells per condition. Data represent mean monocytes bound/mm^2 HIEC monolayer surface area +/- S.D, and are presented for one of three representative experiments. Asterisk (*) indicates significant inhibition of monocyte attachment ($p< 0.05$) versus LPS-stimulated HIEC.


Monocytes/mm2
2000
1750
1500
1250
1000
750
500
250
0
*
*
*
CON
VEGF
x-V
x-I-1
x-I-2
LPS

Next to leukocytes, the vessels and their endothelial lining play the most important role in inflammation

-Ilya Metchnikoff (1845-1916)

Chapter 4

Differential Expression of EphrinB2 by Human Leukocytes: A Potential Mediator of Leukocyte Trafficking and Activation

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ABSTRACT

Purpose: EphrinB2 interacts with the EphB4 receptor tyrosine kinase and regulates the migration of various cell types (e.g., neural and endothelial cells; EC). This is accomplished by inducing a bi-directional signal into both cells and results in cell adhesion, repulsion or attraction by regulating intracellular signaling pathways that influence the cytoskeleton. During inflammation, activated EC interact with leukocytes and promote in the transmigration of the leukocytes into the inflamed tissue. We hypothesize that EphrinB2/EphB4 interactions also play a role in mediating leukocyte trafficking during ocular inflammation.

Methods: Unfractionated peripheral blood leukocytes (PBLs; monocyte depleted), $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells, and polymorphonuclear cells (PMNs) were isolated from human blood, and treated with TNFα, SDF1α, IL-8 or α-IgM antibody from 30 min to 24 hours. RNA or protein was isolated from these cells and EphrinB2 expression measured. We next analyzed human iris tissue and purified iris ECs (HIEC) for the expression of EphB4 by immunohistochemistry, RT-PCR, or western blotting in response to TNFα, IL-1α or endotoxin (LPS). Lastly, confluent monolayers of HIEC were exposed to soluble EphrinB2 from 0 to 60 minutes and phospho-Erk1/2 signaling molecules were analyzed by western blot.

Results: Our studies reveal several novel findings: 1) Human PBLs constitutively express EphrinB2. 2) TNFα and SDF1α stimulated PBLs upregulate their EphrinB2 expression. 3) Human $CD4^+$ T cells upregulate EphrinB2 expression after ~6 hours of

TNFα stimulation. 4) $CD8^+$ and $CD19^+$ cells express EphrinB2 upon isolation. 5) PMNs upregulate EphrinB2 in response to TNFα, but not IL-8. 6) Some, but not all, blood vessels of the human iris are $EphB4^+$. 7) HIEC and iris explants constitutively express EphB4, and TNFα, IL-1α, or LPS does not alter its expression. 8) Soluble EphrinB2/Fc modulates the phosphorylation levels of Erk1/2 in HIEC.

Conclusions: These observations indicate that a ligand expressed by stimulated lymphocytes activates iris EC. These observations suggest a novel mechanism by which iris EC can regulate lymphocyte trafficking during inflammation and a potential mechanism by which EC could activate lymphocytes.

Introduction:

The uveal tract is a highly vascularized region of the eye and consists of the iris, ciliary body, and choroid tissues. We are interested in the pathogenic mechanism(s) involved in a collection of uveal inflammatory diseases, termed anterior uveitis (AU). At the onset of AU, endothelial cells (EC) in the iris and/or leukocytes become "activated," resulting in the transmigration of leukocytes from the blood into the iris stroma and anterior chamber of the eye[67,68,70]. This phenomenon is orchestrated by chemokines and adhesion molecules on the surfaces of the iris EC and leukocytes[69,70]. Inflammatory pathologies of the eye share many of the molecular mechanisms employed during inflammation elsewhere in the body. Leukocytes circulating in the blood stream are recruited to the affected tissue and this process consists of a well-characterized, multi-step process that begins with leukocytes coming into contact with and rolling along the luminal surface of the EC[29-32]. The leukocytes, with an appropriate signal, stop rolling, become firmly adhered, and transmigrate through the EC and into the surrounding tissue[143]. Interestingly, only a fraction of the total number of rolling leukocytes become firmly adhered to the EC[144,145]. Furthermore, a significant portion of leukocytes that are firmly adhered to EC, apparently ready to begin the diapedesis process, will detach from the EC and return into circulation[145-148]. This observation implies that regulatory mechanisms exist to counteract the action of inflammatory mediators and prevent the activated leukocyte from extravasating. Indeed, leukocyte-expressed integrins (e.g., LFA, Mac-1, VLA-4 and VLA-5) change their affinity for EC-expressed adhesion molecules (e.g., ICAM-1, ICAM-2 and VCAM-1) upon engaging them. This is one of many

mechanisms to regulate their firm adhesion to EC[149-151]; however, other mechanisms are likely to exist.

Eph receptors and their Ephrin ligands comprise the largest family of receptor tyrosine kinases known to date. Eph receptors are grouped into two classes (A or B), depending on whether they interact with an EphrinA ligand (no cytoplasmic tail) or an EphrinB ligand (possess cytoplasmic tail; http://cbweb.med.harvard.edu/eph-nomenclature). Furthermore, when an $EphB^+$ cell comes in contact with an $EphrinB^+$ cell, these molecules engage one another and initiate a simulataneous "bi-directional" signal into both cells[152,153]. The generation of this signal can result in modulating integrin activity and reorganization of the actin cytoskeleton to generate adhesive or repulsive signals[154-158]. Ephrin/Eph's were first studied with respect to axon targeting during neural development[159] and have since been implicated in vasculogenesis due to their ability to regulate EC proliferation and migration[116,160-167]. We are specifically interested in EphrinB2 and EphB4 because these molecules are uniquely expressed within the vasculature and are well recognized as regulators of cell migration. EphrinB2 is expressed by arterial EC, while EphB4 is expressed by venule EC. Their expression is required to prevent arterial and venule EC from mixing and allow proper formation of artery-capillary-vein boundaries[27,116]. It is interesting to note that EphrinB2 and EphB4 are key regulators of vasculogenesis and hematopoiesis, both of which share the hemangioblast as a common precursor cell [168-170].

The functional studies of Ephrin/Eph molecules in immune cells are just beginning to be appreciated[158,171-194]. Little is known about the expression profiles of EphrinB2 and EphB4 in differentiated leukocytes of humans. EphrinB2 has been shown

to be expressed on human monocytes and in bone marrow stromal cells[195-197], whereas EphB4 is expressed on human hematopoietic progenitor cells[197,198]. In the mouse, Yu et al. (2003) recently demonstrated that EphrinB2 is expressed on mouse T cells and on monocytes/macrophages, but not on B cells. Yu et al. further showed that EphrinB2 was capable of co-stimulating mouse T cells[174]. Since EphrinB2 and EphB4 can modulate the adhesive and repulsive activities of migrating cells, we hypothesize that these molecules also function to regulate the migratory properties of leukocyte trafficking. In support of this notion, it has been demonstrated that some Ephrin ligands of A and B classes can modify the migratory properties of T cells by *in vitro* assays[158]. Noting that leukocytes generally attach to and extravasate from postcapillary venules during inflammation[29,30,32,37,199,200] and that EphB4 is typically expressed on venule EC[27,113,114,200], we set forth to determine: if human peripheral blood leukocytes (PBLs) express EphrinB2 and if so, can it modulate signaling pathways in human iris ECs (HIEC).

Results:

Expression of EphrinB2 Ligand by Human Peripheral Blood Leukocytes

The EphrinB2 and EphB4 signaling system is well established in the regulation of cell migration. However, little is known about the expression and function of these molecules in human immune cells. Here, we analyzed for the expression of EphrinB2 and EphB4 in human PBLs and PBL subsets in response to TNFα and SDF1α. TNFα is well known as a general inflammatory mediator of PBLs[201-204] and has been shown to be adhesion-strengthening and a stop signal for T cells migrating toward SDF1α[60]. Furthermore, TNFα is highly expressed during inflammation of the iris [205,206]. PBLs were stimulated for 5 hours with TNFα and compared with normal unstimulated PBLs for the expression of EphrinB2 and EphB4 by RT-PCR (Figure 1A). We detected the expression of EphB4 transcript in control PBLs, and TNFα did not appear to change its expression levels. In contrast, EphrinB2 transcript was undetectable in unstimulated control PBLs, but was seen after 5 hours of TNFα treatment. This observation prompted us to focus on EphrinB2 expression by PBLs. We extended the time course to examine the temporal expression of EphrinB2 in TNFα stimulated PBLs. The PBLs rapidly upregulated their expression of EphrinB2 in response to TNFα (Figure 1B). Transcript was detected as soon as 30 minutes after stimulation and peaked between 1 - 6 hours. By 24 hours, EphrinB2 transcripts were undetectable.

We next wanted to confirm that the upregulation of EphrinB2 message translated into an upregulation in EphrinB2 protein. To this end, we examined TNFα stimulated PBLs by western blot analysis (Figure 1C) and observed that EphrinB2 protein regulation matched their transcript profile. We focused on 1-6 hour stimulations since EphrinB2

transcript levels appeared to peak in that interval. As expected, EphrinB2 protein was detected at 4 and 8 hours after TNFα stimulation, but not in untreated PBLs (Figure 1C).

We hypothesize that the expression of EphrinB2 by PBLs regulates their trafficking during inflammation and tested whether PBLs would also upregulate EphrinB2 in response to the chemokine SDF1α. Thus, PBLs were stimulated with SDF1α up to 6 hours and analyzed for EphrinB2 expression by RT-PCR. SDF1α induced EphrinB2 in PBLs within a more restricted timeframe than TNFα (Figure 2). EphrinB2 was upregulated after 3 hours of SDF1α stimulation, but not at the earlier or later time periods studied (Figure 2). This result was obtained in two independent experiments. The observations in this section indicate that EphrinB2 is upregulated in response to inflammatory stimuli, but did not provide information as to which PBL subset(s) were responsible for this observation.

Expression of EphrinB2 Ligand by Human $CD4^+$, $CD8^+$, $CD19^+$, and PMN cells

To determine EphrinB2's expression profile in lymphocyte subsets, PBLs were isolated from whole blood by Ficoll-Paque separation, monocyte depleted and labeled for FACS sorting as follows: $CD4^+$ T cells were targeted with anti-CD3-FITC and anti-CD4-PE conjugated antibodies; $CD8^+$ T cells were targeted with anti-CD3-FITC and anti-CD8-PE antibodies; and $CD19^+$ B cells were targeted with anti-CD19-TRI-COLOR conjugated antibodies and sorted FACS analysis. Individual subsets were separated from contaminating cell types (Figure 3A, C, and E), and an aliquot of sorted, purified cells was re-scanned to confirm purity (Figure 3B, D, and F). This process yielded $CD4^+$ and

$CD8^+$ cell populations of greater than 90% purity, while a purity of ~88% was obtained for $CD19^+$ cells.

Lymphocyte subsets were then cultured for 24 hours prior to experimentation to allow cells to become quiescent in case the selection process activated them. CD4+, CD8+, and CD19+ cells had unique profiles of EphrinB2 expression and they all differed from unfractionated PBLs. EphrinB2 mRNA was not detectable at 0 and 3 hours, but was present after 6 hours of TNFα stimulation (Figure 4A). Unstimulated control $CD8^+$ cells did express EphrinB2 (Figure 4A) and this expression remained after 3 hours of TNFα stimulation but was undetectable after 6 hours. EphrinB2 expression was detected in unstimulated control $CD19^+$ cells, but was undetectable at 3 and 6 hours after activation with anti-human IgM F(ab') fragments. We first attributed the detection of EphrinB2 in our control $CD8^+$ and $CD19^+$ cells to our positive cell selection process, which perhaps activated these cells. Therefore, we implemented negative selection columns to circumvent this activity, but similar results were obtained (data not shown). We then varied the time in culture before starting the assay, surmising that the cells were perhaps upregulating EphrinB2 upon being in culture for the 24 hour time period. Yu et al. (2003) reportedly observed an upregulation of EphrinB2 protein expression in mouse T cells that were in culture overnight. We conducted experiments in which both positive and negative selection methods were implemented to isolate $CD8^+$ and $CD19^+$ cells, and the assays were started either immediately after cell isolation or were delayed until the cells had been in culture for 48 hours. For all of these conditions we still obtained similar results (data not shown) to those demonstrated in figure 4A.

Lastly, we examined EphrinB2 expression in PMNs after stimulation with TNFα or IL-8. TNFα and IL-8 are well characterized modulators of PMN function. IL-8 potently chemoattracts PMNs to sites of inflammation[207-210] where they may be activated by TNFα[211-214]. Isolated PMNs were stimulated with either TNFα or IL-8 for up to 6 hours. EphrinB2 expression was upregulated after 1.5 hours of TNFα stimulation, but not at 0.5, 3 or 6 hours (Figure4B). In contrast, unstimulated PMNs did not express EphrinB2, and IL-8 treatment did not appear to upregulate its expression.

Expression of EphB4 Receptor in Human Iris Tissue and HIEC

To test the hypothesis that leukocyte-expressed EphrinB2 modulates trafficking of these cells during inflammation, we set forth to determine if blood vessels of the iris express its activating receptor, EphB4. This result would suggest the presence of true venules in the human iris, according to existing molecular criteria [27,113,114,200]. Serial sections of human iris were immunostained for EphB4 (Figure 5B and E) or von Willebrand Factor (vWF; Figure 5C and F). All of the blood vessels stained with anti-vWF, but EphB4 was detected in only a subset, presumably venules, stained with anti-Eph4. High magnification analysis of $EphB4^+$ vessels revealed that staining localized to the EC lining of the vessels (compare EphB4: Figure 5E, arrow to vWF: Figure 5F, arrow). Interestingly, EphB4 was also detected in non-endothelial structures of the iris, such as the sphincter papillary muscle (Asterisk: Figure 5B) and various stromal cells (Asterisk: Figure 5E).

We then wanted to determine if HIEC maintained their expression of EphB4 in culture, and if TNFα and/or IL-1α inflammatory mediators could regulate this

expression. EphB4 was detected in control unstimulated HIEC, and neither TNFα or IL-1α altered its expression over a 24 hour time period (Figure 6A). To confirm that HIEC expressed EphB4 protein, we tested HIEC cell lysates by western blot analysis. As shown in figure 6B, EphB4 protein is expressed by cultured HIEC and its expression is not altered by TNFα at 6 hours. Another potent stimulator of PMNs, lipopolysaccharide (LPS) also did not alter EphB4 expression in HIEC or in iris explants (Figure 6C). The data gathered here indicate that EphB4 expression by HIEC is not lost during an inflammatory situation and could potentially interact with leukocyte-EphrinB2 in these conditions.

In line with our hypothesis, we next determined if EphrinB2 is capable of regulating signaling pathways in HIEC, which would suggest a functional role for the EphrinB2/EphB4 signaling axis in iris-derived EC. The chimeric EphrinB2/Fc protein is a soluble ligand for the EphB4 receptor that has been shown to regulate signaling pathways in other cell types [161,167,174]. Confluent monolayers of HIEC were stimulated with soluble EphrinB2/Fc for up to 1 hour. Cell lysates were then analyzed simultaneously for phosho-Erk1 (p44) and phospho-Erk2 (p42) by western blot analysis. Phosphorylation levels of Erk2 remained unchanged in HIEC after 5 and 15 minutes of EphrinB2/Fc stimulation (Figure 7: HIEC bottom band). However, these levels were reduced after 30 minutes and began increasing by 60 minutes. Erk1 phosphorylation levels in HIEC appeared to increase within the first 5 minutes and remained unchanged after 15 minutes of stimulation (Figure 7: HIEC, top band). These levels dropped to below the level of detection by 30 minutes and remained undetectable after 60 minutes of EphrinB2/Fc stimulation. EphrinB2 was reported to modulate the phospho-Erk1/2 levels

of human mesenteric venule EC (HMesVEC) by interacting with EphB4[167]. Therefore, HMesVEC were obtained and utilized here as a positive control cell type (data not shown). Indeed, EphrinB2/Fc similarly regulated the phosphorylation levels of both Erk1/2 in these cells within the same timeframe. TNFα was used as a positive control stimulant, and as expected, it induced an increase in phospho-Erk1/2 in these EC.

Discussion:

The extravasation of leukocytes is an essential process in normal and pathological conditions (e.g., inflammation), and the molecules that regulate this process are well studied. EphrinB2 and EphB4 are important signaling molecules that modulate the migratory properties of various cells (e.g. neural and ECs)[215,216], and we hypothesize that these molecules contribute in the signaling events during leukocyte trafficking in the iris. Little is known as to the expression and function of EphrinB2 and EphB4 in human immune cells so we analyzed the expression of EphrinB2 in human PBLs. In line with our hypothesis, we also investigated the expression of EphB4 in human iris tissue and HIEC.

The most significant finding of this study was that human PBLs rapidly express EphrinB2 in response to TNFα (0.5 hours), and its expression is modulated over a 24 hour period. This observation is unique in that it is the first known report of modulated EphrinB2 expression by human PBLs in response to inflammatory stimuli. Given that EphrinB2 is well characterized in regulating such activities as cell adhesion, repulsion, and migration, these data suggested that PBL expressed EphrinB2 may also function in this context. In support of this notion, Hamada et al. (2003) recently reported that mouse B cells transfected with either EphrinB2 or EphB4 will undergo heterophilic cell aggregation upon co-incubating these cells in culture[162].

Our studies on SDF1α stimulated PBLs revealed an upregulation of EphrinB2 within a limited timeframe compared to TNFα stimulated PBLs. EphrinB2 was expressed after approximately 3 hours of SDF1α treatment, but not at earlier or later times. The differential expression pattern of EphrinB2 between TNFα and SDF1α stimulants is

likely due to the general nature of these stimulants. TNFα is an autocrine stimulator, as well as a potent paracrine inducer of many other inflammatory cytokines, including IL-1, IL-6, IL-8, and GM-CSF[217-219]. The production of these cytokines by one or all of our PBL subsets could potentially influence EphrinB2's expression in these cells. In contrast, SDF1α stimulated immune cells have been shown to selectively upregulate IL-8 production, without affecting TNFα, IL-1β, IL-6, GM-CSF, IFN-γ or RANTES levels[220,221]. SDF1α is a potent chemoattractant of human T cells, monocytes, B cells, and $CD34^+$ progenitor cells[49,222-227], and low concentrations of SDF1α can attract T cells, whereas high concentrations can repel some subgroups[228]. In other studies, Lu et al. (2001) demonstrated that SDF1α/CXCR4 signaling can be inhibited by soluble EphB receptor, while Sharfe et al, (2002) demonstrated a substantial reduction in Jurkat and T cells migration after EphrinA1 stimulation. These studies provided the first clues as to how Ephrin/Eph interactions can regulate the chemoattractive properties of SDF1. Exactly, how differential SDF1α concentrations regulate attraction or repulsion of T cells remains unknown, but such a change in SDF1α sensitivity may be regulated by EphrinB/EphB interactions. It is interesting to note the lack of EphrinB2 expression by unstimulated PBLs. These observations suggest that under normal *in vivo* conditions, PBLs do not require EphrinB2 expression while performing routine surveillance activities within the vascular tree, but can upregulate it in response to inflammatory stimuli.

Most forms of uveitis are mainly characterized by activated $CD4^+$ T cells at inflammatory foci, although activated $CD8^+$ cells are also present in lesser numbers[229-233]. Furthermore, $CD19^+$ positive B cells do not generally infiltrate the iris, but are known to play a role in the humoral response during bouts of uveitis[234-236]. In our studies, we found

that human $CD4^+$ T cells upregulated EphrinB2 after 6 hours of TNFα stimulation. It is unclear how EphrinB2's expression profile by $CD4^+$ T cells fits into context with *in vivo* T cell activation during uveitis, but indicates a potential role for it during T cell activation. Of particular interest is the finding that $CD8^+$ and $CD19^+$ cells express EphrinB2 upon isolation. The results obtained in these studies suggest either that both the positive and negative selection procedures activated $CD8^+$ and $CD19^+$ cells or that an inhibiting factor of EphrinB2 expression is present in whole PBL fractions. In either scenario, it is interesting that stimulation of $CD8^+$ cells with TNFα and $CD19^+$ cells with α-IgM antibodies down regulates their EphrinB2 expression at 3 and/or 6 hours. We believe this to be the case since down regulation of EphrinB2 was stimulant, not time in culture dependent. Nonetheless, results presented here on EphrinB2 expression by $CD4^+$, $CD8^+$, and $CD19^+$ cells indicate they can express EphrinB2 and warrants further investigation.

It is interesting to note that amongst the different leukocyte subsets, the PMN is the only cell type that does not extravasate in the absence of a pathological condition. However, when an inflammatory insult occurs, the PMN is the first cell to extravasate from the blood via EC interactions[237]. The PMN is not the primary infiltrating cell in human forms of uveitis, but it does play a significant role in various animal models of uveits[238]. We found that PMNs upregulated EphrinB2 after 1.5 hours of TNFα treatment, but not at later time points. In contrast, the PMN chemoattractant IL-8 did not seem to induce EphrinB2 expression. These results are intriguing in that IL-8 is a potent chemoattractor of PMNs and induces their migration across EC monolayers. The

complex signaling molecules that influence an EC-adhered PMN during diapedesis make it difficult to assess the role of EphrinB2.

A unique feature of iris vasculature, which is different from other parts of the body, is that blood flow velocity differs minimally between its arteries and veins[239]. In general, depending on the vascular bed of interest, blood flow in arterioles is much faster than within the post-capillary venules[240-243]. In the iris, vessel composition is generally homogenous, regardless of diameter. The vessels lack the typical cellular structure that defines the arteries and veins of other tissues[244]. Only the capillaries are designated as true capillaries based on their diameter (10-15 μm), while the terms arteriole and venule generally refers to pre- and post-capillary vessels[244]. As a result, iris vasculature is currently characterized into arterioles and venules based on the direction of blood flow (artery=blood flow towards pupil; vein=blood flow away from pupil). Nonetheless, rolling, sticking, and extravasation of activated leukocytes typically occur within the venules of the iris[239]. Our finding that EphB4 is expressed by some, but not all iris vessels indicates that the human iris does possess true venules according to existing molecular criteria[114,245]. Furthermore, this result supports the idea that leukocyte-expressed EphrinB2 can interact with EphB4 on iris EC. The observation that HIEC and iris tissue express EphB4 and remains unaltered after TNFα, IL-1α, or LPS indicates that it is present under inflammatory conditions and in position to interact with leukocyte expressed EphrinB2. Furthermore, the Erk1/2 signaling pathway is engaged in HIEC in response to EphrinB2. Indeed, the Erk1/2 signaling pathway is involved in the regulation of leukocyte trafficking, and inhibiting this pathway prevents leukocyte extravasation[246]. Preliminary results from our lab indicate that soluble EphrinB2 (2 μg/ml for 5 hours) can

induce HIEC to upregulate their expression of various genes that regulate EC-EC junctions (e.g., MMP1, MMP2, paxillin, zyxin, rhoB, rho-GDP, rho-GAP; data not shown), further implicating a role for EphrinB2 in the modulation of EC monolayer integrity.

We propose a hypothetical model for EphrinB2 / EphB4 interaction during inflammation (Figure 7). An inflammatory stimulant (1) induces EphrinB2 expression on the surface of a leukocyte (2). Upon margination and/or firm adhesion of the leukocyte, EphrinB2 can then interact with iris EC-expressed EphB4 (3) and induce a simultaneous bi-directional signal into both cells. This activity can then influence the outcome of leukocyte trafficking (4; attach vs. detach). In this scenario, a leukocyte will encounter a milieu of cytokines, chemokines, and adhesion molecules, all of which influence a leukocytes decision to extravasate. The relative contribution of EphrinB2 / EphB4 signaling in this scenario remains to be determined.

Acknowledgements:

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Figure 1: RT-PCR and Western blot analysis of Ephrin-B2 in human PBLs. Human PBLs were stimulated with TNFα for 5 hours and their mRNA analyzed for EphB4 and EphrinB2 expression (A). EphB4 transcripts (A, left=top band) were detected in unstimulated, control (Con) PBLs, and did not appear to change in response to TNFα (n=3). In contrast, unstimulated PBLs did not appear to express EphrinB2 mRNA, but did upregulate its message in response to TNFα stimulation (A, right: top bands; n=3). GAPDH was used as an internal control (bottom bands). An extended time course of TNFα stimulated PBLs revealed that mRNA for EphrinB2 was upregulated at 30 minutes, peaked between 1 and 6 hours, and was undetectable by 24 hours (B; n=5). Western blot analysis paralleled RT-PCR data in that EphrinB2 was undetectable in control PBLs, but upregulated its expression in response to TNFα (C; molecular weight ~40 kDa; n=3).

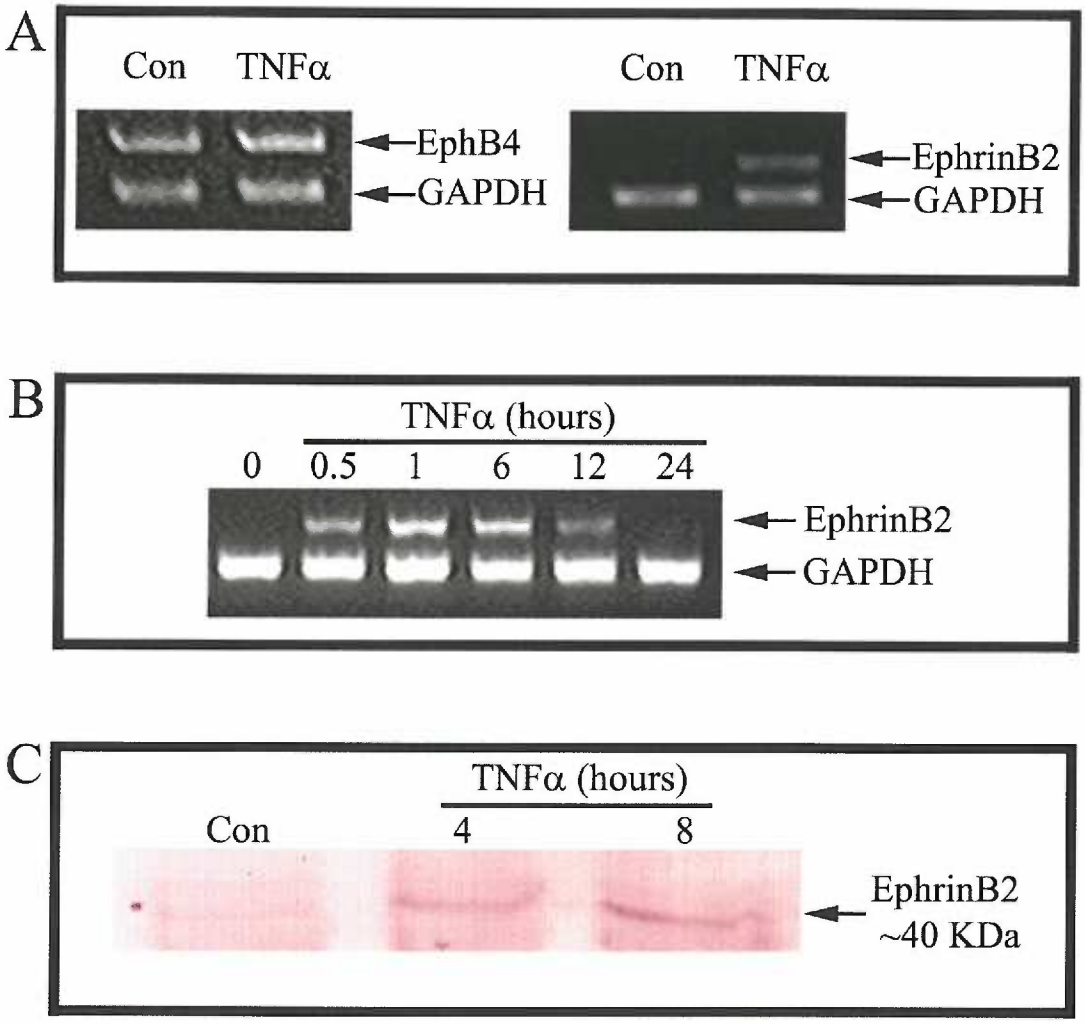

A
Con
TNFα
EphB4
GAPDH
Con
TNFα
EphrinB2
GAPDH
B
TNFα (hours)
0
0.5
1
6
12
24
EphrinB2
GAPDH
C
TNFα (hours)
Con
4
8
EphrinB2
~40 KDa

Figure 2: Upregulation of EphrinB2 transcript in PBLs in response to SDF1α . Human PBLs were stimulated from 0 to 6 hours with SDF1α and analyzed for the expression of EphrinB2 transcript. SDF1α-stimulated PBLs upregulated EphrinB2 after 3 hours of stimulation which indicated that PBLs could differentially regulate mRNA for EphrinB2 depending on inflammatory mediator (compare to figure 1B; n=2).

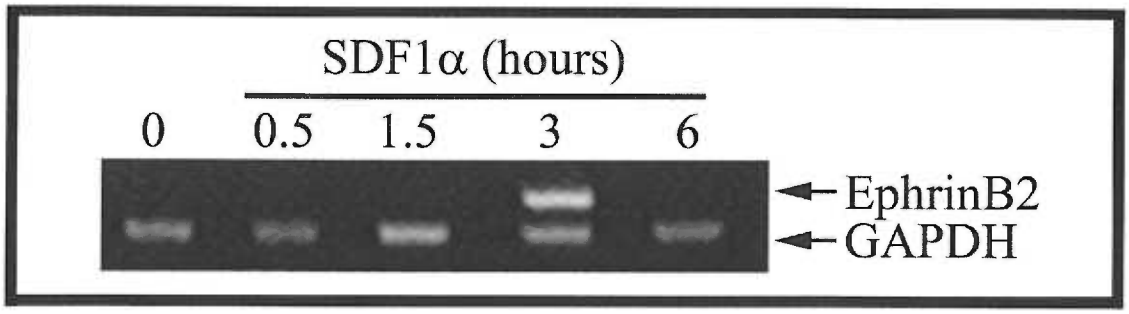

SDF1α (hours)
0
0.5
1.5
3
6
EphrinB2
GAPDH

Figure 3: Positive selection of human lymphocyte subsets. PBLs were FACS sorted after incubating them with fluorochrome-labeled antibodies targeted against CD3, CD4, CD8, and CD19. $CD4^+$ and $CD8^+$ cell populations of greater than 90% purity were obtained, while $CD19^+$ cells were ~88% pure. Panels A, C, and E indicate gating of specified cell types from the monocyte depleted PBL fraction. Panels B, D, and F demonstrate cell purity after FACS sorting.

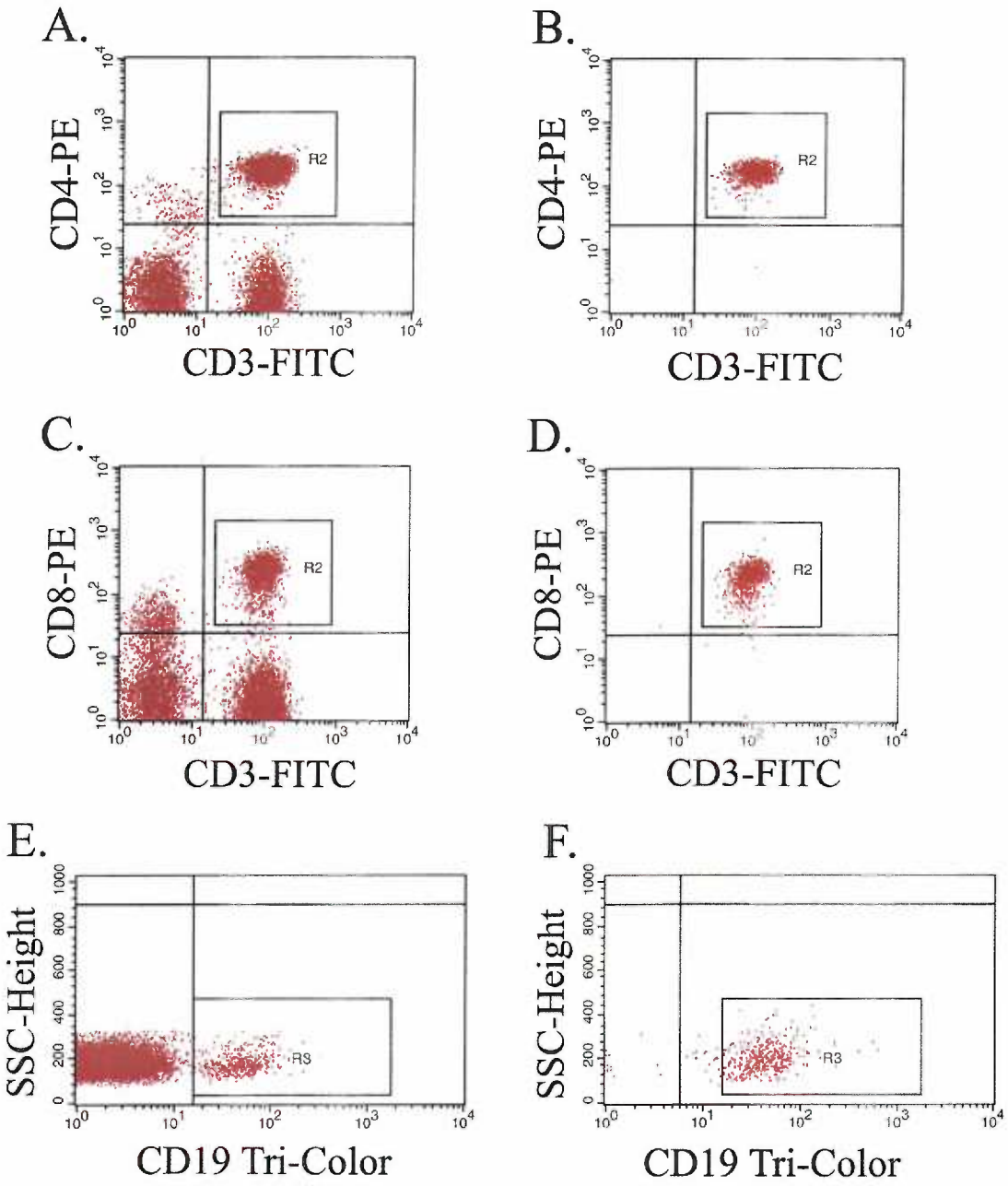

A.
CD4-PE
CD3-FITC
R2
B.
CD4-PE
CD3-FITC
R2
C.
CD8-PE
CD3-FITC
R2
D.
CD8-PE
CD3-FITC
R2
E.
SSC-Height
CD19 Tri-Color
R3
F.
SSC-Height
CD19 Tri-Color
R3

Figure 4: EphrinB2 mRNA expression in PBL and PMN leukocyte subsets. TNFα stimulated $CD4^+$, $CD8^+$, and α-IgM stimulated $CD19^+$ were analyzed for EphrinB2 mRNA by RT-PCR. EphrinB2 transcript was undetectable in unstimulated and 3 hour TNFα-stimulated $CD4^+$ cells, but did upregulate its expression after 6 hours (A: left panel; n=5). Interestingly, unstimulated control $CD8^+$ cells expressed EphrinB2 mRNA, and its expression was maintained after 3 hours of TNFα stimulation. However, EphrinB2 mRNA was undetectable in these cells after 6 hours of TNFα stimulation (A: middle panel; n=4). Similar to $CD8^+$ cells, unstimulated control $CD19^+$ cells expressed EphrinB2 mRNA, but was undetectable after 3 and 6 hours of anti-IgM stimulation (A: right panel; n=5). EphrinB2 transcript was detected in PMNs only after 1.5 hours of TNFα stimulation and was undetectable at 3 and 6 hours (B; n=2). IL-8 did not upregulate EphrinB2 mRNA expression.

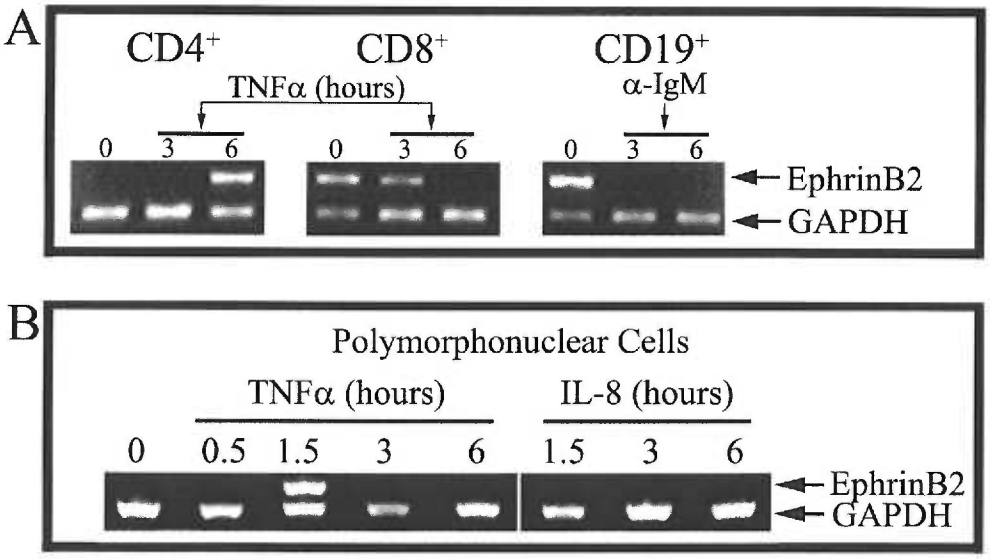

A
CD4+
CD8+
CD19+
TNFα (hours)
α-IgM
0 3 6
0 3 6
0 3 6
EphrinB2
GAPDH
B
Polymorphonuclear Cells
TNFα (hours)
IL-8 (hours)
0 0.5 1.5 3 6
1.5 3 6
EphrinB2
GAPDH

Figure 5: Immunohistology of EphB4 in human iris tissue. Serial sections of human iris were immunostained for either EphB4 (B & E) or von Willebrand Factor (vWF; C & F). Arrows in B&E indicate vessels that are positive for EphB4 and vWF (E & F). Arrowhead in B demonstrates a vessel that is negative for EphB4, but positive for vWF (C; arrowhead). Asterisks indicate positive EphB4 staining in sphincter papillae muscle (B) and stromal cells (E). A and D are representative images of sections incubated with pre-immune, control antibodies. In A, B, and C, the pupil is located on the left, while the ciliary body region (unseen) is located to the right. In D, E, and F, orientation of iris tissue is the same as in A, B, and C, but at higher magnification. Original magnifications: A-C=200X; D-F=400X (n=3).

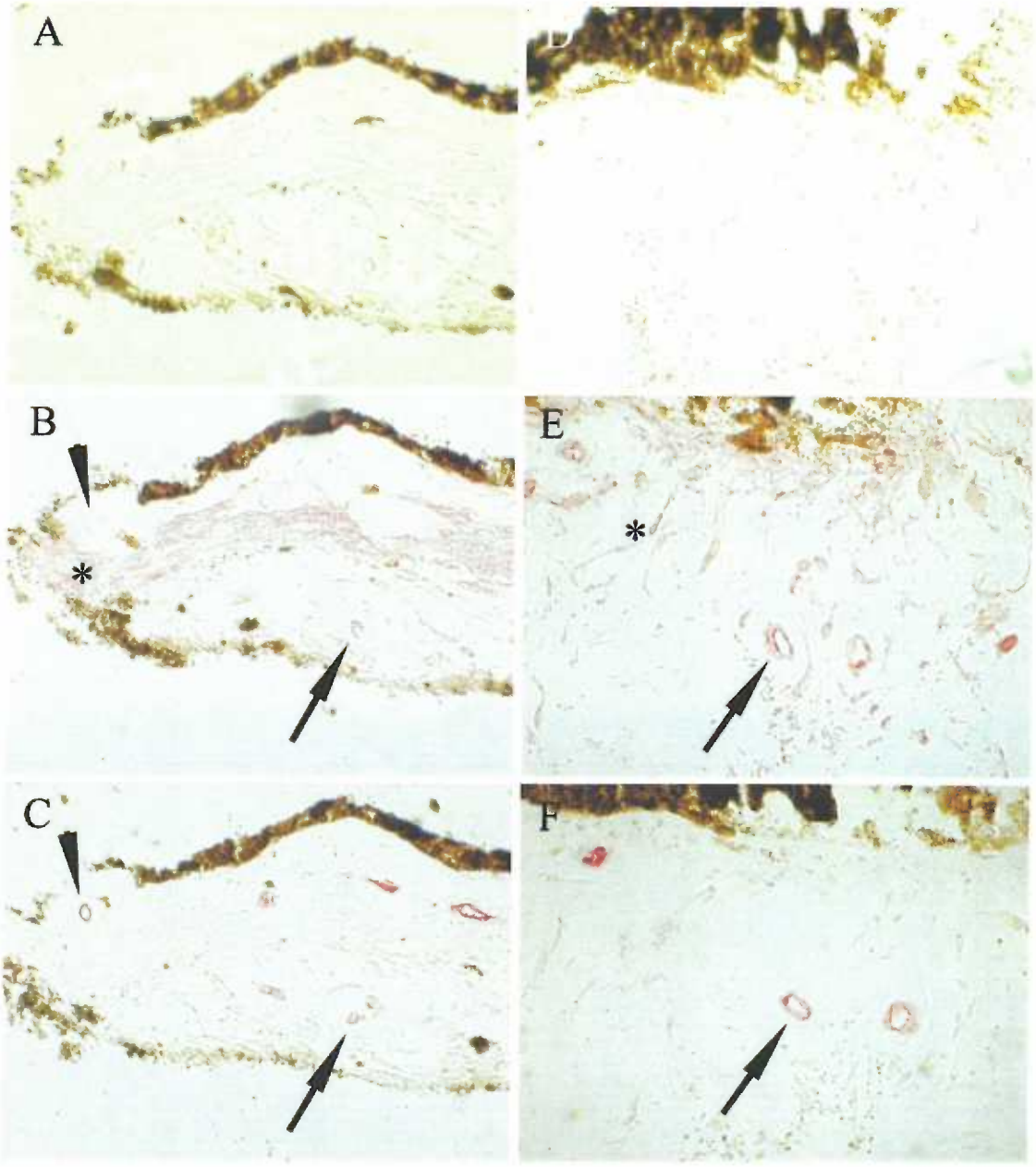

A
B
*
C
E
*
F

Figure 6: EphB4 mRNA and protein expression in HIEC and iris explants. HIEC expressed EphB4 transcripts under normal, unstimulated culture conditions and these levels did not change in response to either TNFα or IL-1α over a 24 hour period (A; n=3). Likewise, western blotting detected EphB4 protein in unstimulated HIEC (B) and did not change after 6 hours of TNFα stimulation (n=2). Lastly, LPS was used as an exogenous stimulant to activate HIEC and human iris explants (C), and neither changed their EphB4 expression levels in response to the stimulus (n=3).

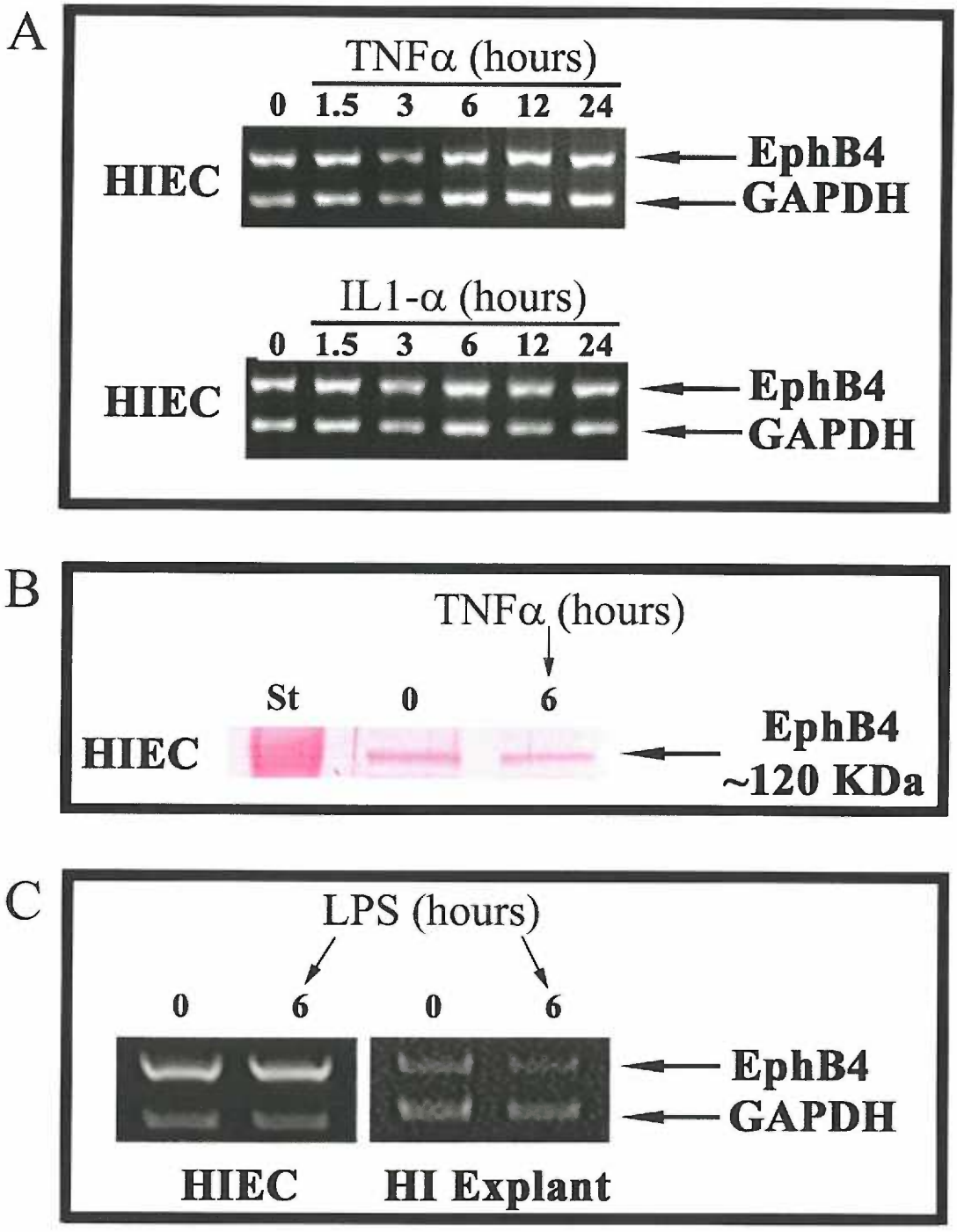

A
TNFα (hours)
0 1.5 3 6 12 24
HIEC
EphB4
GAPDH
IL1-α (hours)
0 1.5 3 6 12 24
HIEC
EphB4
GAPDH
B
TNFα (hours)
St 0 6
HIEC
EphB4
~120 KDa
C
LPS (hours)
0 6 0 6
EphB4
GAPDH
HIEC
HI Explant

Figure 7: Soluble EphrinB2/Fc modulates signaling pathways in HIEC. Erk2 phosphorylation levels remained constant in HIEC after 5 and 15-minute stimulations with EphrinB2/Fc. After 30 minutes, detectable levels diminished and then began increasing by 60 minutes. Phospho-Erk1 levels increased within 5 minutes and were unchanged after 15 minutes of stimulation. Phospho-Erk1 levels decreased below the level of detection at 30 minutes and remained undetectable after 60 minutes. *TNFα is a potent inducer of Erk1/2 phosphorylation in various cell types. TNFα was used here as a positive stimulant for HIEC (n=2) to confirm that their phospho-Erk1/2 levels could be regulated.

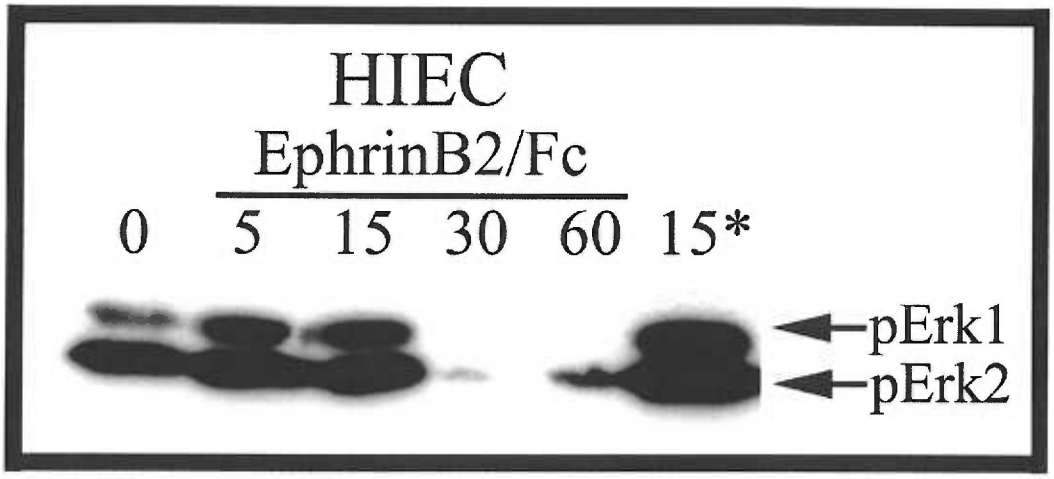
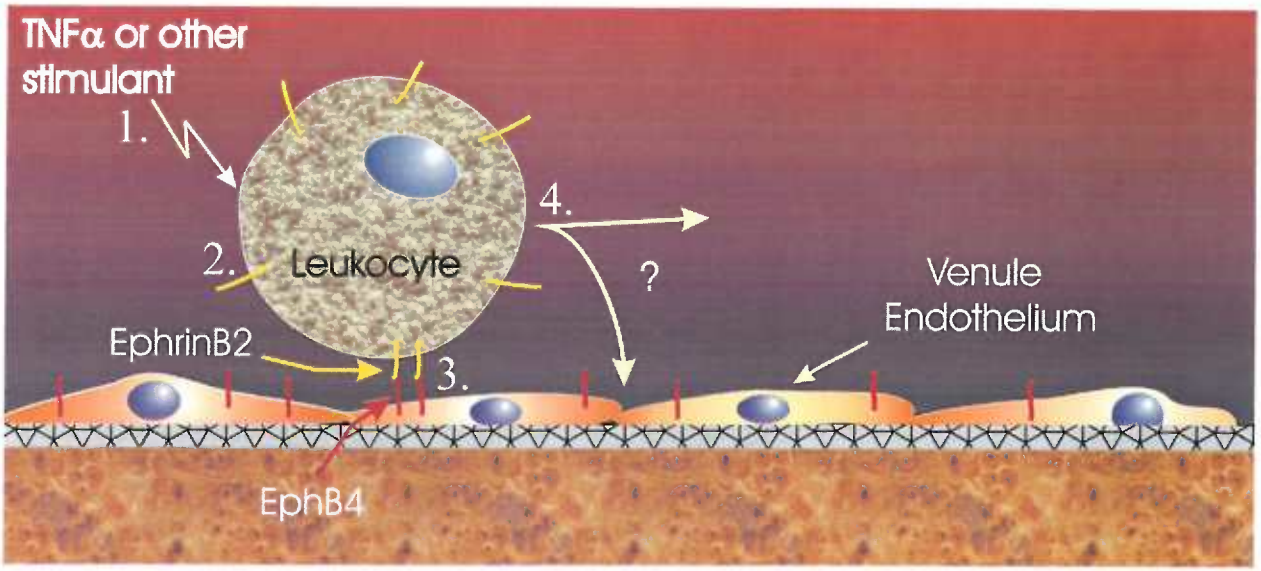

HIEC
EphrinB2/Fc
0
5
15
30
60
15*
pErk1
pErk2

Figure 8: Proposed model of EphrinB2 / EphB4 in leukocyte trafficking during inflammation. 1. Circulating leukocytes are activated in response to inflammatory mediators (e.g., TNFα). 2. Activated leukocytes upregulate EphrinB2 on their cell surface in response to stimuli. 3. Leukocyte EphrinB2 and endothelial EphB4 engage one another to initiate a "bi-directional" signal into both cells and influence leukocyte trafficking (4.) at sites of inflammation.


TNFα or other
stimulant
1.
2.
Leukocyte
4.
?
Venule
Endothelium
EphrinB2
3.
EphB4

"The nearer the arteries are to the heart, the more they differ from the veins in their constitution, and are more robust . . . but in the furthest dispersions . . . they are so like in their constitution, that . . . it is a hard business to know one from the other"

-William Harvey (1578-1657)

Chapter 5

Soluble Forms of EphrinB2 and EphB4 Reduce Neovascular Tuft Formation in the Mouse Model of ROP

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The manuscript that comprises this chapter will be submitted for consideration for publication in Investigative Ophthalmology and Visual Science (July 2004). The schematic in Figure 1 was created by DOZ. The experiments for Figures 2-6 were a joint effort of DOZ and MHD.

Abstract:

Purpose: EphrinB2 ligands, and their EphB4 receptors, are key regulators of EC (EC) proliferation, migration, adhesion, and repulsion during mammalian vascular development. Arteriole ECs express EphrinB2 while venule ECs express EphB4, and during vessel formation, these molecules engage one another to modulate proper artery-capillary-vein boundaries. We hypothesize that these molecules also play a role in pathological neovascularization (NV) in the mouse model of oxygen-induced retinopathy and that soluble versions of these molecules can alter this neovascularization.

Methods: C57BL/6 mice at postnatal day 7 (P7) were exposed to 75% oxygen (O_2) for 5 days (until P12) and allowed to recover in room air to induce retinal NV. Retinas were dissected from unexposed and O_2 exposed mice between P7-24 and analyzed for EphrinB2 and EphB4 transcript expression by semiquantitative RT-PCR. Some O_2 exposed mice had one eye-injected intravitreally with 150 ng/1.5 µls of soluble EphrinB2/Fc or EphB4/Fc chimeras during transition from high O_2 to room air (P12) and reinjected on P14. Contralateral eyes were injected with human IgG antibodies as controls. All injected eyes were collected during peak disease (P17) and processed for quantification of preretinal nuclei and blood vessels.

Results: EphrinB2 was constitutively expressed in the developing retina and hyperoxia treatment did not alter this expression. In contrast, EphB4 expression was modulated during normal retinal development and was altered by hyperoxia. Furthermore, intravitreally injecting soluble versions of these molecules significantly reduced the

pathological neovascularization in this model. The number of preretinal nuclei in hyperoxia treated mice was reduced by 66% ($p<0.05$) in EphrinB2-injected eyes, while EphB4 treatment yielded a 69% reduction ($p<0.05$), compared to control injections. Intraretinal vessel development was not altered by the injections.

Conclusions: EphrinB2 and EphB4 are expressed in the developing mouse retina, and hyperoxia treatment differentially affects EphB4 expression. Furthermore, intravitreal treatment with soluble EphrinB2/Fc or EphB4/Fc significantly reduces the extent of oxygen-induced retinopathy without affecting intraretinal vessel development. The exact mechanism of action of the soluble molecules remains unknown, but we hypothesize that they interfere with endogenous EphrinB2 / EphB4 interactions between EC-EC and/or EC-stromal cells to affect proliferation and migration. These results suggest that endogenous EphrinB2 and EphB4 are regulators of retinal NV during oxygen-induced retinopathy and may serve as novel targets for therapeutic intervention.

Introduction:

The erythropoietin-producing hepatocellular (Eph) receptor family is the largest group of receptor tyrosine kinases known to date. These receptors are further subdivided into groups A and B, depending on whether they bind an Eph-interacting (Ephrin) ligand of the A or B class (http://cbweb.med.harvard.edu/eph-nomenclature). All Ephrin ligands contain an extracellular and transmembrane domain; however, Ephrin ligands of the B class possess a cytoplasmic tail that the A class lacks [153]. In general, Ephrin homodimers will engage Eph homodimers to regulate such cellular events as cell migration, proliferation, attraction, and repulsion. A unique feature of the B class Ephrin/Eph interaction is "bi-directional" signaling [152]. That is, since Ephrins and Ephs of the B class contain cytoplasmic domains, interaction with each another induces simultaneous signaling into both the EphrinB and EphB bearing cells [216,247-249].

EphrinB2 ligands and EphB4 receptors have emerged as key regulators of vascular development and are primarily expressed by the endothelial cells (EC) of developing arteries and veins, respectively. Specifically, EC of developing arteries express EphrinB2 ligands, while EphB4 receptors are expressed by venous EC [27,113-115]. During vessel formation, arteriole and venule EC undergoing proliferation and migration come into proximity of each another. Engagement of venule EphB4 by arteriole EphrinB2 causes the EC to stop proliferating and migrating and thus prevents these cell types from intermingling[116]. Mouse embryos lacking EphrinB2 or EphB4 exhibit lethal defects in early angiogenic remodeling. [27,113,114,250]. These mice improperly formed capillary beds and poorly differentiated arteries and veins. Therefore, signaling resulting from EphrinB2/EphB4 interaction is necessary for proper vascular network development

by allowing correct boundary formation between arterioles and venules [113,159]. Furthermore, recent studies indicate that these molecules may also be critical regulators of postnatal neovascularization [115,163,251,252].

Diseases of the retina are a major cause of vision loss in developed countries (i.e., retinopathy of prematurity, diabetic retinopathy, age-related macular degeneration). A critical component of these disease processes involves neovascularization of the retina [107]. As a result, several animal models have been developed and utilized to study both normal and pathological vascularization of the retina. These models have allowed direct identification of several critical molecular factors that are directly involved in modulating retinal neovascularization and have furthered the understanding of this biological process [108,109,253].

Recent studies have indicated that the developing retinal vascular system of the mouse express EphrinB2 and EphB4 in the forming arterioles and venules, respectively [254]. Furthermore, we and others have observed the expression of EphrinB2 and EphB4 in pure cultures of human retinal ECs, suggesting that these cell cultures may be of arteriole and venule in origin [160]. In addition, stimulating these cultures of retinal EC, as well as EC derived from other tissues, with soluble EphrinB2/Fc or EphB4/Fc can modulate their proliferation and migration *in vitro* [116,160]. These data indicate that EphrinB2 and/or EphB4 may be therapeutic targets in the regulation of retinal neovascularization. In support of this idea, other studies have shown that blockade of various Eph receptors can reduce postnatal angiogenesis [255,256]. Taken together, we hypothesize that EphrinB2 and EphB4 contribute to the neovascularization process that occurs in oxygen-induced retinopathy and that intravitreal treatment with soluble forms of these molecules can

modulate this neovascularization. In this study, we describe the expression patterns of EphrinB2 and EphB4 in normal retinal development and in retinal development after oxygen-injury. We further assess the pathological neovascularization in oxygen-injured retinas after intravitreal treatment with soluble forms of EphrinB2 or EphB4.

Results:

EphrinB2 and EphB4 expression in normal and oxygen-injured retinas

To determine the endogenous expression levels of EphrinB2 and EphB4 mRNA in normal and oxygen-injured retinas, we exposed neonatal C57BL/6 pups to 75% oxygen for 5 days between postnatal (P) days P7 and P12 (Figure 1) and allowed them to recover in room air for 12 days (until P24) [109,257]. We then dissected the retinas, isolated the total RNA, and performed semi-quantitative RT-PCR (Figure 2). By this method, we were able to detect the expression of EphrinB2 and EphB4 in the retinas of P7 mice. Interestingly, we were unable to detect any changes in the levels of EphrinB2 mRNA during normal retinal development or during retinal development after oxygen-injury. In contrast, EphB4 mRNA levels were modulated during normal retinal development and reached peak levels between P12 and P14. Furthermore, after five days of hyperoxia treatment, EphB4 mRNA was downregulated to below the levels of untreated, normal mice on P12. However, upon allowing hyperoxia-exposed mice to recover in room air, EphB4 mRNA levels immediately increased and peaked between P14 and P21. The levels of EphB4 mRNA then equalized to those of normal untreated mice by P24. These data demonstrate that these molecules are expressed under these experimental conditions and can potentially modulate the neovascularization that occurs in hyperoxia-injured retinas.

EphrinB2/Fc or EphB4/Fc Treatment of Oxygen-Injured Retinas

We next wanted to determine if treating oxygen-injured retinas *in vivo* with soluble forms of EphrinB2 or EphB4 could alter the neovascularization that occurs upon room air

recovery. The chimeric EphrinB2/Fc and EphB4/Fc proteins employed in this study are dimerized in their active form by the Fc portion of human IgG. The dimers bind to their respective receptors on the cell surface and induce signaling within that cell which would have occurred normally by cell-cell contact. Our control injections consisted of human IgG. We exposed mice to high oxygen as described (see Figure 1), and allowed the mice to recover in room air for 5 days (P17), the day in which peak disease occurs. During room air recovery, we injected intravitreally 150ng of either EphrinB2/Fc or EphB4/Fc on P12, and gave a second injection on P14. The eyes were then processed for routine histological analysis. We first assessed the pathology that occurred in the oxygen-injured retinas to confirm the validity of our model. As expected, we observed a high number of pre-retinal neovascular tufts in non-injected eyes after oxygen-induced injury (Figure 3A). We then compared pre-retinal tuft formation in the non-injected eyes to the pre-retinal tufts in the injected eyes. We found that control IgG injected eyes (Figure 3B) had slightly reduced pre-retinal tuft formation compared to uninjected eyes (Figure 3A). However, upon comparing control IgG injected eyes (Figure 3B) to experimentally injected eyes, we observed a vast reduction in the presence of pre-retinal tufts in the EphB4/Fc (Figure 3C) and EphrinB2/Fc (Figure 3D) injected eyes. To confirm this observation, neovascular nuclei anterior to the inner-limiting membrane were quantified in a masked fashion and the data statistically analyzed (Figure 4). EphrinB2/Fc injections yielded a 66% reduction over control IgG injections, while EphB4 yielded a 69% reduction (n=12-25).

Intraretinal vessel development after EphrinB2/Fc or EphB4/Fc treatment

Since the experimental injections exhibited a profound effect on pre-retinal tuft formation, we became curious as to whether the injections also affected the development of the intraretinal vascular beds (superficial and deep) in this disease model. Control and experimental tissue sections generated in the previous study were immunostained for type IV collagen (a blood vessel marker) and we then analyzed the superficial and deep vascular networks. As is demonstrated in the representative micrographs shown in Figure 5, we did not observe a marked change in staining pattern in the superficial and deep vascular beds of mice receiving EphrinB2/Fc (Figure 5B), EphB4 (Figure 5D) or their respective controls (Figures 5A and 5C). This observation was further confirmed upon quantitating these vascular beds. Neither experimental nor control injections significantly affected the number of vessels in the superficial (Figure 6A) or deep (Figure 6B) vascular beds.

Discussion:

Here we report that, 1) EphrinB2 is constitutively expressed in normal developing retinas and exposure to hyperoxia does not alter this expression; 2) EphB4 expression is modulated during normal retinal development, and hyperoxia treatment can alter this expression; 3) intravitreal injection of soluble mouse EphrinB2/Fc or EphB4/Fc significantly reduces the number of pre-retinal cells that gather within the pathological tufts; 4) intravitreal injections do not affect the developing intraretinal vascular network from P12 to P17.

Expression gradients of various classes of Ephrins and Ephs have previously been reported in the retina [258-261]. Specifically, EphrinB2 expression is detected in the neural cells of the retina and plays an important role during retinotectal mapping of axons during visual system development [261-264]. Interestingly, EphB4 is not detected in the neural cells of the retina [254,265]. However, both EphB4 and EphrinB2 are expressed in the developing retinal vasculature of the mouse, and this expression is localized to the developing arteries and veins at P3, P7, and P12 by Saint-Geniez *et al.*[254]. This report was limited to three timepoints P3, P7, and P12. Here we report on the expression of EphrinB2 and EphB4 over a broader developmental period (P7-P24) in normal and oxygen-injured retinas.

Although we did not detect changes in EphrinB2 mRNA levels, it is possible that there were some changes in the vascular EC that were masked by a background of neural transcripts. If indeed the venules of the retina are the only location that EphB4 is expressed, then this would make detection of subtle modulation of vascular EphB4 more sensitive. Furthermore, the EphB4 PCR data gathered here would be reflective of the

amount of EphB4 positive vessels that were present in the retina. Therefore, down regulation of EphB4 transcript in oxygen-injured retinas compared to normal unexposed retinas presumably reflects the halting of vessel growth that results from hyperoxia treatment. Furthermore, the shift in peak levels of EphB4 transcript from P12 through P14 in normal uninjured retinas, to P14 through P21 in oxygen-injured retinas may presumably reflect the regrowth of vessels upon ischemic conditions due to room air recovery. These data demonstrate that EphrinB2 and EphB4 are expressed under these experimental conditions and can potentially modulate the neovascularization that occurs in hyperoxia-injured retinas.

The most significant finding of this study was that intravitreal injection of soluble EphrinB2/Fc or EphB4/Fc into hyperoxia-injured eyes resulted in a 66-69% reduction of pathological pre-retinal tuft formation. Interestingly, we found that our control IgG injected eyes had a slightly reduced pre-retinal tuft formation compared to the uninjected control eyes (Figure 3A & 3B). This phenomenon may have occurred as a result of the release of anti-angiogenic factors from the intraocular puncture wound and has been previously described by the Penn lab for the rat model of oxygen-induced retinopathy [266,267]. The exact mechanism by which soluble EphrinB2/Fc or EphB4/Fc reduce pre-retinal neovascularization remains unknown.

Angiogenesis can be divided into three distinct phases: initiation (induction of sprouting), invasion (cell proliferation, migration and matrix degradation), and maturation (remodeling, lumen formation and differentiation of EC) [268]. Several lines of evidence indicate that EphrinB2 / EphB4 "forward" and "reverse" signaling can influence each of these angiogenic phases. In general, forward signaling (EphrinB2 to EphB4)

decreases the proliferation and migration of EphB4 bearing cells, while reverse signaling (EphB4 to EphrinB2) increases the proliferation and migration of EphrinB2 bearing cells [116,161-167]. These activities have been well studied in various tissues and cell culture models, and are just now being explored in the eye[160]. VEGF, the primary initiator of angiogenesis in the retina [269-271], is detected in the mouse retina as early as one day after birth [272], and regulates EC sprouting (initial phase of angiogenesis) [165]. In vitro, VEGF induces the proliferation, sprouting and migration of EC (1st and 2nd phases of angiogenesis), and co-stimulation of the EC with VEGF and EphrinB2/Fc suppresses these activities [116]. This phenomenon is attributed to inhibition of both VEGF induced phosphorylation of ERK1/2 and suppression of Ras activity. Furthermore, Sturz et al., [273] have provided direct evidence implicating EphrinB2/Fc in the inhibition of EC migration by directly activating EphB4's kinase activity. Other studies have shown that EphrinB2/Fc can suppress EC spreading in culture due to inhibition of focal contacts, suggesting that EphrinB2 signaling affects EC-EC interactions via cytoskeletal regulation [162]. It is possible that intravitreally injecting EphrinB2/Fc allows it to bind EC as they penetrate through the inner-limiting membrane of the retina and reduces their proliferation and migration.

The observation that EphB4/Fc injections reduced angiogenesis in our model is contrary to the general outcome of reverse signaling because stimulation of endogenous EphrinB2 with soluble EphB4/Fc induces the migration and proliferation of retinal and other types of EC *in vitro* [116,160-167]. However, a growing body of literature suggests that the outcome of "forward" or "reverse" signaling is dependent on several factors. EC from different vascular beds might possess a unique complement of Eph receptors and Ephrin

ligands, which can produce different responses upon activation [160]. Microenvironment might also alter EC Ephrin/Eph signaling systems. For example, Maekawa *et al.*[163] showed that EphrinB2/Fc saturated pellets surgically implanted into the mouse cornea cause a significant amount of angiogenesis. This result is opposite of what we show here and what others have reported for EphrinB2/Fc forward signaling[116,161,162]. The discrepancy in these data might stem from a variety of conditions that directly or indirectly affect the outcome of exogenous EphrinB2 treatment of mice such as 1) VEGF levels in the oxygen-induced retinopathy model vs. in the avascular cornea, 2) tissue specific EphrinB2/EphB4 expression, 3) age of mice (P7-P24 in our study vs. 8-10wk old mice Maekawa *et al.*[163]). These dichotomous results have an *in vitro* parallel: Maekawa *et al.*, also demonstrated that EphrinB2/Fc induces the migration of HUVECs in a dose dependent manner, while Kim *et al.* [116] reported that EphrinB2/Fc inhibited VEGF induced EC proliferation and migration. A difference in the Kim *et al.* [116] and Maekawa *et al.*[163] studies are that the former group stimulated their HUVEC with VEGF, while the latter did not. It has been suggested that growth factor activated EC, as occurs during angiogenesis and vessel organization, are particularly responsive to EphrinB2 and EphB4[273].

Acknowledgements:

We thank Greg Seitz and Kiera Garman for their technical assistance in developing our ocular injection technique. We also thank Lindsay Simmons and Mark Montanaro for their individual contributions to the project.

Figure 1: Timeline of retinal vessel formation in normal development and oxygen-induced retinopathy in the mouse. This scheme was created based on the protocol developed by Smith *et al.* (1994)[109], and further characterized by Davies *et al.*[257]. Experimental mice placed in 75% O_2 (hyperoxia) on P7 exhibit interrupted development of superficial, transitional, and deep vascular networks of the retina. Some superficial vessels become obliterated during hyperoxia. Mice are moved back to room air on P12, which triggers a relative ischemic condition in the retina. The retina then exhibits pathological growth of vessels in the superficial layer, with pre-retinal vascular tufts extending through the inner-limiting membrane into the vitreous.
(dashed line = interrupted vessel growth; thin line = normal vessel growth; thick line = pathological vessel growth).

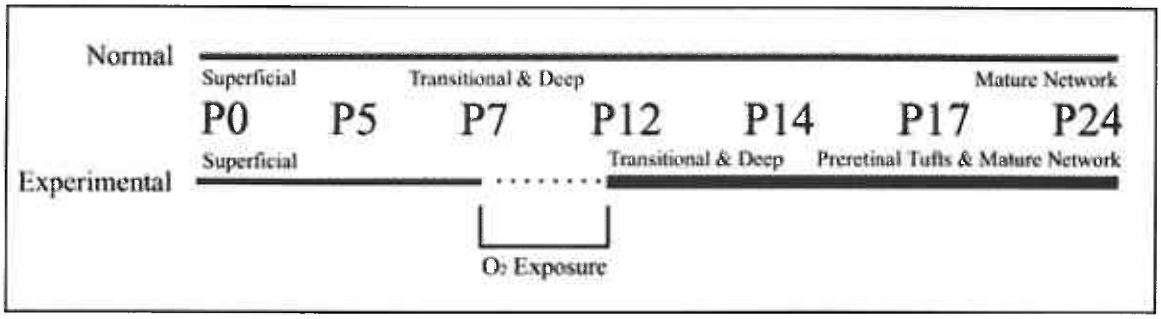

Normal
Superficial
Transitional & Deep
Mature Network
P0
P5
P7
P12
P14
P17
P24
Experimental
Superficial
Transitional & Deep
Preretinal Tufts & Mature Network
O2 Exposure

Figure 2: RT-PCR analysis of EphrinB2 and EphB4 expression in the retina during normal development and in oxygen-induced retinopathy. Total mRNA was isolated from the retinas at various stages of postnatal development (P7-P24) and analyzed by semi-quantitative PCR. EphrinB2 transcript levels remained unchanged in normal developing retinas (N) and hyperoxia-injured retinas (O_2). In contrast to EphrinB2, EphB4 transcript levels were modulated during normal development and five days (P7-12) of O_2 exposure altered this expression pattern. The retinas of four mice/condition were pooled for total RNA isolation. Micrograph is representative of one of five experiments conducted.

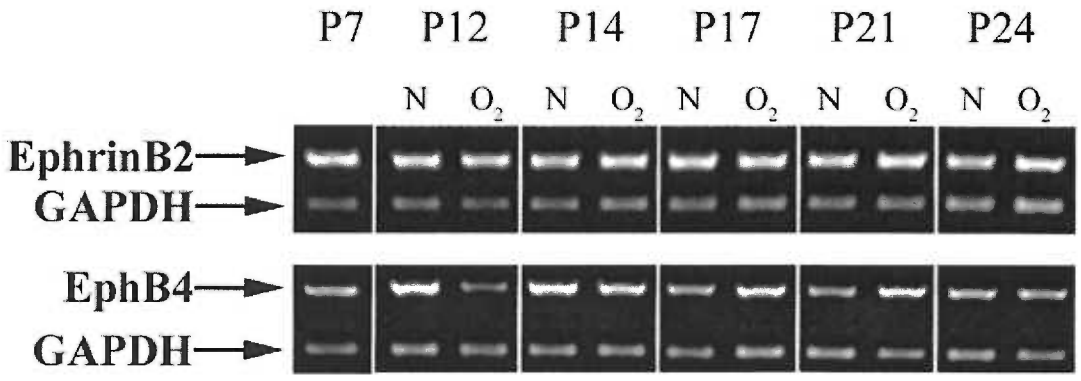

P7
P12
P14
P17
P21
P24
N
O2
EphrinB2
GAPDH
EphB4
GAPDH

Figure 3: Mice were exposed to 75% O2 from P7-P12. On P12 the mice were injected upon return to room air, with either control IgG, soluble EphrinB2/Fc, or EphB4/Fc and then re-injected on P14. The mice were then allowed to develop to peak disease (P17) before enucleating and fixing of eyes. Sections in these micrographs are H&E stained and representative of non-injected (A), control IgG-injected (B), and EphB4/Fc (C) or EphrinB2/Fc (D)-injected retinas. Brackets in each panel identify neovascular tufts just anterior the inner-limiting membrane. A slight reduction in neovascular tufts occurred between non-injected (A) eyes and control IgG-injected (B) eyes. EphB4/Fc (C) and EphrinB2/Fc (D) injected eyes demonstrated a large reduction in neovascular tufts compared to control IgG (B)-injected eyes. (Original magnification=400X).

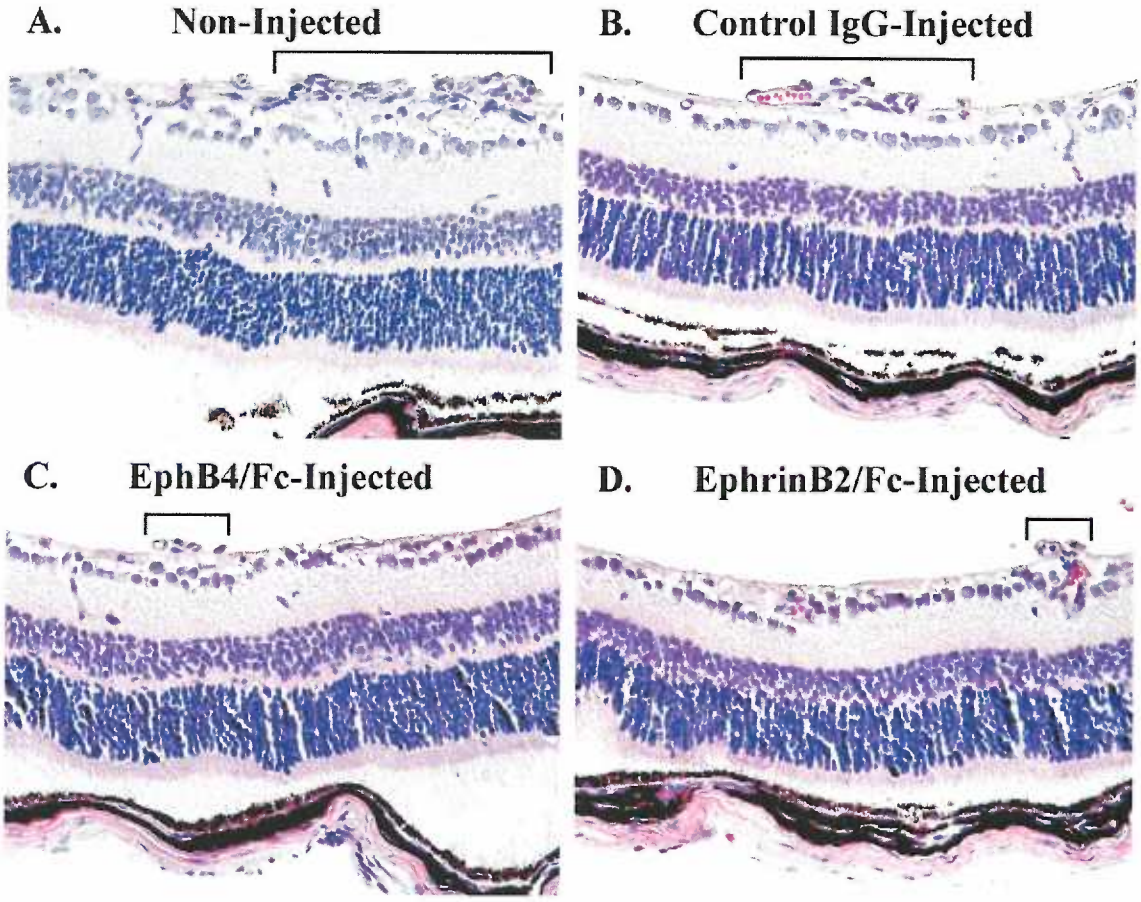

A. Non-Injected
B. Control IgG-Injected
C. EphB4/Fc-Injected
D. EphrinB2/Fc-Injected

Figure 4: Pre-retinal nuclei were counted in a masked fashion and data the statistically analyzed. Intravitreal injection of EphrinB2/Fc reduced the number of pre-retinal nuclei by 66%, in comparison to control IgG-injections ($P<0.05$). Similarly, EphB4 injections reduced the number of preretinal nuclei by 69% ($P<0.05$). Statistics performed were based on the median of the averages using Kruskal-Wallis one-way analysis of variance with a Dunn's method of multiple comparison procedure (n=12-25 mice /condition and represent approximately 5 different litters of mice).

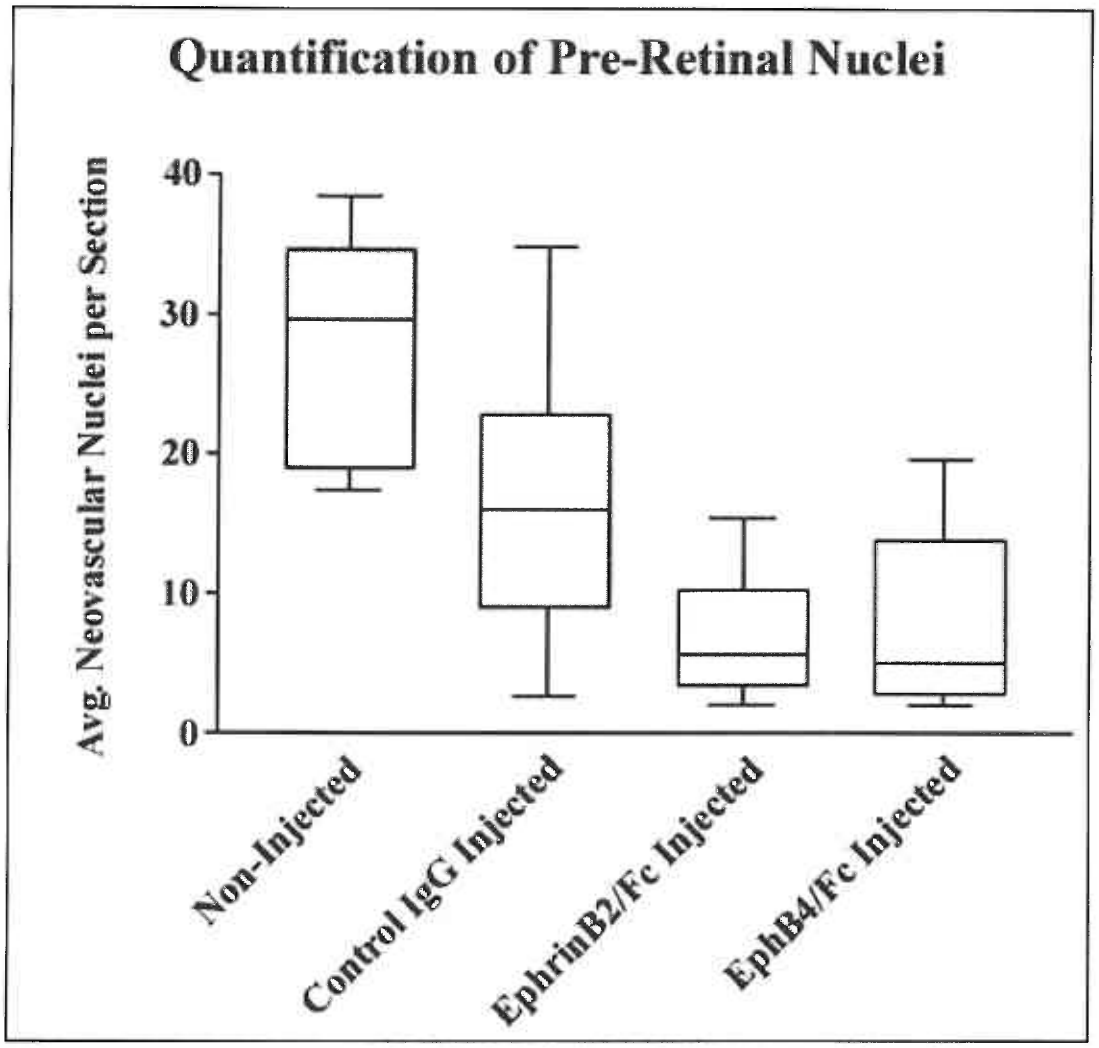

Quantification of Pre-Retinal Nuclei
Avg. Neovascular Nuclei per Section
40
30
20
10
0
Non-Injected
Control IgG Injected
EphrinB2/Fc Injected
EphB4/Fc Injected

Figure 5: Immunodetection of blood vessels in tissue sections (P17) from EphrinB2/Fc (B), EphB4/Fc (D) injected eyes, and their respective control injections (A, C). Blood vessels in the superficial (*) and deep ([ ) layers are labeled in red. There was no apparent difference in superficial and deep vascular bed formation. (Original magnification=400X).

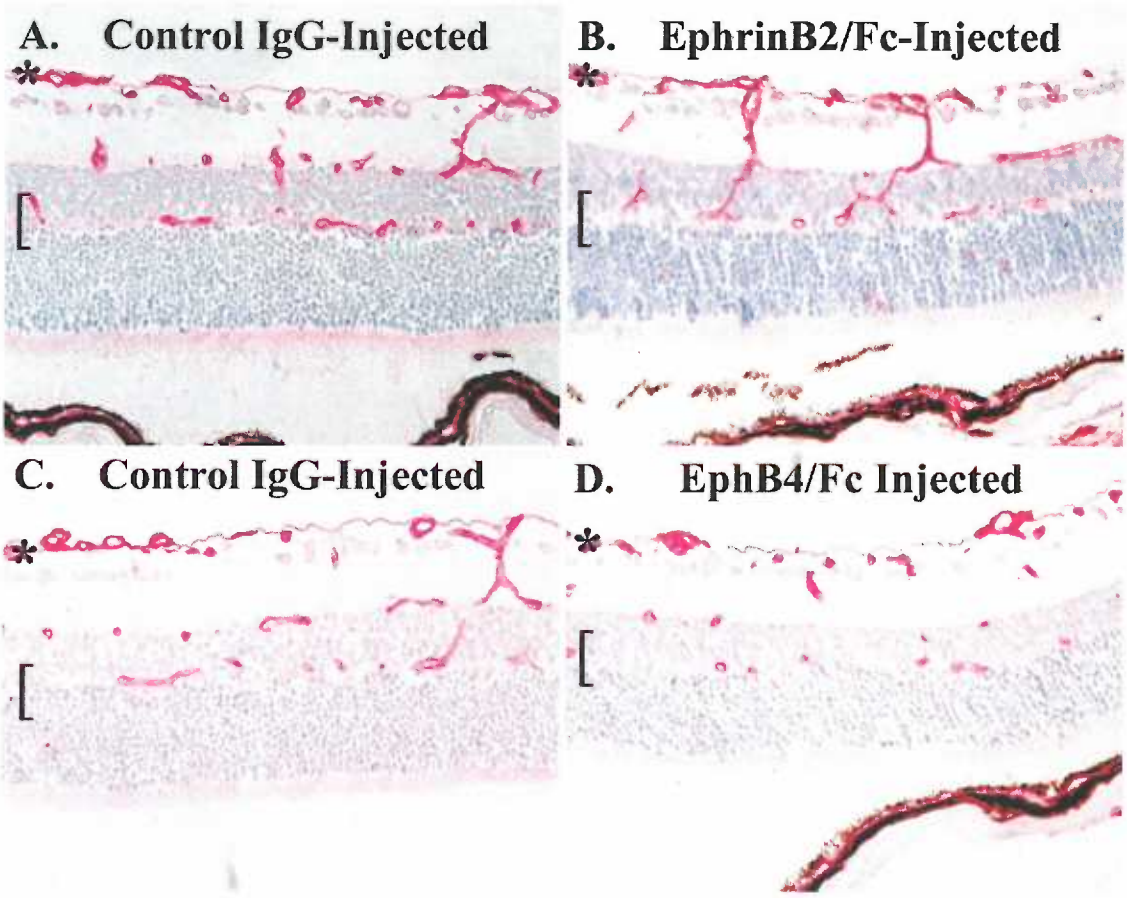
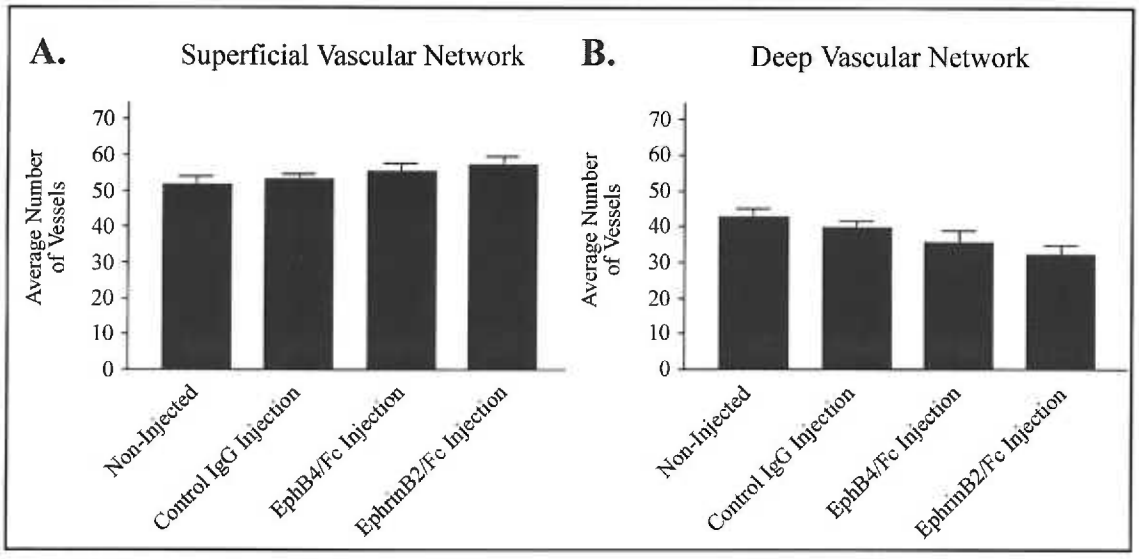

A. Control IgG-Injected
B. EphrinB2/Fc-Injected
C. Control IgG-Injected
D. EphB4/Fc Injected

Figure 6: Quantification of blood vessels in the superficial (A) and deep (B) vascular beds of the retina on P17. EphrinB2/Fc and EphB4/Fc injections did not significantly alter the amount of blood vessels in either superficial (A) or deep (B) vascular beds, compared to control IgG injections. Blood vessels in superficial and deep regions were counted and analyzed using a one-way ANOVA with a Tukey's multiple comparison test (n=12-16 mice/condition and represent approximately 5 different litters of mice).


A.
Superficial Vascular Network
Average Number of Vessels
70
60
50
40
30
20
10
0
Non-Injected
Control IgG Injection
EphB4/Fc Injection
EphrinB2/Fc Injection
B.
Deep Vascular Network
Average Number of Vessels
70
60
50
40
30
20
10
0
Non-Injected
Control IgG Injection
EphB4/Fc Injection
EphrinB2/Fc Injection

Chapter 6: Summary and Conclusions

Summary

Ocular inflammatory diseases are a leading cause of blindness. Although the understanding of the underlying molecular mechanisms has increased over the past decade, it nonetheless remains incomplete. Given the ethical and technical barriers in conducting mechanistic studies of AU in humans, a variety of investigative animal models have been developed[71]. These systems have provided some insight into the biology of ocular inflammation, but the translation of data from animal studies into humans remains difficult.

It was initially hypothesized that the basic inflammatory properties of HIEC contribute to leukocyte trafficking during anterior uveitis. I set forth to test this hypothesis by first isolating the EC of human iris tissue. A protocol was developed to reliably isolate pure HIEC, which could then be propagated in culture for study. The basic inflammatory properties of these cells were then studied. It was determined that HIEC constitutively express mRNA and protein for ICAM-1 and ICAM-2, and little to no expression of VCAM-1 or E-selectin. However, upon stimulation of HIEC with LPS or TNFα, protein and mRNA levels of ICAM-1, VCAM-1 and E-selectin were potently upregulated and were sensitive to stimulants in a time and dose manner. These data are in line with their previously suspected roles in uveitis[69,70,133-136]. In contrast, ICAM-2 protein and mRNA were slowly down regulated by inflammatory agents over time, but nonetheless remained present and functional. Furthermore, this is the first report to demonstrate the regulated expression of ICAM-2 in any ocular microvascular cell. Lastly, the functional ability of these cells to bind leukocytes was then analyzed, and it was determined that cytokine- or LPS-activated HIEC increased their adhesiveness for

leukocytes. These data suggest that the inflammatory properties of HIEC are similar in function to those of EC from other parts of the body. However, subtle differences in expression of inflammatory mediators between HIEC, choroidal EC and retinal EC remain to be determined. Furthermore, if subtle differences do exist, then it will be important to determine if these differences translate into making one tissue more or less susceptible to inflammation.

The extravasation of leukocytes is a complex process. Numerous molecular interactions occur between the leukocyte and EC that lead to intracellular signaling in both cells. Given that the Ephrin/Eph signaling system regulates the migratory properties of various cell types, it was hypothesized that they could also function in regulating leukocyte extravasation during AU. To date, little is known on the expression and function of EphrinB2 in leukocytes. To this end, human PBLs were examined for the expression and potential modulation of EphrinB2 by various inflammatory stimuli. The studies of this section revealed several novel findings on the expression of EphrinB2 by immune cells. The most significant finding was that unstimulated human PBLs do not express EphrinB2, but rapidly upregulate its expression in response to TNFα or SDF1α, both of which are potent inflammatory stimulators of PBLs. Interestingly, unstimulated PBLs also expressed EphB4, but its expression did not seem change in response to TNFα. Since cellular infiltration during AU is characterized by $CD4^+$ cells, and to some extent $CD8^+$ T cells, the ability of individual PBL subsets to express EphrinB2 was investigated. It was determined that unstimulated $CD4^+$ T cells do not express EphrinB2, but rapidly upregulate its expression after 6 hours of TNFα stimulation. Unexpectedly, unstimulated $CD8^+$ T cells and $CD19^+$ B cells expressed EphrinB2 and its expression appeared to be

down regulated upon stimulation. The ability of PMNs to express EphrinB2 was also investigated. It was determined that TNFα did upregulate PMNs expression of EphrinB2s expression after 1.5 hours of stimulation, but not at earlier or later times. Interestingly, IL-8, a potent chemoattractant for PMNs, did not induce them to express EphrinB2.

In line with the hypothesis, I next wanted to discern if human iris tissue and/or cultured HIEC express EphB4. The finding that EphB4 is expressed by some, but not all iris vessels indicates that the human iris does possess true venules according to existing molecular criteria[114,245]. Furthermore, the observation that iris tissue and HIEC constitutively express EphB4, even after stimulation, indicate that it is in position to interact with leukocyte expressed EphrinB2. This notion is further supported by the observation that the HIEC Erk1/2 signaling pathway is activated in response to EphrinB2. Indeed, the Erk1/2 signaling pathway has already been implicated in the regulation of leukocyte trafficking, and inhibition of this pathway prevents leukocyte extravasation[246]. These observations suggest that a ligand expressed by stimulated lymphocytes activates iris EC and suggest a novel mechanism by which iris EC can regulate lymphocyte trafficking during inflammation.

Lastly, the functional role of EphrinB2 and EphB4 was explored in pathological NV using a mouse model of oxygen-induced ROP. It was hypothesized that since EphrinB2 and EphB4 are potent regulators of angiogenesis, inundating the posterior region of the eye with soluble EphrinB2 or EphB4 could alter neovascularization in this system. First, it was determined that EphrinB2 was constitutively expressed in the developing retina , and that hyperoxia treatment did not alter its expression. In contrast, EphB4 expression was modulated throughout normal retinal development, and its

expression was altered in response to hyperoxia. These data are in line with current known functions of EphrinB2/EphB4 in the regulation of angiogenesis[113,114]. It was observed that soluble, chimeric versions of EphrinB2 and EphB4 reduced neovascular tuft formation in this model, without affected intraretinal vessel development. The exact mechanism of action of these soluble molecules remains unknown. However, it is hypothesized that they are blocking endogenous EphrinB2/EphB4 binding between EC-EC and/or EC-stromal cells, thus interfering with the progression of this disease. These results support the hypothesis that endogenous EphrinB2 and EphB4 are regulators of retinal NV during oxygen-induced retinopathy and may serve as novel targets for therapeutic intervention.

Conclusions

The isolation and culture of HIEC has yielded a critical, invaluable reagent in the study of ocular EC biology. Until the published report on HIEC isolation, described in chapter 3 of this dissertation, the iris endothelium remained one of the last major tissues of the human eye to have their EC isolated and studied. Since their isolation and characterization in 2001, HIEC have been used in a various studies involving, ocular EC heterogeneity, their expression of a novel chemokine called Fractalkine[123], and now in the biological implication of their expression of Ephrin/Ephs. There is much anticipation on determining the underlying mechanism on why iris vascular beds become inflamed during bouts of AU, while those of choroid and retina do not. Equally important is the possible existence of a unique vascular "address" on iris endothelium that can be utilized to target therapeutic drugs to this tissue.

The finding that human PBLs upregulate EphrinB2 in response to inflammatory stimuli is intriguing. Currently, little information exists regarding EphrinB2 expression in immune cells. The fact that Ephrin/Eph signaling modulates the migration of different cell types suggests that this system may also function in similar fashion by human leukocytes. Various experimental reagents (i.e., blocking antibodies and soluble adhesion molecules, receptor antagonists against cytokines), aimed at preventing/reducing leukocyte-EC interactions, are currently available. Similar reagents could be developed should the Ephrin/Eph system function in leukocyte extravasation.

Lastly, the finding that soluble EphrinB2 and EphB4 reduce pathological NV in the mouse model of ROP suggests that these molecules play a significant role in this disease process. Currently, the predominant clinical treatment for neovascular retinopathies is laser photocoagulation. This treatment has shown some efficacy at reducing disease, but can often lead to such adverse effects as a reduction in peripheral and night vision. Other lines of treatment have focused on VEGF and its various signaling molecules. However, the targeting of the Ephrin/Eph system may prove to be a more specific inhibitor of ocular angiogenesis. Alternatively, combination (i.e., Ephrin/Eph and VEGF systems) antiangiogenic therapies could be implemented for synergistic effects to occur.

Appendices

The following abstracts are from co-authored, peer-reviewed manuscripts, of which I contributed my critical scientific and/or technical laboratory skills.

Appendix A

Murine endotoxin-induced uveitis, but not immune complex-induced uveitis, is dependent on the IL-8 receptor homolog.

PURPOSE: To determine the roles of the murine interleukin-8 receptor homolog (mIL-8Rh, neutrophil chemokine CXC receptor 2) and macrophage inflammatory protein-1alpha (MIP-1alpha, a CC chemokine) in two eye inflammation models: endotoxin-induced uveitis (EIU) and immune complex-induced uveitis (reverse passive Arthus reaction (RPAR) uveitis).

METHODS: For the EIU model, 250 ng *E.coli* endotoxin was injected into the vitreous of mIL-8Rh-/- mice or heterozygous littermate mIL-8Rh+/- controls and into MIP-1alpha-/- mice or congenic MIP-1alpha+/+ controls. Eyes were harvested after 24 h for histologic characterization of infiltrating cells and IL-6 bioassays. For the RPAR model, mouse antiserum against human serum albumin (HSA) was injected into the vitreous of mIL-8Rh-/-, mIL-8Rh+/-, MIP-1alpha-/-, and MIP-1alpha+/+ mice. Twenty-four hours later, animals were challenged with intravenous HSA. Eyes were harvested after 4 h for analysis.

RESULTS: RPAR resulted in the deposition of immune complexes at the ciliary area and iris with the subsequent development of uveitis. Genetic deficiency of mIL-8Rh reduced the median number of infiltrating cells in EIU by 63% ($p < 0.01$) but had no effect on RPAR-induced inflammation. In the EIU model, macrophages comprised a much higher percentage (45%) of infiltrating cells in mice lacking mIL-8Rh than in controls (17%). Loss of the MIP-1alpha gene had no apparent effect on RPAR uveitis and a 39% reduction of infiltrating cells in EIU that was not statistically significant. IL-6 activity in aqueous humor was much less in mice with RPAR uveitis than in those with EIU. Neither gene deletion had a significant impact on IL-6 levels in either disease model.

CONCLUSIONS: Chemokines acting via mIL-8Rh have a significant role in the induction of neutrophil infiltration during EIU but not during RPAR uveitis. MIP-1alpha is not critical for either EIU or RPAR-induced uveitis. The differential dependence on IL-8-like chemokines is in accord with the two forms of uveitis having different etiologies and, therefore, potentially different optimal therapies.

Reference:
Brito, B.E., O'Rourke, L.M., Pan, Y., Huang, X., Park, J.M., **Zamora, D.O.** Cook, D.N., Planck, S.R., and Rosenbaum, J.T. "Murine endotoxin-induced uveitis, but not immunocomplex-induced uveitis, is dependent on the IL-8 receptor homolog." *Curr Eye Res* 1999; 19(1): 76-85.

Appendix B

Constitutive and Inflammatory Mediator-Regulated Fractalkine Expression in Human Ocular Tissues and Cultured Cells.

PURPOSE: Fractalkine (FKN) is a dual adhesion molecule-chemokine that plays a role in inflammation but has not been explored in the eye. In the current study, constitutive expression of FKN was identified in human iris and retina, and its regulation by various cytokines by endothelial cells (ECs) and stromal cells from human iris, retina, and choroid was investigated.

METHODS: Human iris and retina explants were evaluated for FKN mRNA and protein expression using RT-PCR and immunohistochemistry, respectively. Cultured ocular ECs and stromal cells were stimulated with various inflammatory mediators (endotoxin; TNFα; interferon-gamma; interleukin (IL)-1alpha, -4, -10, -13, -17, and -18; and/or CD40 ligand, or combinations thereof), with FKN mRNA being subsequently evaluated by cDNA array and/or RT-PCR and FKN protein by enzyme-linked immunoculture assay (ELICA) and/or by Western blot analysis.

RESULTS: Iris and retina explants constitutively expressed FKN protein in microvascular ECs and also in several stromal cell types. Iris and retina both express FKN mRNA. TNFα upregulated FKN in iris explants. All ocular microvascular ECs and stromal cultures expressed low FKN mRNA and/or protein levels, which were variably upregulated by endotoxin, TNFα, interferon-gamma, IL-1alpha, and/or CD40 ligand, but not by IL-18. In ECs, the Th2 cytokines IL-4 and -13, but not IL-10, reduced TNFα-induced FKN protein. IL-17, usually considered pro-inflammatory, reduced TNFα-induced FKN protein in ocular ECs.

CONCLUSIONS: FKN is expressed in various ocular tissues and cells. Inflammatory mediator modulation of ocular FKN expression suggests that this adhesive chemokine may play important roles in regulating leukocyte efflux in inflammatory eye diseases, such as anterior uveitis and retinochoroiditis.

Reference:
Silverman, M.D., **Zamora, D.O.**, Pan, Y., Texeira, P.V., Baek, S.H., Planck, S.R., and Rosenbaum, J.T. "Constitutive and Inflammatory Mediator-Regulated Fractalkine Expression in Human Ocular Tissues and Cultured Cells." *Invest Ophthalmol Vis Sci* 2003; 44(4): 1608-1615.

Appendix C

Susceptibility of Retinal Vascular Endothelium to Infection with Toxoplasma gondii Tachyzoites

PURPOSE: Retinochoroiditis infection with the protozoan parasite *Toxoplasma gondii* is the most common cause of posterior uveitis worldwide. Tachyzoites spread throughout the body through the blood stream and lymphatics, but preferentially encyst in the eye and other parts of the central nervous system (CNS). It s unknown whether *T. gondii* penetrates the CNS selectively or whether these sites of immune privilege have limited capacity to eradicate the parasite.

METHODS: Human vascular cell lines, including retinal (three lines from three different donors), aortic, umbilical vein, and dermal microvascular endothelium, as well as human foreskin fibroblasts, were grown to confluence in 24-well plates. Cells were incubated with RH-strain *T. gondii* tachyzoites in the presence of [^{3}H]-uracil. Trichloroacetic acid-insoluble radioactivity was measured as an index of *T. gondii* proliferation, because tachyzoites, but not human cells, incorporate uracil directly through pyrimidine salvage.

RESULTS: Tachyzoites showed higher [^{3}H]-uracil incorporation after incubation with retinal vascular ECs in comparison with aortic (55% more), umbilical vein (33% more) and dermal (34% more) ECs. In eight separate assays, significantly greater radioactivity was measured for tachyzoites cultured with retinal versus other cell subtypes ($P < 0.05$), except for one assay in which differences reached only borderline significance ($P \leq 0.07$). In contrast, experiments comparing different retinal endothelial lines revealed no difference between any pair. Growth of the tachyzoites was approximately 2.8-fold higher in retinal endothelium than in foreskin fibroblasts, the cell subtype often used to investigate processes of *T. gondii* infection.

CONCLUSIONS: Enhanced susceptibility of retinal vascular endothelium to infection by *T. gondii* tachyzoites may explain, at least in part, preferential localization of *T. gondii* to the retina. Susceptibility may relate to preferential binding of tachyzoites to the retinal vascular endothelial surface, relative ease of penetration into the cell, rate or replication within the cell and/or cell response to infection.

Reference:

Smith, J.R., Franc, D.T., Carter, N.S., **Zamora, D.O.**, Planck, S.R., Rosenbaum, J.T. "Susceptibility of Retinal Vascular Endothelium to Infection with Toxoplasma gondii Tachyzoites." *Invest Ophthalmol Vis Sci* 2004; 45(4): 1157-1161.

Appendix D

Toll-Like Receptor 4 and CD14 Expression in Human Ciliary Body and TLR-4 in Human Iris ECs

We investigated the expression of the functional endotoxin receptor proteins Toll-like receptor-4 and CD14 in human eyes. Toll-like receptor-4 and CD14 proteins were detected by immunohistochemical analysis of sections of whole human eyes embedded in paraffin with monoclonal antibodies against human toll-like receptor-4 (HTA-125), human CD14 (RPA-M1), or as a control, an irrelevant mouse IgG1k (MOPC-21). Incubation of explants with a neutralizing anti-toll-like receptor-4 monoclonal antibody was used to determine if lipopolysaccharide stimulation of tumor necrosis factor or interleukin-6 secretion was dependent on Toll-like receptor-4 activity. Reverse transcription-polymerase chain reaction was used to detect mRNAs for toll-like receptor-4, tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8, 3 hours after stimulation of cultured iris microvascular ECs. By immunohistochemistry, human ciliary body non-pigmented epithelial cells showed strong expression of the endotoxin receptor proteins, toll-like receptor-4 and CD14. Toll-like receptor-4 antibodies significantly inhibited lipopolysaccharide-stimulated tumor necrosis factor secretion by the ciliary body. Toll-like receptor-4 mRNA was constitutively expressed in iris ECs and slightly down-regulated by endotoxin. mRNA levels for tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8 were all increased by endotoxin treatment. This is the first report that shows intraocular (ciliary body and iris) expression of toll-like receptor-4, other than in cornea. Our results show that the ciliary body also expresses CD14, which is anatomically colocalized with toll-like receptor-4. This suggests a potential interaction between both molecules during endotoxin activation of ciliary body cells. The juxtaposition of toll-like receptor-4 and CD14 in the anterior uveal tract helps to explain the sensitivity of the iris/ciliary body to bacterial endotoxin as seen in the standard animal model of endotoxin-induced uveitis.

Reference:

Brito, B.E., **Zamora, D.O.**, Bonnah, R.A., Pan, Y., Planck, S.R., and Rosenbaum, J.T. "Toll-Like Receptor 4 and CD14 Expression in Human Ciliary Body and TLR-4 in Human Iris Endothelial Cells." *Exp Eye Res* 2004; 79: 203-8.

Appendix E

β3-Adrenergic Receptors Mediate Choroidal EC Migration and Tube Formation with No Effect on Cell Proliferation

PURPOSE: To determine the expression pattern and role of β_3-adrenergic receptor signaling in human choroidal ECs in culture

METHODS: Human choroidal ECs were stained with antibodies to determine β_3-adrenergic receptor expression using immunofluorescence. Choroidal ECs were stimulated with BRL37344, a specific β_3-adrenergic receptor agonist, and cell proliferation, migration and cord formation were measured. Cell proliferation, migration, and tube formation were also investigated in the presence of BRL37344 with pre-treatment with inhibitors to the extracellular signal-regulated kinase (ERK1/2), Src, and phosphatidylinositol-3-kinase (PI3K) cell signaling cascades.

RESULTS: β_3-adrenergic receptor expression was greater in choroidal ECs than in mesenteric ECs. Stimulation of human choroidal ECs with BRL37344 produced a 203% increase in cell migration relative to control values. Migration was inhibited by prior administration of inhibitors to Src, PI3K, and Akt. Cord formation was increased by 300% in BRL37344-treated cells, and was blocked by pre-treatment with Src and PI3K inhibitors. Proliferation was significantly increased following BRL37344, but not to the extent of migration or cord formation.

CONCLUSIONS: These results demonstrate the presence of β_3-adrenergic receptors in choroidal ECs. These receptors mediate the three primary stages of angiogenesis *in vitro* of cell proliferation, cell migration, and tube formation. Thus, modulation of β_3-adrenergic receptors may be a novel approach to address vascular growth noted in choroidal neovascularization.

Reference:

Steinle, J.J., **Zamora, D.O.**, Rosenbaum, J.T., and Granger, H.J. "β3-Adrenergic Receptors Mediate Choroidal Endothelial Cell Migration and Tube Formation with No Effect on Cell Proliferation." (Manuscript In Preparation: To Be Submitted to *Curr Eye Res*).

Bibliography

1. Thilo-Korner, D.G.S. & Heinrich, D. *Historical Development of Endothelial Cell Research*, 1-206 (Karger 1983, Paris, France, 1983).
2. Harrison, R.G. Observation on the living developing nerve fibre. *Proc. Soc. Exp. Biol., N.Y.*. 4, 140-143 (1907).
3. Carrel, A. & Burrows, M.T. Cultivation of adult tissues and organs outside the body. *JAMA* 55, 1379-1381 (1910).
4. Lewis, W.H. Smooth muscle and endothelium in tissue cultures. *Anat. Rec.* 21, 72 (1921).
5. Lewis, W.H. The outgrowth of endothelium and capillaries in tissue culture. *Johns Hopkins Med. J.* 48, 242-253 (1931).
6. Shibuya, M. On the pure culture of endothelial cells from aorta and their differentiation. *Kitasato Arch. Exp. Med.* 8, 68-88 (1931).
7. Lazzarini-Robertson, A.A. Studies of the effect of lipid emulsion on arterial intimal cells in tissue culture in relation to atherosclerosis. Ph.D. thesis, The Graduate School of Cornell University. in *Biology of Endothelial Cells* (ed. Jaffe, E.) 1-456 (Martinus Nijhoff Publishers, Boston, 1959).
8. Maruyama, Y. The human endothelial cell in tissue culture. *Z Zellforsch Mikrosk Anat* 60, 69-79 (1963).
9. Fryer, D.G., Birnbaum, G. & Luttrell, C.N. Human endothelium in cell culture. *J Atheroscler Res* 6, 151-63 (1966).
10. Jaffe, E., Nachman, R.L. & Becker, C.G. Culture of human endothelial cells derived from umbilical cord veins. *J. Clin. Invest.* 51, 46A (1972).
11. Jaffe, E., Nachman, R.L., Becker, C.G. & Minick, C.R. Culture of human endothelial cells derived from human umbilical cord veins. *Circulation* 46 (Suppl. II), 252 (1972).
12. Jaffe, E.A., Nachman, R.L., Becker, C.G. & Minick, C.R. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 52, 2745-56 (1973).
13. Sakamoto, T. et al. In vitro studies of human choroidal endothelial cells. *Curr Eye Res* 14, 621-7 (1995).
14. Grant, M.B. et al. Matrix metalloproteinase expression in human retinal microvascular cells. *Diabetes* 47, 1311-7 (1998).
15. Silverman, M.D. et al. Cell adhesion molecule expression in cultured human iris endothelial cells. *Invest Ophthalmol Vis Sci* 42, 2861-6 (2001).
16. Jaffe, E.A. Physiologic functions of normal endothelial cells. *Ann N Y Acad Sci* 454, 279-91 (1985).
17. Boeynaems, J.-M. & Pirotton, S. Multiplicity of Endothelial Cell Functions and Regulatory Mediators. in *Regulation of the vascular endothelium: signals and transduction mechanisms (Molecular Biology Intelligence Unit)* (ed. Kerkaporta, C.) 1-117 (R.G. Landes Company, Austin, 1994).
18. Garlanda, C. & Dejana, E. Heterogeneity of endothelial cells. Specific markers. *Arterioscler Thromb Vasc Biol* 17, 1193-202 (1997).
19. Thorin, E. & Shreeve, S.M. Heterogeneity of vascular endothelial cells in normal and disease states. *Pharmacol Ther* 78, 155-66 (1998).

20. Pasqualini, R., Arap, W. & McDonald, D.M. Probing the structural and molecular diversity of tumor vasculature. *Trends Mol Med* 8, 563-71 (2002).
21. Suter, E.R. & Majno, G. Passage of lipid across vascular endothelium in newborn rats. An electron microscopic study. *J Cell Biol* 27, 163-77 (1965).
22. Abbott, N.J. Astrocyte-endothelial interactions and blood-brain barrier permeability. *J Anat* 200, 629-38 (2002).
23. Kmiec, Z. Cooperation of liver cells in health and disease. *Adv Anat Embryol Cell Biol* 161, III-XIII, 1-151 (2001).
24. Auerbach, R. Vascular endothelial cell differentiation: organ-specificity and selective affinities as the basis for developing anti-cancer strategies. *Int J Radiat Biol* 60, 1-10 (1991).
25. Rajotte, D. & Ruoslahti, E. Membrane dipeptidase is the receptor for a lung-targeting peptide identified by in vivo phage display. *J Biol Chem* 274, 11593-8 (1999).
26. Essler, M. & Ruoslahti, E. Molecular specialization of breast vasculature: a breast-homing phage-displayed peptide binds to aminopeptidase P in breast vasculature. *Proc Natl Acad Sci U S A* 99, 2252-7 (2002).
27. Wang, H.U., Chen, Z.F. & Anderson, D.J. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* 93, 741-53 (1998).
28. Ryan, G.B. & Majno, G. Historic Highlights. in *Inflammation* (ed. Thomas, B.A.) 6-23 (The Upjohn Company, Kalamazoo, 1977).
29. Springer, T.A. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76, 301-14 (1994).
30. von Andrian, U.H. & Mackay, C.R. T-cell function and migration. Two sides of the same coin. *N Engl J Med* 343, 1020-34 (2000).
31. Ley, K. Integration of inflammatory signals by rolling neutrophils. *Immunol Rev* 186, 8-18 (2002).
32. Alon, R., Chen, S., Fuhlbrigge, R., Puri, K.D. & Springer, T.A. The kinetics and shear threshold of transient and rolling interactions of L-selectin with its ligand on leukocytes. *Proc Natl Acad Sci U S A* 95, 11631-6 (1998).
33. Lawrence, M.B. & Springer, T.A. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* 65, 859-73 (1991).
34. Lindbom, L., Xie, X., Raud, J. & Hedqvist, P. Chemoattractant-induced firm adhesion of leukocytes to vascular endothelium in vivo is critically dependent on initial leukocyte rolling. *Acta Physiol Scand* 146, 415-21 (1992).
35. Carlos, T.M. & Harlan, J.M. Leukocyte-endothelial adhesion molecules. *Blood* 84, 2068-101 (1994).
36. Abbassi, O., Kishimoto, T.K., McIntire, L.V., Anderson, D.C. & Smith, C.W. E-selectin supports neutrophil rolling in vitro under conditions of flow. *J Clin Invest* 92, 2719-30 (1993).
37. Ley, K. & Gaehtgens, P. Endothelial, not hemodynamic, differences are responsible for preferential leukocyte rolling in rat mesenteric venules. *Circ Res* 69, 1034-41 (1991).

38. von Andrian, U.H. et al. Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins in vivo. *Proc Natl Acad Sci U S A* 88, 7538-42 (1991).
39. Dore, M., Korthuis, R.J., Granger, D.N., Entman, M.L. & Smith, C.W. P-selectin mediates spontaneous leukocyte rolling in vivo. *Blood* 82, 1308-16 (1993).
40. Mayadas, T.N., Johnson, R.C., Rayburn, H., Hynes, R.O. & Wagner, D.D. Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. *Cell* 74, 541-54 (1993).
41. Olofsson, A.M. et al. E-selectin mediates leukocyte rolling in interleukin-1-treated rabbit mesentery venules. *Blood* 84, 2749-58 (1994).
42. Ley, K. et al. Sequential contribution of L- and P-selectin to leukocyte rolling in vivo. *J Exp Med* 181, 669-75 (1995).
43. Ley, K., Tedder, T.F. & Kansas, G.S. L-selectin can mediate leukocyte rolling in untreated mesenteric venules in vivo independent of E- or P-selectin. *Blood* 82, 1632-8 (1993).
44. Kunkel, E.J. & Ley, K. Distinct phenotype of E-selectin-deficient mice. E-selectin is required for slow leukocyte rolling in vivo. *Circ Res* 79, 1196-204 (1996).
45. Alon, R. & Feigelson, S. From rolling to arrest on blood vessels: leukocyte tap dancing on endothelial integrin ligands and chemokines at sub-second contacts. *Semin Immunol* 14, 93-104 (2002).
46. Constantin, G. et al. Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* 13, 759-69 (2000).
47. Weber, C., Kitayama, J. & Springer, T.A. Differential regulation of beta 1 and beta 2 integrin avidity by chemoattractants in eosinophils. *Proc Natl Acad Sci U S A* 93, 10939-44 (1996).
48. Mackay, C.R. Chemokines: immunology's high impact factors. *Nat Immunol* 2, 95-101 (2001).
49. Luster, A.D. Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med* 338, 436-45 (1998).
50. Miller, M.D. & Krangel, M.S. Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. *Crit Rev Immunol* 12, 17-46 (1992).
51. Johnston, B. & Butcher, E.C. Chemokines in rapid leukocyte adhesion triggering and migration. *Semin Immunol* 14, 83-92 (2002).
52. Tanaka, Y., Adams, D.H. & Shaw, S. Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol Today* 14, 111-5 (1993).
53. Middleton, J. et al. Transcytosis and surface presentation of IL-8 by venular endothelial cells. *Cell* 91, 385-95 (1997).
54. van Hinsbergh, V.W. & van Nieuw Amerongen, G.P. Intracellular signalling involved in modulating human endothelial barrier function. *J Anat* 200, 549-60 (2002).

55. Goeckeler, Z.M. & Wysolmerski, R.B. Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. *J Cell Biol* 130, 613-27 (1995).
56. Moy, A.B., Shasby, S.S., Scott, B.D. & Shasby, D.M. The effect of histamine and cyclic adenosine monophosphate on myosin light chain phosphorylation in human umbilical vein endothelial cells. *J Clin Invest* 92, 1198-206 (1993).
57. Moy, A.B. et al. Histamine and thrombin modulate endothelial focal adhesion through centripetal and centrifugal forces. *J Clin Invest* 97, 1020-7 (1996).
58. Sheldon, R., Moy, A., Lindsley, K., Shasby, S. & Shasby, D.M. Role of myosin light-chain phosphorylation in endothelial cell retraction. *Am J Physiol* 265, L606-12 (1993).
59. Dejana, E. Endothelial cell-cell junctions: happy together. *Nat Rev Mol Cell Biol* 5, 261-70 (2004).
60. Vaday, G.G. et al. Combinatorial signals by inflammatory cytokines and chemokines mediate leukocyte interactions with extracellular matrix. *J Leukoc Biol* 69, 885-92 (2001).
61. Marchesi, V.T. Some Electron Microscopic Observations on Interactions between Leukocytes, Platelets, and Endothelial Cells in Acute Inflammation. *Ann N Y Acad Sci* 116, 774-88 (1964).
62. Marchesi, V.T. & Gowans, J.L. The Migration of Lymphocytes through the Endothelium of Venules in Lymph Nodes: An Electron Microscope Study. *Proc R Soc Lond B Biol Sci* 159, 283-90 (1964).
63. Feng, D., Nagy, J.A., Pyne, K., Dvorak, H.F. & Dvorak, A.M. Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. *J Exp Med* 187, 903-15 (1998).
64. Liu, L. & Kubes, P. Molecular mechanisms of leukocyte recruitment: organ-specific mechanisms of action. *Thromb Haemost* 89, 213-20 (2003).
65. Corbacho, A.M. et al. Tissue-specific gene expression of prolactin receptor in the acute phase response induced by lipopolysaccharides. *Am J Physiol Endocrinol Metab*, 00522.2003 (2004).
66. Rosenbaum, J.T., Martin, T.M. & Planck, S.R. Anterior uveitis: clinical and research perspectives. *Springer Semin Immunopathol* 21, 135-45 (1999).
67. Rosenbaum, J.T., McDevitt, H.O., Guss, R.B. & Egbert, P.R. Endotoxin-induced uveitis in rats as a model for human disease. *Nature* 286, 611-3 (1980).
68. Stevens, G., Jr., Chan, C.C., Wetzig, R.P., Nussenblatt, R.B. & Palestine, A.G. Iris lymphocytic infiltration in patients with clinically quiescent uveitis. *Am J Ophthalmol* 104, 508-15 (1987).
69. La Heij, E. et al. Adhesion molecules in iris biopsy specimens from patients with uveitis. *Br J Ophthalmol* 82, 432-7 (1998).
70. Wakefield, D., McCluskey, P. & Palladinetti, P. Distribution of lymphocytes and cell adhesion molecules in iris biopsy specimens from patients with uveitis. *Arch Ophthalmol* 110, 121-5 (1992).

71. Smith, J.R., Hart, P.H. & Williams, K.A. Basic pathogenic mechanisms operating in experimental models of acute anterior uveitis. *Immunol Cell Biol* 76, 497-512 (1998).
72. Carmeliet, P. Angiogenesis in health and disease. *Nat Med* 9, 653-60 (2003).
73. Weinstein, B.M. What guides early embryonic blood vessel formation? *Dev Dyn* 215, 2-11 (1999).
74. Carmeliet, P. & Collen, D. Role of vascular endothelial growth factor and vascular endothelial growth factor receptors in vascular development. *Curr Top Microbiol Immunol* 237, 133-58 (1999).
75. Flamme, I. & Risau, W. Induction of vasculogenesis and hematopoiesis in vitro. *Development* 116, 435-9 (1992).
76. Risau, W. Mechanisms of angiogenesis. *Nature* 386, 671-4 (1997).
77. Yoder, M.C. Blood cell progenitors: insights into the properties of stem cells. *Anat Rec* 276A, 66-74 (2004).
78. Risau, W. & Flamme, I. Vasculogenesis. *Annu Rev Cell Dev Biol* 11, 73-91 (1995).
79. Cormier, F., de Paz, P. & Dieterlen-Lievre, F. In vitro detection of cells with monocytic potentiality in the wall of the chick embryo aorta. *Dev Biol* 118, 167-75 (1986).
80. Olah, I., Medgyes, J. & Glick, B. Origin of aortic cell clusters in the chicken embryo. *Anat Rec* 222, 60-8 (1988).
81. Risau, W. Differentiation of endothelium. *Faseb J* 9, 926-33 (1995).
82. Asahara, T. et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275, 964-7 (1997).
83. Folkman, J. & D'Amore, P.A. Blood vessel formation: what is its molecular basis? *Cell* 87, 1153-5 (1996).
84. Sottile, J. Regulation of angiogenesis by extracellular matrix. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 1654, 13-22 (2004).
85. Bicknell, R. *Mechanistic insights into tumour angiogenesis.*, 1-381 (Oxford University Press, Oxford, 1997).
86. Patan, S., Haenni, B. & Burri, P.H. Implementation of intussusceptive microvascular growth in the chicken chorioallantoic membrane (CAM): 1. pillar formation by folding of the capillary wall. *Microvasc Res* 51, 80-98 (1996).
87. Lindahl, P. et al. Paracrine PDGF-B/PDGF-Rbeta signaling controls mesangial cell development in kidney glomeruli. *Development* 125, 3313-22 (1998).
88. Carmeliet, P. et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380, 435-9 (1996).
89. Ferrara, N. et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380, 439-42 (1996).
90. Dumont, D.J. et al. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* 282, 946-9 (1998).
91. Leung, D.W., Cachianes, G., Kuang, W.J., Goeddel, D.V. & Ferrara, N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246, 1306-9 (1989).

92. Tischer, E. et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 266, 11947-54 (1991).
93. Dickson, M.C. et al. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* 121, 1845-54 (1995).
94. Oshima, M., Oshima, H. & Taketo, M.M. TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. *Dev Biol* 179, 297-302 (1996).
95. Leavesley, D.I., Schwartz, M.A., Rosenfeld, M. & Cheresh, D.A. Integrin beta 1- and beta 3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. *J Cell Biol* 121, 163-70 (1993).
96. Brooks, P.C., Clark, R.A. & Cheresh, D.A. Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science* 264, 569-71 (1994).
97. Brooks, P.C. et al. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* 79, 1157-64 (1994).
98. Soldi, R. et al. Role of alphavbeta3 integrin in the activation of vascular endothelial growth factor receptor-2. *Embo J* 18, 882-92 (1999).
99. Brooks, P.C. et al. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* 85, 683-93 (1996).
100. Vittet, D., Buchou, T., Schweitzer, A., Dejana, E. & Huber, P. Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. *Proc Natl Acad Sci U S A* 94, 6273-8 (1997).
101. Carmeliet, P. et al. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* 98, 147-57 (1999).
102. Phelps, D.L. Retinopathy of prematurity. *Pediatr Rev* 16, 50-6 (1995).
103. Penn, J.S., Rajaratnam, V.S., Collier, R.J. & Clark, A.F. The effect of an angiostatic steroid on neovascularization in a rat model of retinopathy of prematurity. *Invest Ophthalmol Vis Sci* 42, 283-90 (2001).
104. Phelps, D.L. Retinopathy of prematurity. *Pediatr Clin North Am* 40, 705-14 (1993).
105. Watanabe, T. & Raff, M.C. Retinal astrocytes are immigrants from the optic nerve. *Nature* 332, 834-7 (1988).
106. Stone, J. et al. Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. *J Neurosci* 15, 4738-47 (1995).
107. Lee, P., Wang, C.C. & Adamis, A.P. Ocular neovascularization: an epidemiologic review. *Surv Ophthalmol* 43, 245-69 (1998).
108. Madan, A. & Penn, J.S. Animal models of oxygen-induced retinopathy. *Front Biosci* 8, d1030-43 (2003).
109. Smith, L.E. et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci* 35, 101-11 (1994).

110. Dodelet, V.C. & Pasquale, E.B. Eph receptors and ephrin ligands: embryogenesis to tumorigenesis. *Oncogene* 19, 5614-9 (2000).
111. Nakamoto, M. Eph receptors and ephrins. *Int J Biochem Cell Biol* 32, 7-12 (2000).
112. Kullander, K. & Klein, R. Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol* 3, 475-86 (2002).
113. Adams, R.H. et al. Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev* 13, 295-306 (1999).
114. Gerety, S.S., Wang, H.U., Chen, Z.F. & Anderson, D.J. Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol Cell* 4, 403-14 (1999).
115. Gale, N.W. et al. Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. *Dev Biol* 230, 151-60 (2001).
116. Kim, I. et al. EphB ligand, ephrinB2, suppresses the VEGF- and angiopoietin 1-induced Ras/mitogen-activated protein kinase pathway in venous endothelial cells. *FASEB J* 16, 1126-8 (2002).
117. Hoyer, L.W., De los Santos, R.P. & Hoyer, J.R. Antihemophilic factor antigen. Localization in endothelial cells by immunofluorescent microscopy. *J Clin Invest* 52, 2737-44 (1973).
118. Voyta, J.C., Via, D.P., Butterfield, C.E. & Zetter, B.R. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J Cell Biol* 99, 2034-40 (1984).
119. Folkman, J. & Haudenschild, C. Angiogenesis in vitro. *Nature* 288, 551-6 (1980).
120. Kinsella, J.L., Grant, D.S., Weeks, B.S. & Kleinman, H.K. Protein kinase C regulates endothelial cell tube formation on basement membrane matrix, Matrigel. *Exp Cell Res* 199, 56-62 (1992).
121. Planck, S.R. et al. The effect of genetic deficiency of adhesion molecules on the course of endotoxin-induced uveitis. *Curr Eye Res* 17, 941-6 (1998).
122. Kanda, K., Hayman, G.T., Silverman, M.D. & Lelkes, P.I. Comparison of ICAM-1 and VCAM-1 expression in various human endothelial cell types and smooth muscle cells. *Endothelium* 6, 33-44 (1998).
123. Silverman, M.D. et al. Constitutive and inflammatory mediator-regulated fractalkine expression in human ocular tissues and cultured cells. *Invest Ophthalmol Vis Sci* 44, 1608-15 (2003).
124. Becker, M.D. et al. Immunohistology of antigen-presenting cells in vivo: a novel method for serial observation of fluorescently labeled cells. *Invest Ophthalmol Vis Sci* 44, 2004-9 (2003).
125. Careless, D.J. & Inman, R.D. Acute anterior uveitis: clinical and experimental aspects. *Semin Arthritis Rheum* 24, 432-41 (1995).
126. Fabbri, M., Bianchi, E., Fumagalli, L. & Pardi, R. Regulation of lymphocyte traffic by adhesion molecules. *Inflamm Res* 48, 239-46 (1999).

127. Lehmann, I., Brylla, E., Sittig, D., Spanel-Borowski, K. & Aust, G. Microvascular endothelial cells differ in their basal and tumour necrosis factor-alpha-regulated expression of adhesion molecules and cytokines. *J Vasc Res* 37, 408-16 (2000).
128. Craig, L.E., Spelman, J.P., Strandberg, J.D. & Zink, M.C. Endothelial cells from diverse tissues exhibit differences in growth and morphology. *Microvasc Res* 55, 65-76 (1998).
129. Chung-Welch, N., Patton, W.F., Yen-Patton, G.P., Hechtman, H.B. & Shepro, D. Phenotypic comparison between mesothelial and microvascular endothelial cell lineages using conventional endothelial cell markers, cytoskeletal protein markers and in vitro assays of angiogenic potential. *Differentiation* 42, 44-53 (1989).
130. Ritterband, D.C. & Friedberg, D.N. Virus infections of the eye. *Rev Med Virol* 8, 187-201 (1998).
131. Brown, S.M., Jampol, L.M. & Cantrill, H.L. Intraocular lymphoma presenting as retinal vasculitis. *Surv Ophthalmol* 39, 133-40 (1994).
132. Silverman, M.D. et al. Evaluating tissue-specific expression of inflammatory mediators in cultured human iris, choroid, and retinal endothelial cells: Utility of DNA array technology as a screening tool. *Invest Ophthalmol Vis Sci* 41, S570 ARVO Abstract 3030. (2000).
133. Kanagawa, T. et al. Role of ICAM-1 and LFA-1 in endotoxin-induced uveitis in mice. *Jpn J Ophthalmol* 40, 174-80 (1996).
134. Whitcup, S.M. et al. Monoclonal antibodies against CD54 (ICAM-1) and CD11a (LFA-1) prevent and inhibit endotoxin-induced uveitis. *Exp Eye Res* 60, 597-601 (1995).
135. Martin, C.M. et al. Levels of soluble ICAM-1 and soluble IL-2R in the serum and aqueous humor of uveitis patients. *Curr Eye Res* 20, 287-92 (2000).
136. Suzuma, I. et al. Contribution of E-selectin to cellular infiltration during endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci* 39, 1620-30 (1998).
137. McLaughlin, F., Ludbrook, V.J., Kola, I., Campbell, C.J. & Randi, A.M. Characterisation of the tumour necrosis factor (TNF)-(alpha) response elements in the human ICAM-2 promoter. *J Cell Sci* 112 (Pt 24), 4695-703 (1999).
138. Reiss, Y., Hoch, G., Deutsch, U. & Engelhardt, B. T cell interaction with ICAM-1-deficient endothelium in vitro: essential role for ICAM-1 and ICAM-2 in transendothelial migration of T cells. *Eur J Immunol* 28, 3086-99 (1998).
139. Issekutz, A.C., Rowter, D. & Springer, T.A. Role of ICAM-1 and ICAM-2 and alternate CD11/CD18 ligands in neutrophil transendothelial migration. *J Leukoc Biol* 65, 117-26 (1999).
140. Kotovuori, A., Pessa-Morikawa, T., Kotovuori, P., Nortamo, P. & Gahmberg, C.G. ICAM-2 and a peptide from its binding domain are efficient activators of leukocyte adhesion and integrin affinity. *J Immunol* 162, 6613-20 (1999).

141. Klok, A.M., Luyendijk, L., Zaal, M.J., Rothova, A. & Kijlstra, A. Soluble ICAM-1 serum levels in patients with intermediate uveitis. *Br J Ophthalmol* 83, 847-51 (1999).
142. Barile, G.R. et al. Soluble cellular adhesion molecules in proliferative vitreoretinopathy and proliferative diabetic retinopathy. *Curr Eye Res* 19, 219-27 (1999).
143. Muller, W.A. Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol* 24, 327-34 (2003).
144. Von Andrian, U.H. et al. L-selectin function is required for beta 2-integrin-mediated neutrophil adhesion at physiological shear rates in vivo. *Am J Physiol Heart Circ Physiol* 263, H1034-1044 (1992).
145. Oliani, S.M., Paul-Clark, M.J., Christian, H.C., Flower, R.J. & Perretti, M. Neutrophil interaction with inflamed postcapillary venule endothelium alters annexin 1 expression. *Am J Pathol* 158, 603-15 (2001).
146. Hatanaka, K. & Katori, M. Detachment of polymorphonuclear leukocytes adhered on venular endothelial cells. *Microcirculation Ann* 8, 129-130 (1992).
147. Mancuso, F., Flower, R.J. & Perretti, M. Leukocyte transmigration, but not rolling or adhesion, is selectively inhibited by dexamethasone in the hamster post-capillary venule. Involvement of endogenous lipocortin 1. *J Immunol* 155, 377-86 (1995).
148. Lim, L.H.K., Solito, E., Russo-Marie, F., Flower, R.J. & Perretti, M. Promoting detachment of neutrophils adherent to murine postcapillary venules to control inflammation: Effect of lipocortin 1. *PNAS* 95, 14535-14539 (1998).
149. Laudanna, C., Kim, J.Y., Constantin, G. & Butcher, E. Rapid leukocyte integrin activation by chemokines. *Immunol Rev* 186, 37-46 (2002).
150. Wang, J. & Springer, T.A. Structural specializations of immunoglobulin superfamily members for adhesion to integrins and viruses. *Immunol Rev* 163, 197-215 (1998).
151. Dejana, E., Breviario, F. & Caveda, L. Leukocyte-endothelial cell adhesive receptors. *Clin Exp Rheumatol* 12 Suppl 10, S25-8 (1994).
152. Holland, S.J. et al. Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands. *Nature* 383, 722-5 (1996).
153. Murai, K.K. & Pasquale, E.B. 'Eph'ective signaling: forward, reverse and crosstalk. *J Cell Sci* 116, 2823-32 (2003).
154. Davy, A. & Robbins, S.M. Ephrin-A5 modulates cell adhesion and morphology in an integrin-dependent manner. *Embo J* 19, 5396-405 (2000).
155. Haddad, E. et al. The interaction between Cdc42 and WASP is required for SDF-1-induced T-lymphocyte chemotaxis. *Blood* 97, 33-8 (2001).
156. Kaibuchi, K., Kuroda, S. & Amano, M. Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annu Rev Biochem* 68, 459-86 (1999).
157. Wahl, S., Barth, H., Ciossek, T., Aktories, K. & Mueller, B.K. Ephrin-A5 induces collapse of growth cones by activating Rho and Rho kinase. *J Cell Biol* 149, 263-70 (2000).

158. Sharfe, N., Freywald, A., Toro, A., Dadi, H. & Roifman, C. Ephrin stimulation modulates T cell chemotaxis. *Eur J Immunol* 32, 3745-55 (2002).
159. Flanagan, J.G. & Vanderhaeghen, P. The ephrins and Eph receptors in neural development. *Annu Rev Neurosci* 21, 309-45 (1998).
160. Steinle, J.J., Meininger, C.J., Chowdhury, U., Wu, G. & Granger, H.J. Role of ephrin B2 in human retinal endothelial cell proliferation and migration. *Cell Signal* 15, 1011-7 (2003).
161. Fuller, T., Korff, T., Kilian, A., Dandekar, G. & Augustin, H.G. Forward EphB4 signaling in endothelial cells controls cellular repulsion and segregation from ephrinB2 positive cells. *J Cell Sci* 116, 2461-70 (2003).
162. Hamada, K. et al. Distinct roles of ephrin-B2 forward and EphB4 reverse signaling in endothelial cells. *Arterioscler Thromb Vasc Biol* 23, 190-7 (2003).
163. Maekawa, H. et al. Ephrin-B2 induces migration of endothelial cells through the phosphatidylinositol-3 kinase pathway and promotes angiogenesis in adult vasculature. *Arterioscler Thromb Vasc Biol* 23, 2008-14 (2003).
164. Oike, Y. et al. Regulation of vasculogenesis and angiogenesis by EphB/ephrin-B2 signaling between endothelial cells and surrounding mesenchymal cells. *Blood* 100, 1326-33 (2002).
165. Helbling, P.M., Saulnier, D.M. & Brandli, A.W. The receptor tyrosine kinase EphB4 and ephrin-B ligands restrict angiogenic growth of embryonic veins in Xenopus laevis. *Development* 127, 269-78 (2000).
166. Zhang, X.Q. et al. Stromal cells expressing ephrin-B2 promote the growth and sprouting of ephrin-B2(+) endothelial cells. *Blood* 98, 1028-37 (2001).
167. Steinle, J.J. et al. Eph B4 receptor signaling mediates endothelial cell migration and proliferation via the phosphatidylinositol 3-kinase pathway. *J Biol Chem* 277, 43830-5 (2002).
168. Choi, K. Hemangioblast development and regulation. *Biochem Cell Biol* 76, 947-56 (1998).
169. Suda, T., Takakura, N. & Oike, Y. Hematopoiesis and angiogenesis. *Int J Hematol* 71, 99-107 (2000).
170. Wang, Z. et al. Receptor tyrosine kinase, EphB4 (HTK), accelerates differentiation of select human hematopoietic cells. *Blood* 99, 2740-7 (2002).
171. Yu, G., Luo, H., Wu, Y. & Wu, J. Mouse ephrinB3 augments T-cell signaling and responses to T-cell receptor ligation. *J Biol Chem* 278, 47209-16 (2003).
172. de Saint-Vis, B. et al. Human dendritic cells express neuronal Eph receptor tyrosine kinases: role of EphA2 in regulating adhesion to fibronectin. *Blood* 102, 4431-40 (2003).
173. Wohlfahrt, J.G. et al. Ephrin-A1 suppresses Th2 cell activation and provides a regulatory link to lung epithelial cells. *J Immunol* 172, 843-50 (2004).
174. Yu, G., Luo, H., Wu, Y. & Wu, J. Ephrin B2 induces T cell costimulation. *J Immunol* 171, 106-14 (2003).
175. Andres, A.C. et al. Expression of two novel eph-related receptor protein tyrosine kinases in mammary gland development and carcinogenesis. *Oncogene* 9, 1461-7 (1994).

176. Ciossek, T., Lerch, M.M. & Ullrich, A. Cloning, characterization, and differential expression of MDK2 and MDK5, two novel receptor tyrosine kinases of the eck/eph family. *Oncogene* 11, 2085-95 (1995).
177. Lickliter, J.D., Smith, F.M., Olsson, J.E., Mackwell, K.L. & Boyd, A.W. Embryonic stem cells express multiple Eph-subfamily receptor tyrosine kinases. *Proc Natl Acad Sci U S A* 93, 145-50 (1996).
178. Gurniak, C.B. & Berg, L.J. A new member of the Eph family of receptors that lacks protein tyrosine kinase activity. *Oncogene* 13, 777-86 (1996).
179. Munoz, J.J. et al. Expression and function of the Eph A receptors and their ligands ephrins A in the rat thymus. *J Immunol* 169, 177-84 (2002).
180. Fox, G.M. et al. cDNA cloning and tissue distribution of five human EPH-like receptor protein-tyrosine kinases. *Oncogene* 10, 897-905 (1995).
181. Luo, H., Yu, G., Wu, Y. & Wu, J. EphB6 crosslinking results in costimulation of T cells. *J Clin Invest* 110, 1141-50 (2002).
182. Shimoyama, M. et al. Developmental expression of EphB6 in the thymus: lessons from EphB6 knockout mice. *Biochem Biophys Res Commun* 298, 87-94 (2002).
183. Shimoyama, M. et al. T-cell-specific expression of kinase-defective Eph-family receptor protein, EphB6 in normal as well as transformed hematopoietic cells. *Growth Factors* 18, 63-78 (2000).
184. Aasheim, H.C., Terstappen, L.W. & Logtenberg, T. Regulated expression of the Eph-related receptor tyrosine kinase Hek11 in early human B lymphopoiesis. *Blood* 90, 3613-22 (1997).
185. Rissoan, M.C. et al. Subtractive hybridization reveals the expression of immunoglobulin-like transcript 7, Eph-B1, granzyme B, and 3 novel transcripts in human plasmacytoid dendritic cells. *Blood* 100, 3295-303 (2002).
186. Wicks, I.P., Wilkinson, D., Salvaris, E. & Boyd, A.W. Molecular cloning of HEK, the gene encoding a receptor tyrosine kinase expressed by human lymphoid tumor cell lines. *Proc Natl Acad Sci U S A* 89, 1611-5 (1992).
187. Steube, K.G., Meyer, C., Habig, S., Uphoff, C.C. & Drexler, H.G. Expression of receptor tyrosine kinase HTK (hepatoma transmembrane kinase) and HTK ligand by human leukemia-lymphoma cell lines. *Leuk Lymphoma* 33, 371-6 (1999).
188. Luo, H., Wan, X., Wu, Y. & Wu, J. Cross-linking of EphB6 resulting in signal transduction and apoptosis in Jurkat cells. *J Immunol* 167, 1362-70 (2001).
189. Shao, H., Pandey, A., O'Shea, K.S., Seldin, M. & Dixit, V.M. Characterization of B61, the ligand for the Eck receptor protein-tyrosine kinase. *J Biol Chem* 270, 5636-41 (1995).
190. Davis, S. et al. Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. *Science* 266, 816-9 (1994).
191. Freywald, A., Sharfe, N., Rashotte, C., Grunberger, T. & Roifman, C.M. The EphB6 receptor inhibits JNK activation in T lymphocytes and modulates T cell receptor-mediated responses. *J Biol Chem* 278, 10150-6 (2003).

192. Cheng, N. & Chen, J. Tumor necrosis factor-alpha induction of endothelial ephrin A1 expression is mediated by a p38 MAPK- and SAPK/JNK-dependent but nuclear factor-kappa B-independent mechanism. *J Biol Chem* 276, 13771-7 (2001).
193. Vergara-Silva, A., Schaefer, K.L. & Berg, L.J. Compartmentalized Eph receptor and ephrin expression in the thymus. *Gene Expr Patterns* 2, 261-5 (2002).
194. Chiari, R. et al. Identification of a tumor-specific shared antigen derived from an Eph receptor and presented to CD4 T cells on HLA class II molecules. *Cancer Res* 60, 4855-63 (2000).
195. Bennett, B.D. et al. Molecular cloning of a ligand for the EPH-related receptor protein-tyrosine kinase Htk. *Proc Natl Acad Sci U S A* 92, 1866-70 (1995).
196. Inada, T. et al. Selective expression of the receptor tyrosine kinase, HTK, on human erythroid progenitor cells. *Blood* 89, 2757-65 (1997).
197. Suenobu, S. et al. A role of EphB4 receptor and its ligand, ephrin-B2, in erythropoiesis. *Biochem Biophys Res Commun* 293, 1124-31 (2002).
198. Bennett, B.D. et al. Cloning and characterization of HTK, a novel transmembrane tyrosine kinase of the EPH subfamily. *J Biol Chem* 269, 14211-8 (1994).
199. Tozeren, A. & Ley, K. How do selectins mediate leukocyte rolling in venules? *Biophys J* 63, 700-9 (1992).
200. Thurston, G., Baluk, P. & McDonald, D.M. Determinants of endothelial cell phenotype in venules. *Microcirculation* 7, 67-80 (2000).
201. Cope, A. Studies of T-cell activation in chronic inflammation. *Arthritis Res* 4, S197 - S211 (2002).
202. Kollias, G. & Kontoyiannis, D. Role of TNF/TNFR in autoimmunity: specific TNF receptor blockade may be advantageous to anti-TNF treatments. *Cytokine Growth Factor Rev* 13, 315-21 (2002).
203. Yokota, S., Geppert, T.D. & Lipsky, P.E. Enhancement of antigen- and mitogen-induced human T lymphocyte proliferation by tumor necrosis factor-alpha. *J Immunol* 140, 531-6 (1988).
204. Choy, E.H.S. & Panayi, G.S. Cytokine Pathways and Joint Inflammation in Rheumatoid Arthritis. *N Engl J Med* 344, 907-916 (2001).
205. Mo, J.S., Matsukawa, A., Ohkawara, S. & Yoshinaga, M. Involvement of TNF alpha, IL-1 beta and IL-1 receptor antagonist in LPS-induced rabbit uveitis. *Exp Eye Res* 66, 547-57 (1998).
206. Planck, S.R., Huang, X.N., Robertson, J.E. & Rosenbaum, J.T. Cytokine mRNA levels in rat ocular tissues after systemic endotoxin treatment. *Invest Ophthalmol Vis Sci* 35, 924-30 (1994).
207. Djeu, J.Y., Matsushima, K., Oppenheim, J.J., Shiotsuki, K. & Blanchard, D.K. Functional activation of human neutrophils by recombinant monocyte-derived neutrophil chemotactic factor/IL-8. *J Immunol* 144, 2205-10 (1990).
208. Detmers, P.A. et al. Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. *J Exp Med* 171, 1155-62 (1990).

209. Atta ur, R., Harvey, K. & Siddiqui, R.A. Interleukin-8: An autocrine inflammatory mediator. *Curr Pharm Des* 5, 241-53 (1999).
210. Baggiolini, M., Moser, B. & Clark-Lewis, I. Interleukin-8 and related chemotactic cytokines. The Giles Filley Lecture. *Chest* 105, 95S-98S (1994).
211. Shalaby, M.R. et al. Receptor binding and activation of polymorphonuclear neutrophils by tumor necrosis factor-alpha. *J Leukoc Biol* 41, 196-204 (1987).
212. Akgul, C. & Edwards, S.W. Regulation of neutrophil apoptosis via death receptors. *Cell Mol Life Sci* 60, 2402-8 (2003).
213. Morzycki, W. & Issekutz, A.C. Tumour necrosis factor-alpha but not interleukin-1 induces polymorphonuclear leucocyte migration through fibroblast layers by a fibroblast-dependent mechanism. *Immunology* 74, 107-13 (1991).
214. von Asmuth, E.J., Leeuwenberg, J.F., van der Linden, C.J. & Buurman, W.A. Tumour necrosis factor-alpha induces neutrophil-mediated injury of cultured human endothelial cells. *Scand J Immunol* 34, 197-206 (1991).
215. Wilkinson, D.G. Multiple roles of EPH receptors and ephrins in neural development. *Nat Rev Neurosci* 2, 155-64 (2001).
216. Cheng, N., Brantley, D.M. & Chen, J. The ephrins and Eph receptors in angiogenesis. *Cytokine Growth Factor Rev* 13, 75-85 (2002).
217. Nawroth, P.P. et al. Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. *J Exp Med* 163, 1363-75 (1986).
218. Butler, D.M., Maini, R.N., Feldmann, M. & Brennan, F.M. Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist. *Eur Cytokine Netw* 6, 225-30 (1995).
219. Haworth, C. et al. Expression of granulocyte-macrophage colony-stimulating factor in rheumatoid arthritis: regulation by tumor necrosis factor-alpha. *Eur J Immunol* 21, 2575-9 (1991).
220. Lin, T.J., Issekutz, T.B. & Marshall, J.S. SDF-1 induces IL-8 production and transendothelial migration of human cord blood-derived mast cells. *Int Arch Allergy Immunol* 124, 142-5 (2001).
221. Lin, T.J., Issekutz, T.B. & Marshall, J.S. Human mast cells transmigrate through human umbilical vein endothelial monolayers and selectively produce IL-8 in response to stromal cell-derived factor-1 alpha. *J Immunol* 165, 211-20 (2000).
222. Cherla, R.P. & Ganju, R.K. Stromal cell-derived factor 1 alpha-induced chemotaxis in T cells is mediated by nitric oxide signaling pathways. *J Immunol* 166, 3067-74 (2001).
223. Aiuti, A., Webb, I.J., Bleul, C., Springer, T. & Gutierrez-Ramos, J.C. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med* 185, 111-20 (1997).
224. Hamada, T. et al. Transendothelial migration of megakaryocytes in response to stromal cell-derived factor 1 (SDF-1) enhances platelet formation. *J Exp Med* 188, 539-48 (1998).

225. D'Apuzzo, M. et al. The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4. *Eur J Immunol* 27, 1788-93 (1997).
226. Schabath, R. et al. The murine chemokine receptor CXCR4 is tightly regulated during T cell development and activation. *J Leukoc Biol* 66, 996-1004 (1999).
227. Murdoch, C. CXCR4: chemokine receptor extraordinaire. *Immunol Rev* 177, 175-84 (2000).
228. Poznansky, M.C. et al. Active movement of T cells away from a chemokine. *Nat Med* 6, 543-8 (2000).
229. Norose, K., Yano, A., Aosai, F. & Segawa, K. Immunologic analysis of cerebrospinal fluid lymphocytes in Vogt-Koyanagi-Harada disease. *Invest Ophthalmol Vis Sci* 31, 1210-6 (1990).
230. Wang, X.C., Norose, K., Yano, A., Ohta, K. & Segawa, K. Two-color flow cytometric analysis of activated T lymphocytes in aqueous humor of patients with endogenous vs. exogenous uveitis. *Curr Eye Res* 14, 425-33 (1995).
231. Ohta, K., Norose, K., Wang, X.C., Ito, S. & Yoshimura, N. Abnormal naive and memory T lymphocyte subsets in the peripheral blood of patients with uveitis. *Curr Eye Res* 16, 650-5 (1997).
232. Calder, V.L. et al. Increased CD4+ expression and decreased IL-10 in the anterior chamber in idiopathic uveitis. *Invest Ophthalmol Vis Sci* 40, 2019-24 (1999).
233. Imai, Y. & Ohno, S. Helper T-cell subsets in uveitis. *Int Ophthalmol Clin* 42, 25-32 (2002).
234. Muhaya, M. et al. Characterization of T cells and cytokines in the aqueous humour (AH) in patients with Fuchs' heterochromic cyclitis (FHC) and idiopathic anterior uveitis (IAU). *Clin Exp Immunol* 111, 123-8 (1998).
235. Murray, P.I., Clay, C.D., Mappin, C. & Salmon, M. Molecular analysis of resolving immune responses in uveitis. *Clin Exp Immunol* 117, 455-61 (1999).
236. Murray, P.I., Hoekzema, R., Luyendijk, L., Konings, S. & Kijlstra, A. Analysis of aqueous humor immunoglobulin G in uveitis by enzyme-linked immunosorbent assay, isoelectric focusing, and immunoblotting. *Invest Ophthalmol Vis Sci* 31, 2129-35 (1990).
237. Perretti, M. Endogenous mediators that inhibit the leukocyte-endothelium interaction. *Trends Pharmacol Sci* 18, 418-25 (1997).
238. Smith, J.R. et al. Experimental melanin-induced uveitis in the Fischer 344 rat is inhibited by anti-CD4 monoclonal antibody, but not by mannose-6-phosphate. *Clin Exp Immunol* 115, 64-71 (1999).
239. Becker, M.D., Nobiling, R., Planck, S.R. & Rosenbaum, J.T. Digital video-imaging of leukocyte migration in the iris: intravital microscopy in a physiological model during the onset of endotoxin-induced uveitis. *J Immunol Methods* 240, 23-37 (2000).
240. Russell, J., Cooper, D., Tailor, A., Stokes, K.Y. & Granger, D.N. Low venular shear rates promote leukocyte-dependent recruitment of adherent platelets. *Am J Physiol Gastrointest Liver Physiol* 284, G123-9 (2003).

241. Bienvenu, K. & Granger, D.N. Molecular determinants of shear rate-dependent leukocyte adhesion in postcapillary venules. *Am J Physiol* 264, H1504-8 (1993).
242. Bienvenu, K., Russell, J. & Granger, D.N. Leukotriene B4 mediates shear rate-dependent leukocyte adhesion in mesenteric venules. *Circ Res* 71, 906-11 (1992).
243. Perry, M.A. & Granger, D.N. Role of CD11/CD18 in shear rate-dependent leukocyte-endothelial cell interactions in cat mesenteric venules. *J Clin Invest* 87, 1798-804 (1991).
244. Freddo, T.F. & Raviola, G. The homogeneous structure of blood vessels in the vascular tree of Macaca mulatta iris. *Invest Ophthalmol Vis Sci* 22, 279-91 (1982).
245. Thurston, G. & Yancopoulos, G.D. Gridlock in the blood. *Nature* 414, 163-4 (2001).
246. Stein, B.N., Gamble, J.R., Pitson, S.M., Vadas, M.A. & Khew-Goodall, Y. Activation of endothelial extracellular signal-regulated kinase is essential for neutrophil transmigration: potential involvement of a soluble neutrophil factor in endothelial activation. *J Immunol* 171, 6097-104 (2003).
247. Adams, R.H. Vascular patterning by Eph receptor tyrosine kinases and ephrins. *Semin Cell Dev Biol* 13, 55-60 (2002).
248. Adams, R.H. & Klein, R. Eph receptors and ephrin ligands. essential mediators of vascular development. *Trends Cardiovasc Med* 10, 183-8 (2000).
249. Pasquale, E.B. The Eph family of receptors. *Curr Opin Cell Biol* 9, 608-15 (1997).
250. Gerety, S.S. & Anderson, D.J. Cardiovascular ephrinB2 function is essential for embryonic angiogenesis. *Development* 129, 1397-410 (2002).
251. Yuan, K., Jin, Y.T. & Lin, M.T. Expression of Tie-2, angiopoietin-1, angiopoietin-2, ephrinB2 and EphB4 in pyogenic granuloma of human gingiva implicates their roles in inflammatory angiogenesis. *J Periodontal Res* 35, 165-71 (2000).
252. Shin, D. et al. Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. *Dev Biol* 230, 139-50 (2001).
253. Campochiaro, P.A. & Hackett, S.F. Ocular neovascularization: a valuable model system. *Oncogene* 22, 6537-48 (2003).
254. Saint-Geniez, M., Argence, C.B., Knibiehler, B. & Audigier, Y. The msr/apj gene encoding the apelin receptor is an early and specific marker of the venous phenotype in the retinal vasculature. *Gene Expr Patterns* 3, 467-72 (2003).
255. Cheng, N. et al. Blockade of EphA receptor tyrosine kinase activation inhibits vascular endothelial cell growth factor-induced angiogenesis. *Mol Cancer Res* 1, 2-11 (2002).
256. Brantley, D.M. et al. Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo. *Oncogene* 21, 7011-26 (2002).

257. Davies, M.H., Eubanks, J.P. & Powers, M.R. Increased retinal neovascularization in Fas ligand-deficient mice. *Invest Ophthalmol Vis Sci* 44, 3202-10 (2003).
258. Cheng, H.J., Nakamoto, M., Bergemann, A.D. & Flanagan, J.G. Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. *Cell* 82, 371-81 (1995).
259. Drescher, U. et al. In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. *Cell* 82, 359-70 (1995).
260. Birgbauer, E., Cowan, C.A., Sretavan, D.W. & Henkemeyer, M. Kinase independent function of EphB receptors in retinal axon pathfinding to the optic disc from dorsal but not ventral retina. *Development* 127, 1231-41 (2000).
261. Drescher, U., Bonhoeffer, F. & Muller, B.K. The Eph family in retinal axon guidance. *Curr Opin Neurobiol* 7, 75-80 (1997).
262. Braisted, J.E. et al. Graded and lamina-specific distributions of ligands of EphB receptor tyrosine kinases in the developing retinotectal system. *Dev Biol* 191, 14-28 (1997).
263. Marcus, R.C., Gale, N.W., Morrison, M.E., Mason, C.A. & Yancopoulos, G.D. Eph family receptors and their ligands distribute in opposing gradients in the developing mouse retina. *Dev Biol* 180, 786-9 (1996).
264. Holash, J.A. et al. Reciprocal expression of the Eph receptor Cek5 and its ligand(s) in the early retina. *Dev Biol* 182, 256-69 (1997).
265. Helbling, P.M. et al. Comparative analysis of embryonic gene expression defines potential interaction sites for Xenopus EphB4 receptors with ephrin-B ligands. *Dev Dyn* 216, 361-73 (1999).
266. Penn, J.S., Rajaratnam, V.S. & Bullard, L.E. The angiostatic influence of penetrating ocular injury [ARVO Abstract]. *Invest Ophthalmol Vis Sci* 41, S142Abstract #731 (2000).
267. Rajaratnam, V.S., Koepke, K.A. & Penn, J.S. Identifying retinal genes that respond to penetrating ocuar injury using gene array analysis in a rat model of retinopathy of prematurity [ARVO Abstract]. *Invest Ophthalmol Vis Sci* 44(2001).
268. Bussolino, F., Mantovani, A. & Persico, G. Molecular mechanisms of blood vessel formation. *Trends Biochem Sci* 22, 251-6 (1997).
269. Okamoto, N. et al. Transgenic mice with increased expression of vascular endothelial growth factor in the retina: a new model of intraretinal and subretinal neovascularization. *Am J Pathol* 151, 281-91 (1997).
270. Tobe, T. et al. Evolution of neovascularization in mice with overexpression of vascular endothelial growth factor in photoreceptors. *Invest Ophthalmol Vis Sci* 39, 180-8 (1998).
271. Ozaki, H. et al. Blockade of vascular endothelial cell growth factor receptor signaling is sufficient to completely prevent retinal neovascularization. *Am J Pathol* 156, 697-707 (2000).

272. **Feeney, S.A. et al. Role of vascular endothelial growth factor and placental growth factors during retinal vascular development and hyaloid regression.** ***Invest Ophthalmol Vis Sci*** **44, 839-47 (2003).**
273. **Sturz, A., Bader, B., Thierauch, K.H. & Glienke, J. EphB4 signaling is capable of mediating ephrinB2-induced inhibition of cell migration.** ***Biochem Biophys Res Commun*** **313, 80-8 (2004).**

CASEY EYE INSTITUTE

Department of Ophthalmology
Faculty Meeting Agenda
Thursday, January 6, 2005
6:45 a.m. – 7:45 a.m.
Macdonald Auditorium

Welcome new faculty	Dr. Robertson
After hours care of ophthalmology patients (handout)	Dr. Lauer
School of Medicine budget update	Dr. Robertson
Epic update	Dr. Chung
Epic assessment	Dr. Stout
Discretionary salary components	Dr. Robertson
Pediatrics Division Chief	Dr. Robertson
OR update	Dr. Dailey
Operative reports	Elizabeth Cottle
OHP standard patient referrals from outside metro	Elizabeth Cottle
River Campus Group/Existing CEI floor plan	Dr. Wilson Brent Carreau
Fundraising update	Sigrid Button