REGULATION OF IL-6 SIGNALING IN RETINAL CELLS AND TISSUE IN HEALTH AND DISEASE

By

Brandon A. Coughlin

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ABSTRACT

REGULATION OF IL-6 SIGNALING IN RETINAL CELLS AND TISSUE IN HEALTH AND DISEASE

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The global prevalence of diabetic retinopathy continues to rise as more individuals are diagnosed with diabetes each year. With a rising patient population, the need for a cure is greater than ever. The current available treatment strategies are aimed at treating the more advanced stages of the disease, macular edema and neovascularization, and are not efficacious for a large portion of the patient population. In order to develop more effective and targeted therapies, a better understanding of the molecular mechanisms involved in the pathophysiology of diabetic retinopathy are needed. Chronic low-grade inflammation induced by hyperglycemia is commonly understood to play a role in disease progression. Interleukin-6 (IL-6) is a prominent inflammatory cytokine whose role in disease advancement is not well understood.

The overall of effects of IL-6 are determined by two diverse signaling pathways: classical IL-6 signaling and IL-6 trans-signaling. The expression of the membrane-bound IL-6 receptor (mIL-6R) and glycoprotein 130 (gp130) are needed for classical IL-6 signaling, while gp130 and the soluble IL-6 receptor (sIL-6R) are needed for IL-6 trans-signaling. Activation of these pathways are hypothesized to promote anti-inflammatory or pro-inflammatory functions, respectively. Therefore, the aim of this study was to determine the IL-6 signaling capabilities of Human Müller and retinal endothelial cells, both of which are crucial for maintaining a healthy retina, and its effect on function and

viability. In addition, this dissertation examined the regulation of IL-6 signaling pathways in response to intravitreal treatment with ranibizumab, the most common anti-VEGF therapy in patients with proliferative diabetic retinopathy and diabetic macular edema.

First, we determined that human Müller cells (HMC) express both mIL-6R and gp130, which allows them to signal through both classical IL-6 and IL-6 trans-signaling. Interestingly, treatment with IL-6, the agonist of classical IL-6 signaling, protected HMCs from hyperglycemia-induced cell death. Further, these protective effects were mediated by VEGF-A. Surprisingly, treatment with IL-6/sIL-6R, the agonist of IL-6 trans-signaling, had slight protective effects, but were not dependent on VEGF-A. Second, it was established that human retinal endothelial cells (HREC) express gp130, but do not express mIL-6R. Therefore, HRECs were only responsive to IL-6 trans-signaling but not to IL-6-induced classical signaling. Treatment of HRECs with IL-6/sIL-6R (10 ng/mL) decreased cell viability and led to increased release of inflammatory cytokines. Intriguingly, when treated with a higher concentration of IL-6/sIL-6R (50 ng/mL) HRECs began releasing VEGF-A and forming tubular networks. Finally, our clinical study has shown that intravitreal ranibizumab had a strong impact on aqueous humor levels of soluble cytokine receptors, some of which belonging to IL-6 signaling. Interestingly, changes in VEGF family members (A and C) were correlated with changes in IL-6 signaling members. Taken together, all our findings suggest that an IL-6-VEGF signaling axis may be important for the development of diabetic retinopathy.

To my family, mentors, and friends,
thank you for your unwavering support.

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KEY TO ABBREVIATIONS

AQP- Aquaporin

ADAM-10/17- A disintegrin and metalloproteinase domain-containing protein 10/17

BRB- Blood retinal barrier

BCVA- Best corrected visual acuity

DCCT- Diabetes control and complications trial

DM- Diabetes mellitus

DME- Diabetic macular edema

EDIC- Epidemiology of diabetes interventions complications study

ETDRS- Early treatment for diabetic retinopathy study

DR- Diabetic retinopathy

FA- Fluorescein angiography

GABA- Gamma amino butyric acid

GAPDH- Glyceraldehyde-3-phosphate dehydrogenase

GFAP- Glial fibrillary acidic protein

GLAST- Glutamate aspartate transporter

GLP-1- Glucagon like protein 1

gp130- Glycoprotein 130

HLA- Human leukocyte antigen

HMC- Human Müller cell

HREC- Human retinal endothelial cell

IFNγ- Interferon gamma

IL-1α- Interleukin-1 alpha

IL-1β- Interleukin-1 beta

IL-6- Interleukin-6

IL-8- Interleukin-8

IL-10- Interleukin-10

IL-17A- Interleukin-17A

IL-18- Interleukin-18

IP-10- Interferon gamma-induced protein 10

IRMA- Intraretinal microvascular abnormalities

MCP-1- Monocyte chemoattractant protein-1

mIL-6R- membrane bound IL-6 receptor

NPDR- Non-proliferative diabetic retinopathy

OCT- Optical coherence tomography

OGTT- Oral glucose tolerance test

PDR- Proliferative diabetic retinopathy

RANTES- Regulated on activation, normal T cell expressed and secreted

RPE- Retinal pigmental epithelial cells

sgp130- soluble glycoprotein 130

sIL-1R1- Soluble interleukin-1 receptor 1

sIL-1R2- Soluble interleukin-1 receptor 2

sIL-6R- soluble interleukin-6 receptor

sRAGE- soluble receptor for advanced glycation end-products

STZ- Streptozotocin

TNFα- Tumor necrosis factor alpha

TUNEL- Terminal deoxynucleotidyl transferase

VEGF- Vascular endothelial growth factor

VEGFR- Vascular endothelial growth factor receptor

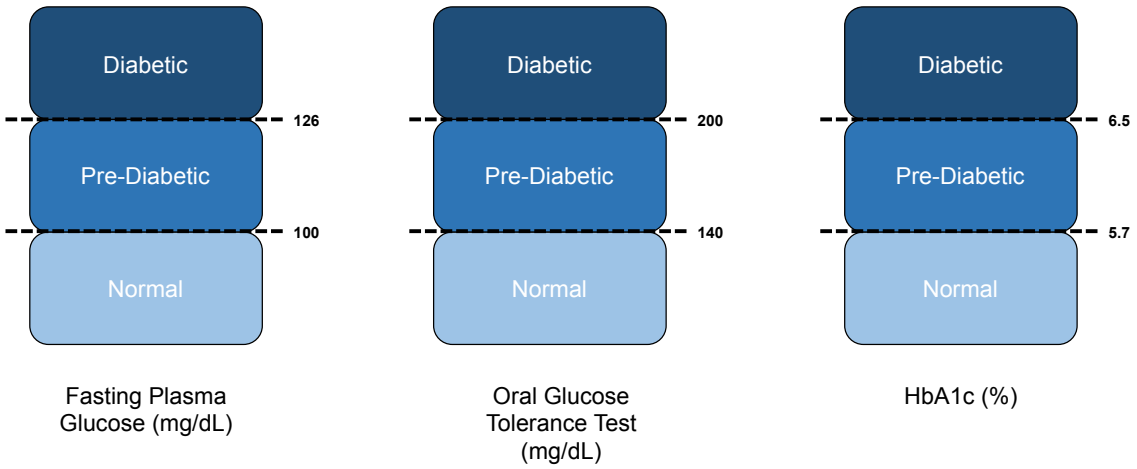
Chapter 1. Introduction

1.1 Diabetes Mellitus

1.1.1 Background and classifications of diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder characterized by elevated blood glucose levels that affects the entire body. According to the World Health Organization (WHO), the diagnostic criteria for patients with DM include: fasting plasma glucose levels ≥126 mg/dl (≥7.0 mmol/l), plasma glucose levels ≥200 mg/dl (≥11.1 mmol/l) two hours following an oral glucose tolerance test (OGTT), and glycated hemoglobin ≥6.5% (Figure 1)[1]. The development of the hyperglycemic environment is either due to the inability of the pancreatic β-cells to produce and secrete insulin and/or the failure to properly respond to insulin. In addition to hyperglycemia, patients may also experience polyuria, polydipsia, polyphagia, dyslipidemia, and blurred vision. If left untreated, chronic hyperglycemia can lead to a variety of secondary diabetic complications as well as diabetic ketoacidosis and ultimately prove to be fatal[2–4]. DM is broken down into three main sub-types: type 1, type 2, and gestational diabetes.

Figure 1. Clinical tests for diagnosing diabetes mellitus. The percentage (%) of glycated hemoglobin (HbA1c) and the measurement of plasma glucose levels when fasted and 2 hours following an oral glucose tolerance test are common clinical tests for the diagnosis of diabetes mellitus.



Type 1 diabetes (T1D), or insulin dependent diabetes mellitus, affects approximately 5-10% of the diabetic population and is due to autoimmune destruction of pancreatic β-cells, which are responsible for producing insulin[5–7]. To date, the initiation of this autoimmune response is unknown, however, both genetic and environmental factors are thought to be major components. One of the main genetic risk factors are variations in genes of the human leukocyte antigen (HLA) class II family, such as HLA-DQA1, -DQB1, and -DRB1[5,8–10]. In contrast, environmental factors such as viral infections and dietary exposure (early introduction to eggs, cow's milk, and root vegetables to name a few) are far less understood[11]. Enteroviruses, such as coxsackie viruses, are most commonly associated with T1D and have been found in the pancreatic islets of T1D patients[12–14]. The complex interaction of genetics and environmental triggers is hypothesized to promote the production of β-cell autoantibodies and insulitis mediated by infiltrating T-cells. This T-cell mediated immune response can lead to β-cell destruction and subsequent loss of insulin production[15,16]. The most common treatment strategy to maintain glycemic control is exogenous insulin administration. Current commercially available forms of insulin include rapid-, short-, intermediate-, and long-acting forms which can be administered using an insulin pen, syringe, or pump[17,18]. Additional treatment strategies currently being developed and tested are pancreas and islet cell transplants. These experimental treatment strategies are currently only used in T1D patients with severe and uncontrollable blood glucose levels due to the extreme side effects and mixed success rates[19–21]. In order to increase the quality of life for patients with T1D new efficient and reliable treatment strategies and delivery options would be absolutely valuable.

Type 2 diabetes (T2D), also known as non-insulin dependent diabetes mellitus, makes up approximately 90-95% of the diabetic population and is a rising public health concern[22]. T2D is characterized by a decrease in insulin sensitivity due to insulin resistance. One of the most well documented contributors to insulin resistance is chronic systemic inflammation originating in adipose tissue. When triggered, adipose tissue derived pro-inflammatory macrophages can produce increased levels of inflammatory cytokines, such as interleukin-6 and tumor necrosis factor-α[23]. In turn, these inflammatory cytokines can initiate a systemic inflammatory response. Increased inflammatory cytokine levels can promote insulin resistance through inhibition or degradation of insulin receptor substrates by serine phosphorylation. Insulin receptor substrates are important signaling adaptor proteins whose inhibition or degradation prevent downstream insulin receptor signaling[22–28]. The pancreas's initial response to insulin resistance is a compensatory increase in insulin production. However, the increased demand for insulin can eventually lead to β-cell dysfunction and a subsequent decrease in insulin production accompanied by β-cell failure[24]. Environmental risk factors associated with increased incidence of T2D and insulin resistance include an unhealthy diet, physical inactivity, obesity, and metabolic syndrome[29,30]. Additionally, genetic mutations, such as CAPN10 and TCF7L2, are associated with a predisposition for T2D[31]. Due to the vast array of mechanisms connected with the pathogenesis of T2D a wide variety of treatment options are available, including life style changes and pharmacological agents. Previous studies have shown that lifestyle modifications like diet adjustments, regular exercise, and abstinence from smoking can significantly reduce the incidence of T2D[32–34]. Furthermore, pharmacological agents, most commonly metformin, are used to decrease hepatic glucose production and

increase insulin sensitivity and glucose uptake[35–37]. Additional agents used to promote insulin release are sulfonylureas, meglitinides, and glucagon-like peptide 1 (GLP-1) analogues[24]. In some cases, the increased demand for insulin leads to a progressive loss of β-cell function and the need for insulin supplementation[38–40].

Although T1D and T2D are the most common forms of DM, gestational diabetes affects many women and infants every year. In 2016, one in every twelve pregnant women were affected by gestational diabetes, and 50% these women would go on to further develop T2D[41,42]. As pregnancy progresses through the second and third trimester, so does insulin resistance[43]. Throughout pregnancy, an increase in insulin resistance is associated with increased release of placental hormones. As the placenta continues to grow, it secretes a wide variety of hormones, such as cortisol and progesterone, and one recently connected with insulin resistance is human chorionic somatomammotropin (HCS), also known as human placental lactogen[44,45]. In early pregnancy, HCS mobilizes free fatty acids to be used as a fuel source and conserves glucose for the fetus. However, during late-term pregnancy the concentration of HCS increases 10-fold and antagonizes insulin induced glucose uptake in the mother[46]. In addition, increased levels of cortisol during pregnancy contribute to maternal insulin resistance accompanied by elevated blood glucose levels[47]. Similar to T2D, the decrease in insulin sensitivity leads to a compensatory reaction by the β-cells to increase insulin production. Consequently, women who cannot combat this decrease in insulin sensitivity will develop gestational diabetes and an increased risk for fetal macrosomia[48]. In order to prevent or treat gestational diabetes blood glucose levels are closely monitored along

with diet and exercise and treated with insulin if necessary[49,50]. Besides T1D, T2D, and gestational diabetes, other subtypes of diabetes include but are not limited to maturity onset diabetes of the young (MODY) and latent autoimmune diabetes of adult hood (LADA), however, these types of diabetes are less common.

1.1.2 Prevalence and global burden

Diabetes, labeled a global epidemic by the WHO, affected approximately 422 million individuals worldwide in 2014[51]. In the United States over 30.3 million Americans (>9% of the U.S. population) are affected by diabetes, and currently diabetes is the seventh leading cause of death according to the American Diabetes Association[52]. In the United States alone, the direct medical costs associated with diabetes has risen from $176 billion in 2012 to $237 billion in 2017. Additionally, when adding in the indirect costs of diabetes, such as unemployment, the overall estimated costs of diabetes in 2017 was $327 billion[53]. Furthermore, it is projected that by the year 2030 more than 54.9 million Americans will be affected by diabetes, an increase of 54% from 2015, elevating the economic burden to nearly $622 billion[54]. The drastic increase in economic burden is due to a wide variety of complications associated with diabetes that affect the entire body.

1.1.3 Diabetic complications

As the duration of diabetes increases, so does the probability of developing secondary diabetic complications. Furthermore, according to the Diabetes Control and Complications Trial (DCCT) and the Epidemiology of Diabetes Interventions and Complications (EDIC) study, the best predictor for the progression of diabetic

complications is glycemic control (mean HbA_{1C})[55]. Diabetic complications can be broken down into macrovascular and microvascular complications. Macrovascular complications are commonly associated with cardiovascular problems and can include coronary artery disease, atherosclerosis, and stroke, all of which can prove to be fatal. On the other hand, common microvascular complications can include diabetic neuropathy, nephropathy, and retinopathy. Side effects of these secondary complications can ultimately lead to but are not limited to foot ulcers, impaired wound healing, heart attack, and blindness[56,57]. Although many different tissues are affected by diabetes, one of the most commonly affected tissues for both T1D and T2D is the retina.

1.2 Diabetic retinopathy

1.2.1 Background and prevalence

Diabetic retinopathy (DR) is one of the most common complications for both type 1 and type 2 diabetics and is the leading cause of acquired blindness for working-aged adults. Risk factors associated with the development of DR include: duration of diabetes, hypertension, and dyslipidemia[58]. Despite significant advances in our understanding of the molecular mechanisms associated with the initiation and progression of DR, there is no cure. Approximately one-third of diabetics world-wide have some form of DR, and roughly 11% have vision threatening DR[59]. Importantly, as the prevalence of DM continues to rise so does the prevalence of DR (Figure 2). DR is a complication of DM that affects the retina and its associated vasculature. It is now very well accepted that chronic hyperglycemia induces chronic-low grade tissue inflammation that affects

neuronal as well as vascular function of retinal cells followed by increased cell death[60]. As a consequence, retinal blood vessels begin to grow uncontrollably, a process known as neovascularization. However, these new blood vessels are immature and can leak blood into the retina and vitreous causing hemorrhages. These hemorrhages prevent light from reaching the retina affecting the patient's vision by seeing black spots. In addition, fluid can accumulate in the macula (diabetic macular edema; DME) causing blurred vision (Figure 3). To increase life quality of diabetic patients there is a pressing need to fully understand mechanisms underlying the disease so better therapies and potentially a cure can be developed.

Figure 2. Global projections for the incidence of diabetic retinopathy. Approximately 35% of individuals with diabetes have some form of diabetic retinopathy, and 11% have vision threatening diabetic retinopathy in the form of macular edema and/or neovascularization. These numbers are expected to rise dramatically by the year 2040 and beyond. Image used with permission from http://atlas.iapb.org/vision-trends/diabetic-retinopathy/

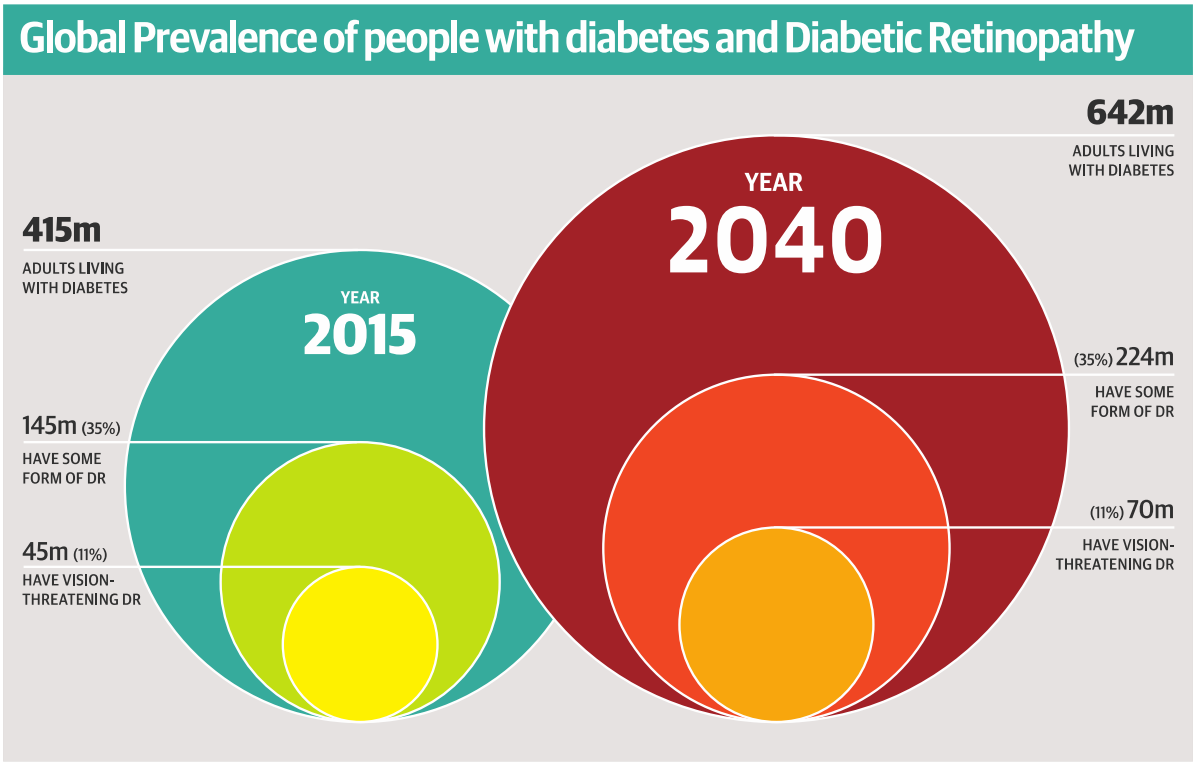
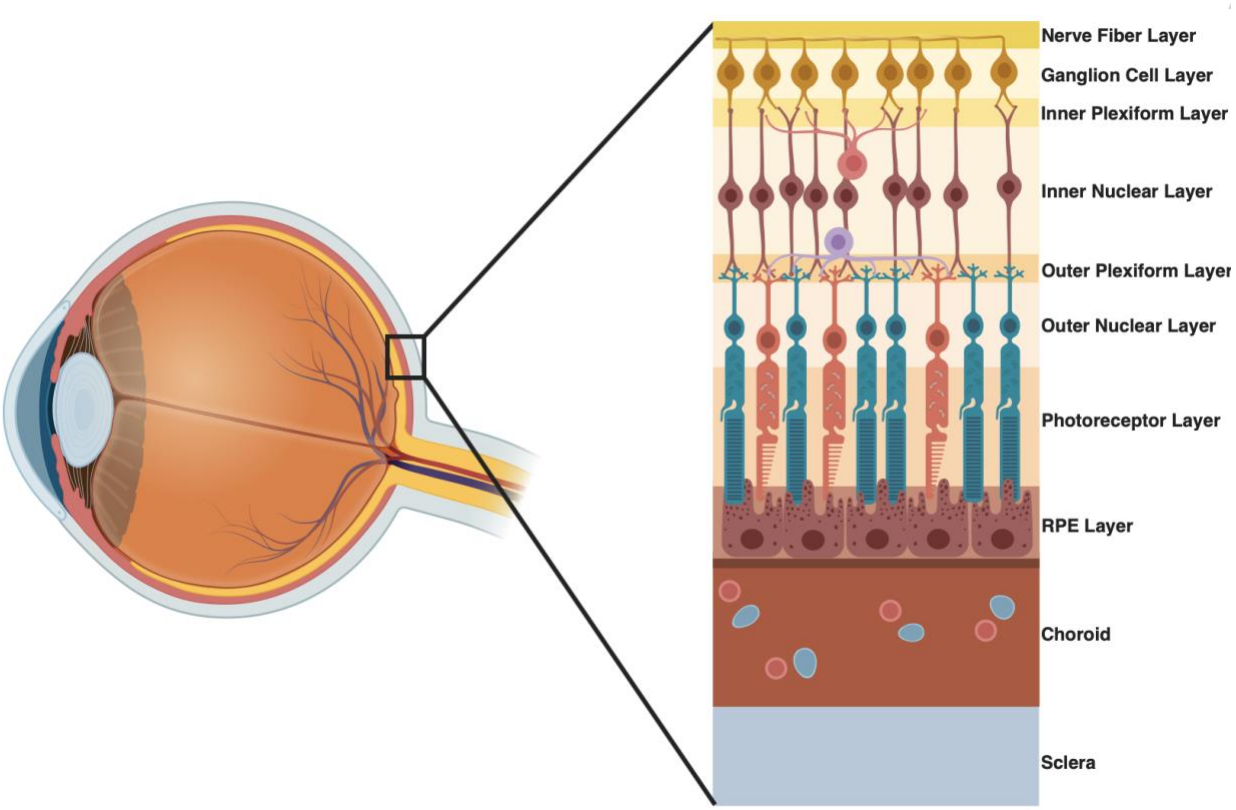


Figure 3. Blurred vision in diabetic retinopathy. Comparison of vision in a patient with normal vision (left) and a patient with diabetic retinopathy (right). Patients with diabetic retinopathy can experience blurred vision due to accumulation of fluid within the retinal layers and floaters due to hemorrhaging.

1.2.2 Retina structure: layers, cell types, and vasculature

The retina is a light-sensitive tissue located at the back of the eye originating from neuroepithelial cells of the neuroectoderm layer[61]. The main purpose of the retina is to receive photons of light from the surrounding environment. The incoming photons activate light-sensitive pigments located in the outermost portion of the retina and convert it into chemical and electrical signals that are carried through the retina and towards the brain to develop a visual image[62]. In order for this intricate visual process to occur, the retina is organized into ten distinct layers. The outermost to the innermost (adjacent to the vitreous body) retinal layers include: (1) retinal pigmented epithelial (RPE) cell layer, (2) photoreceptor layer, (3) outer limiting membrane, (4) outer nuclear layer, (5) outer plexiform layer, (6) inner nuclear layer, (7) inner plexiform layer, (8) ganglion cell layer, (9) nerve fiber layer, and (10) inner limiting membrane (Figure 4)[63].

Figure 4. Anatomy of the retina. Sagittal cross-section of the eye with a magnified cross-section of the retina. Retinal cross-section demonstrates the highly organized retinal cell layers and intricate cell to cell connections. *Created with BioRender.com



The outermost layer, (1) the RPE cell layer, has many responsibilities, some of which include: absorption of scattered light, nutrient exchange between the choroid and photoreceptors, visual pigment recycling, and ion spatial buffering[64,65]. The (2) photoreceptor layer contains the inner and outer segments of the rod and cone photoreceptors. The outer segments of photoreceptors contain the opsin receptors carrying the light-sensitive visual pigment, 11-cis retinal, which initiates phototransduction in response to photons of light. The density of rods and cones varies throughout the retina. The cones, which are responsible for color vision, are the densest within the fovea, an area located within the macula where visual acuity is at its highest. In contrast, rods are responsible for night vision and are most dense surrounding the fovea[66,67]. Directly adjacent to the photoreceptor inner and outer segments is the (3) outer limiting membrane made up of Müller cell end feet. The outer limiting membrane separates the inner and outer segments from the nuclei of the photoreceptors and helps maintain the mechanical strength of the retina[68]. The (4) outer nuclear layer contains the nuclei of rod and cone photoreceptors, which form synapse with bipolar and horizontal cells in the (5) outer plexiform layer. The nuclei of bipolar, horizontal, amacrine, and Müller cells are located in the (6) inner nuclear layer. Bipolar cells are responsible for transmitting the electrical and biochemical information from the photoreceptors to the ganglion cells, while horizontal (outer plexiform) and amacrine (inner plexiform) cells interconnect neurons laterally[69,70]. Although Müller cell nuclei are located in the inner nuclear layer, their extensions reach into every retinal layer and have intimate contact with every retinal cell type[71]. The (7) inner plexiform layer holds the connections between the bipolar cells and ganglion cells in the (8) ganglion cell layer. Ganglion cells are the final order of retinal

neurons in visual phototransduction, and their axons make up the (9) nerve fiber layer and optic nerve which exits the eye and passes through the optic chiasm on the way to the lateral geniculate nucleus located in the thalamus of the brain[72]. The inner most layer of the retina, (10) the inner limiting membrane, is made up of Müller cell end feet and is directly adjacent to the vitreous body and thought to play a prominent role in ion and acid base balance of the inner retina[73].

Due to the retinas high metabolic demand, it consumes large amounts of oxygen and nutrients and, therefore, has a well-organized vasculature[74]. The retina receives its blood supply from two main sources: the central retinal artery and the choroid located between the retina and sclera. The central retinal artery enters the retina through the optic disc alongside the optic nerve and supplies oxygen and nutrients to the innermost layers of the retina. The retinal arterioles that branch off the central retinal artery can be found as deep as the inner two-thirds of the inner nuclear layer[63,75]. On the other hand, the choroidal blood vessels are located sub-retinal, between the retina and the sclera, and supply the highly metabolic RPE and photoreceptor layers as well as the outer nuclear, outer plexiform, and outermost one-third of the inner nuclear layer. Unique within in the retina is the foveal avascular zone, which is supplied solely by the choroidal vasculature[76]. The retina is one of the most metabolically active tissues in the human body and accounts for about 8% of the basal metabolic rate[77]. Due to the high metabolic activity, the delivery of nutrients is tightly regulated by the blood retinal barrier (BRB). The BRB can be further broken into an inner blood retinal barrier (iBRB) and an outer blood retinal barrier (oBRB). The iBRB is established by tight junction proteins, such zonula occludens, between retinal

endothelial cells of arterioles that have branched off the central retinal artery. Furthermore, these vessels are surrounded by pericytes, astrocytes, and Müller cells. In contrast, the oBRB is maintained by tight junctions between RPE cells[78,79]. Loss of the BRB, more specifically the iBRB, contributes to retinal pathology associated with retinal diseases such as diabetic retinopathy.

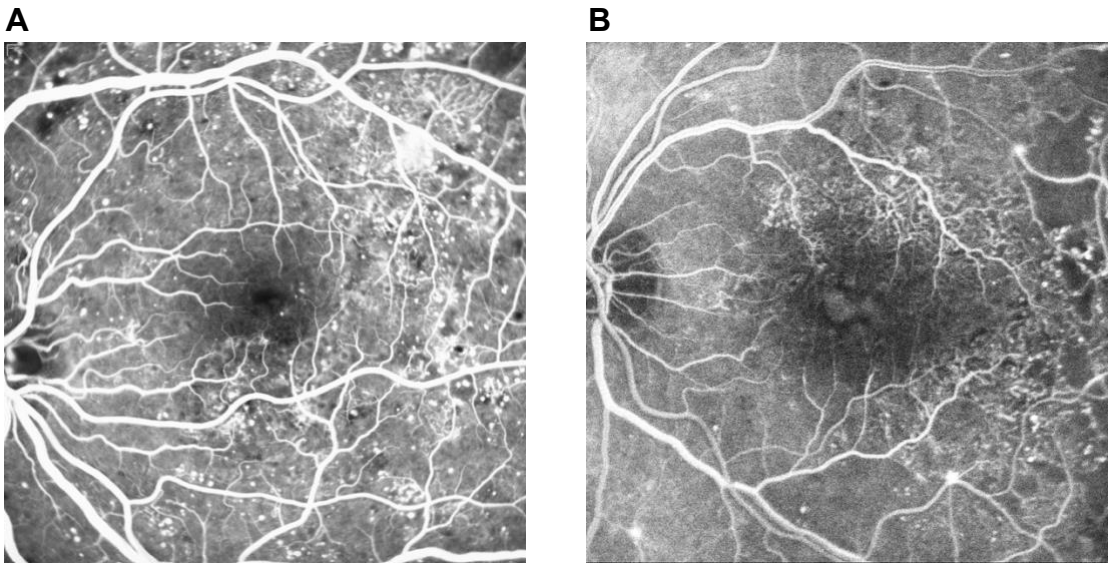
1.2.3 Non-proliferative diabetic retinopathy

DR pathology has long been associated with the duration of hyperglycemia and can be distinguished by the severity of retinal pathology. Non-proliferative diabetic retinopathy (NPDR), or the background stages, is classified by visible clinical findings through fundus photography including: microaneurysms, retina hemorrhages, intraretinal microvascular abnormalities (IRMA), cotton wool spots (infarcts of the never fiber layer), and hard exudates (lipid deposits)[80]. NPDR can further be broken down clinically into mild, moderate, and severe NPDR. Patients are considered to have mild or moderate NPDR if they present with mild or moderate levels of microaneurysms and intraretinal hemorrhaging respectively[81]. Severe NPDR is defined according to the "4-2-1 rule" by the Early Treatment for Diabetic Retinopathy Study (ETDRS): "intraretinal hemorrhaging and microaneurysms in four quadrants, venous beading in $\geq$ 2 quadrants, and IRMA in $\geq$ 1 quadrant[82]." As DR progresses over time it can advance towards the proliferative stage of the disease which is characterized by neovascularization.

1.2.4 Proliferative diabetic retinopathy

Proliferative diabetic retinopathy (PDR), the more advanced stage of the disease, is characterized by the proliferation and growth of new blood vessels in response to oxygen deprivation and low-grade chronic inflammation[83]. The immature and leaky blood vessels can grow along the retina or towards the vitreous humor, and if not properly treated the vessels can hemorrhage and cloud vision. Additionally, vessel growth into the vitreous can lead to tractional retinal detachment and impaired vision[84]. A prominent growth factor known to play a key role in the uncontrolled angiogenesis of PDR is vascular endothelial growth factor-A (VEGF-A). In addition, VEGF-A has also been implicated in increasing vessel permeability through disruption of endothelial cell tight junctions[85,86]. Previous, studies have shown diabetic patients with PDR to have higher vitreous levels of VEGF-A, which was associated with angiogenesis, compared to non-diabetics[87,88]. The discovery of VEGF-A's role in the development and progression of PDR led to the development of anti-VEGF related therapies. In the clinic, funduscopic imaging and fluorescein angiography (FA) are used to distinguish between NPDR and PDR (Figure 5).

Figure 5. Fluorescein angiography of patients with NPDR and PDR. Fluorescein angiography of a patient with A) severe NPDR showing microaneurysms and hemorrhaging and B) PDR with neovascularization of the optic disc.



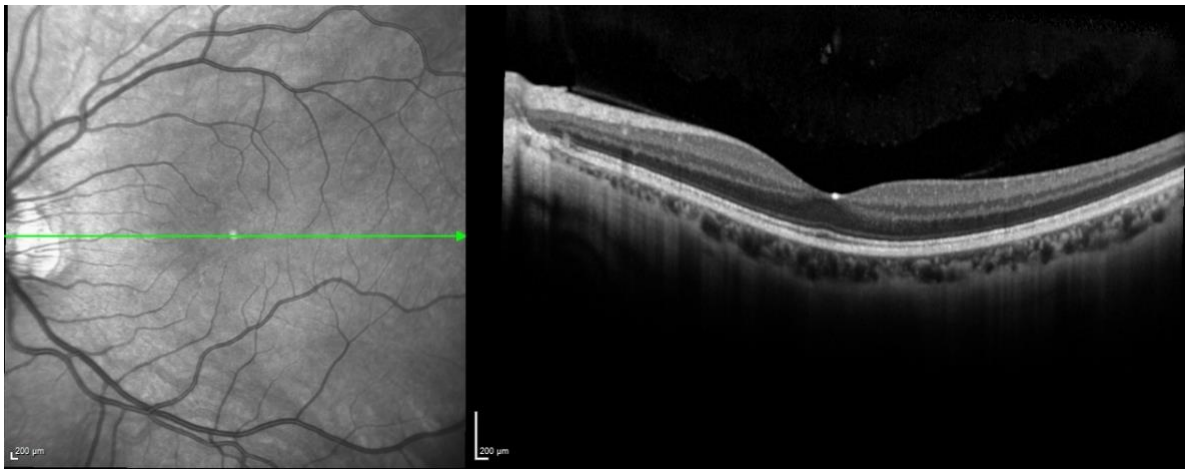
1.2.5 Diabetic macular edema

Among the patients with DR, NPDR or PDR, 7.4% have diabetic macular edema (DME), which is considered to be the most prominent vision threatening complication of DR[89]. The diagnosis and treatment of DME has become easier with advancements in fundus imaging, more specifically optical coherence tomography (OCT). With the use of OCT, DME can be diagnosed as central macular thickness greater than 250 micrometers due to accumulation of fluid within the macula (Figure 6)[90]. The macula, which is responsible for highest visual acuity, is located near the center of the retina and is made up of the fovea, parafovea, and perifovea[63]. Accumulation of fluid within the macula leads to distortion of the retinal layers ultimately leading to retinal dysfunction and blurred vision.

In a 2015 meta-analysis study it was determined that T1D patients are more likely to develop DR compared to T2D patients. Interestingly when looking at the progression towards the vision threatening stages of the disease, T1D patients trended towards developing neovascularization while T2D patients favored developing DME[91]. Whether or not the mechanisms for developing DME are different for T1D and T2D patients and if the efficacy of anti-VEGF therapy favors T2D compared to T1D patients or vice versa remains unclear.

Figure 6. Fundus and OCT Images of DME. A) Fundus (left) and OCT (right) image of a patient with a healthy retina compared to B) a fundus (left) and OCT (right) image of a patient with DME.

A



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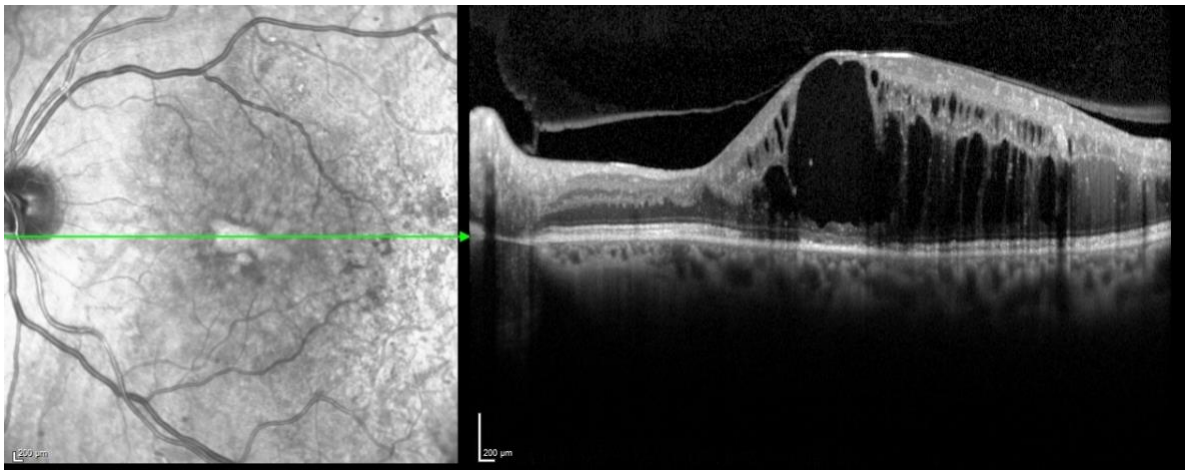


1.2.6 Current treatment strategies for diabetic retinopathy

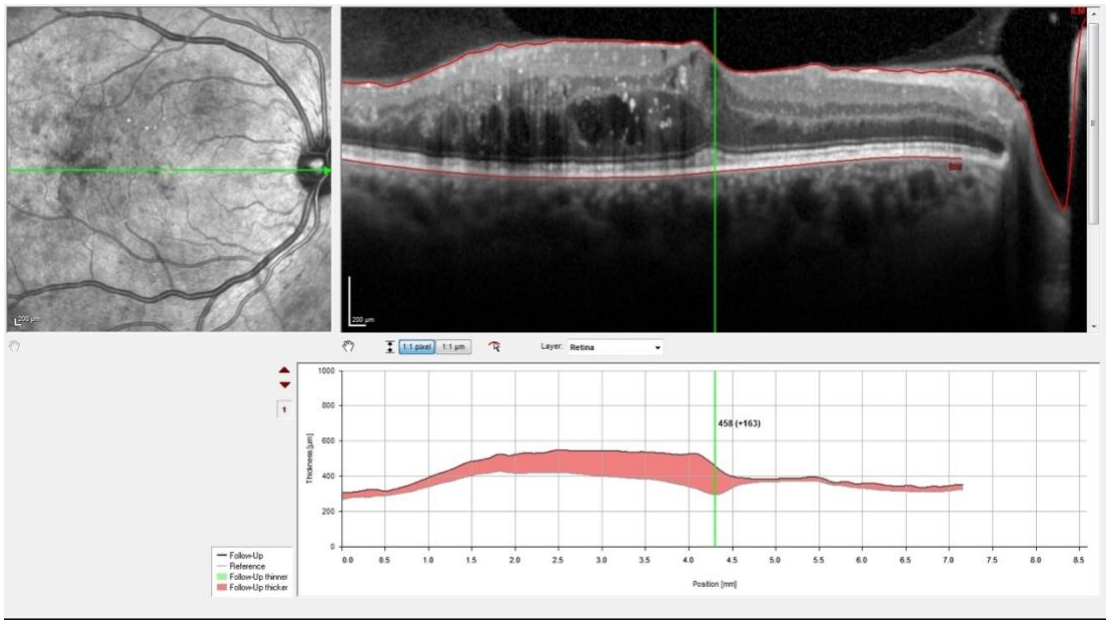
Current therapeutic strategies are aimed at treating PDR and DME, the more advanced and vision threatening stages of the disease. Available treatment strategies include laser photocoagulation, anti-VEGF therapy, pars plana vitrectomy, and intravitreal corticosteroids. The current treatment strategies of choice for patients with PDR and DME are laser photocoagulation and anti-VEGF therapy. According to the American Academy of Ophthalmology, laser photocoagulation is recommended for patients with severe NPDR, PDR, and clinically significant edema outside the fovea[92]. For this therapy, a laser is used to cauterize ocular blood vessels and destroy VEGF secreting cells in the peripheral retina in order to slow down the growth and leakage of new blood vessels. Although laser photocoagulation slows down PDR development, this therapy is associated with many complications, such as reduced night vision, hemorrhaging, and worsened visual acuity[93].

More recently, anti-VEGF therapy (intravitreal injection), such as ranibizumab and aflibercept, is indicated for patients with PDR or DME. Anti-VEGF therapy utilizes an antibody fragment against VEGF-A, known to be upregulated in the vitreous of diabetic patients, and works to prevent vessel growth and permeability[87,94,95]. Anti-VEGF therapy has proven to be effective in reducing central macular thickness (Figure 7). Although anti-VEGF therapy has proven to be effective in ameliorating neovascularization and macular edema, it is only effective for about one-third of the patient population[96]. Additionally, the long-term implications of anti-VEGF therapy have yet to be determined. Previous studies have shown VEGF-A to be protective in the neural component of the retina[97–100].

Therefore, anti-VEGF therapy may have short-term gains but more importantly long-term deficits. The second line of defense for patients who don't respond to laser photocoagulation or anti-VEGF therapy is the use of intravitreal corticosteroids, such as Ozurdex and Iluvien. Further studies are needed to develop newly targeted therapies for the treatment of DR, and more specifically, therapies aimed at targeting the earlier stages of the disease to slow down or prevent progression towards PDR and DME.

Figure 7. Anti-VEGF therapy for patients with DME. Fundus (left) and OCT (right) images of a patient with NPDR and DME A) before anti-VEGF therapy, and B) after anti-VEGF therapy.

A



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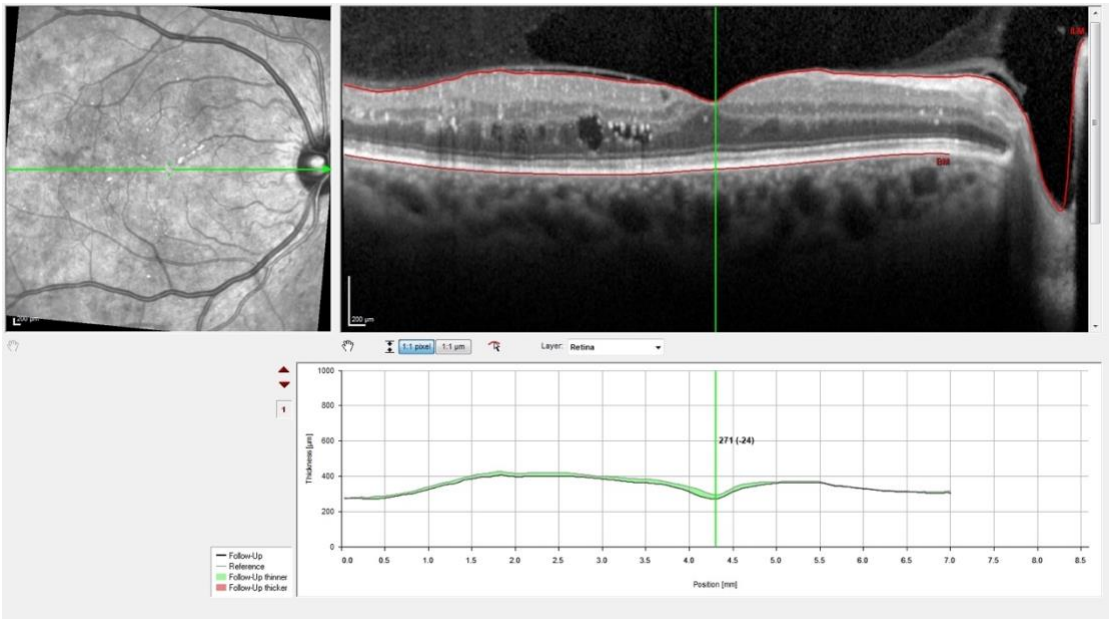
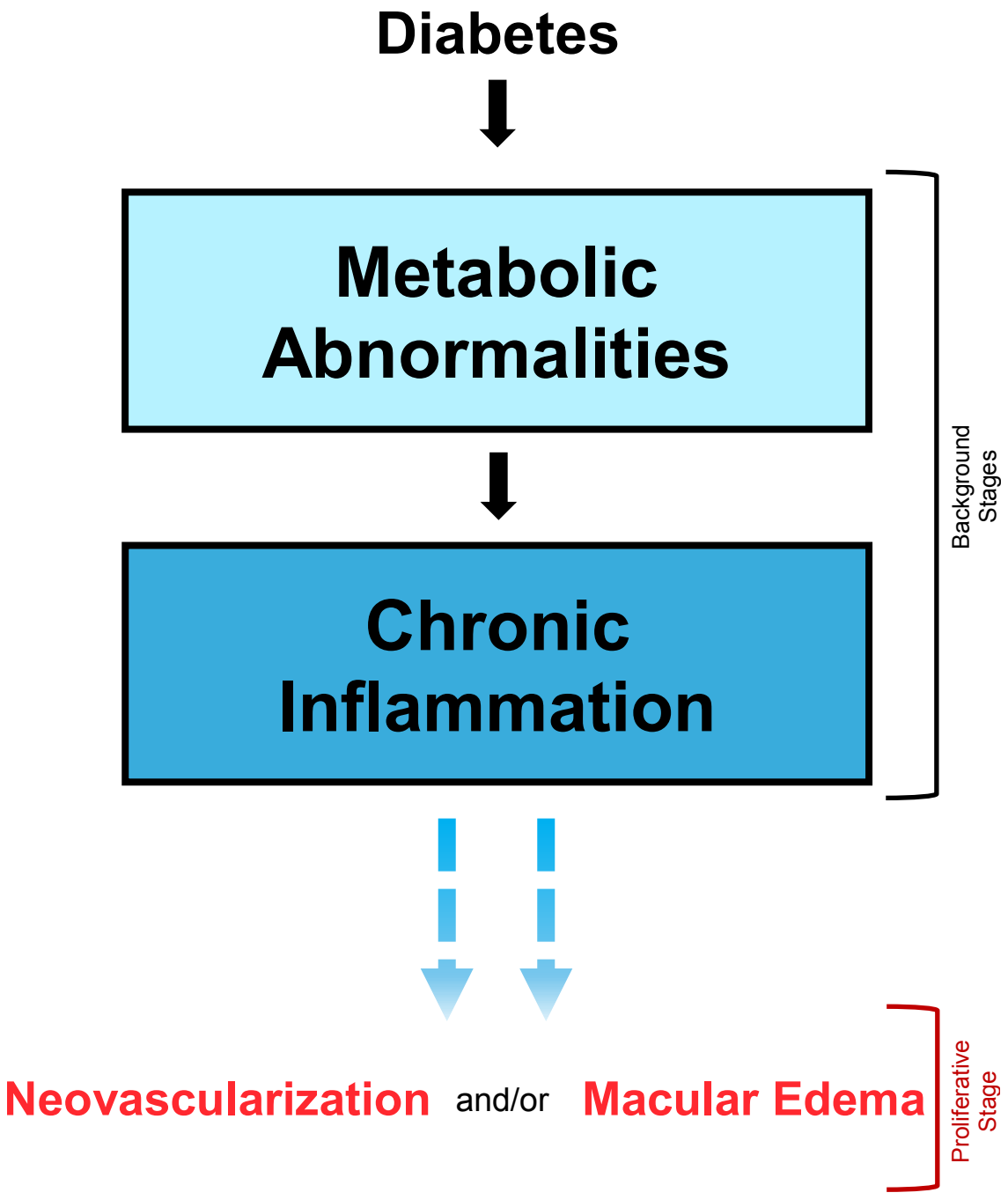


1.2.7 Mechanisms of DR – what do we know?

DR is unique in a way that the background stages of the disease can be present for years before any clinical signs become visible, making the disease difficult to promptly diagnose and treat. There are no known biomarkers identified to date that would allow to predict whether a diabetic patient will develop DR or not. The historic view for the development of DR was that chronic hyperglycemia promotes metabolic dysregulation in the retina, which leads to the formation of acellular capillaries (blood vessels that have no cells and are non-perfused) and subsequent oxygen deprivation. Lower oxygen levels were considered to be the trigger for increased VEGF-A production and neovascularization[101]. For a long time, DR was considered purely as vascular disease. However, newer studies have changed the view about how DR develops. It is more and more accepted that hyperglycemia and metabolic dysregulation within the retina causes a tissue-specific low-grade chronic inflammatory response. It seems that the chronic inflammatory environment is initiated, promoted, and maintained by the neuroretina. As a consequence, chronic inflammation by the neuroretina can directly or indirectly initiate neovascularization and/or macular edema (Figure 8). A wide variety of inflammatory cytokines, such as interleukin-1β (IL-1β), have been detected in the vitreous and aqueous humor of patients with DR[102–106]. Previous studies have shown hyperglycemia-induced chronic inflammation leads to the formation of acellular capillaries in an IL-1β dependent manner[107,108]. Additionally, inflammatory cytokines have been linked with promoting neovascularization by means of VEGF-A production[109,110].

Hyperglycemia-induced retinal inflammation has also been implicated in the development of DME[111]. For example, IL-1β and TNFα are known to decrease endothelial cell viability, alter cell to cell tight junctions, and increase vascular permeability[112–115]. Furthermore, other retinal cell types that are important for maintaining the blood retinal barrier, such as pericytes and Müller cells, are also negatively affected by inflammatory cytokines[116–119]. In addition to vascular permeability and extracellular fluid accumulation, intracellular cell swelling has also been suggested to play a role in DME. Müller cells, one of only two cell types found within the macula, are known to maintain ion and water balance in the retina. A prominent channel on Müller cells that plays a critical role in maintaining retinal water homeostasis is aquaporin 4 (AQP4). In the retinas of diabetic rats, Müller cell expression of AQP4 was upregulated and promoted cell enlargement. In addition, selective inhibition of AQP4 prevented volumetric changes in retinal Müller cells[120–122]. Since Müller cells are a unique cell type (being the only cell that spans the entire retina down to the macula, has contact with almost every retinal cell type, and surrounds the retinal vasculature) they have been discussed as a major player in the initiation and progression of DR.

Figure 8. Working hypothesis for the development of DR. Chronic hyperglycemia causes metabolic dysregulation and the initiation of a low-grade chronic inflammatory response in the retina. Chronic inflammation can then directly or indirectly promote neovascularization and/or macular edema.



1.3 Müller Cells and Diabetic Retinopathy

Authors: Brandon A. Coughlin, Derrick J. Feenstra, Susanne Mohr

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1.3.1 Abstract

Müller cells are one of the primary glial cell types found in the retina and play a significant role in maintaining retinal function and health. Since Müller cells are the only cell type to span the entire width of the retina and have contact to almost every cell type in the retina they are uniquely positioned to perform a wide variety of functions necessary to maintaining retinal homeostasis. In the healthy retina, Müller cells recycle neurotransmitters, prevent glutamate toxicity, redistribute ions by spatial buffering, participate in the retinoid cycle, and regulate nutrient supplies by multiple mechanisms. Any disturbance to the retinal environment is going to influence proper Müller cell function and well-being which in turn will affect the entire retina. This is evident in a disease like diabetic retinopathy where Müller cells contribute to neuronal dysfunction, the production of pro-angiogenic factors leading to neovascularization, the set-up of a chronic inflammatory retinal environment, and eventual cell death. In this review, we highlight the importance of Müller cells in maintaining a healthy and functioning retina and discuss various pathological events of diabetic retinopathy in which Müller cells seem to play a crucial role. The beneficial and detrimental effects of cytokine and growth factor

production by Müller cells on the microvasculature and retinal neuronal tissue will be outlined. Understanding Müller cell functions within the retina and restoring such function in diabetic retinopathy should become a cornerstone for developing effective therapies to treat diabetic retinopathy.

1.3.2 Introduction

Müller cells are the principle glia of the retina. They are the only cells to span the entire width of the retina and have intimate contact with both the retinal blood vessels and retinal neurons. Because of this arrangement, Müller cells have a variety of important functions in the healthy retina. Functions of Müller cells can be divided into 3 major categories: (1) Uptake and recycling of neurotransmitters, retinoic acid compounds, and ions (such as potassium K^+), (2) control of metabolism and supply of nutrients for the retina, and (3) regulation of blood flow and maintenance of the blood retinal barrier.

The extensive contact of Müller cells with retinal neurons allows Müller cells to actively participate in proper neurotransmission. They rapidly take up and clear glutamate and γ-aminobutyric acid (GABA) in the inner plexiform layer[123–126]. Studies have shown that Müller cells take up extracellular glutamate through the Glutamate Aspartate Transporter (GLAST) and indicate that glutamate removal and prevention of neurotoxicity in the retina is achieved primarily by this mechanism[127,128]. Once taken up, glutamate is converted to glutamine by glutamine synthetase and released back to neurons for re-synthesis of glutamate and GABA[129]. This process provides substrate for neurotransmitter synthesis and also prevents glutamate toxicity. Müller cells further maintain proper retinal function by participating in a process known as “potassium spatial buffering”, a process that

redistributes and normalizes K^+ in the surrounding microenvironment to avoid prolonged accumulation of K^+[130]. It has been shown that Müller cells can take up K^+ from the inner and outer plexiform layers where neuronal synapses occur and release the K^+ into the vitreous humor in an effort to redistribute K^+ ions[131]. This process is also involved in retinal fluid removal. Müller cells act as potassium shuttle by taking up potassium from the extracellular fluid through Kir2.1 potassium channels and depositing the potassium into the vasculature using Kir4.1 channels that are found on the Müller cell processes that encompass the blood vessels[132,133]. This leads to osmotic fluid removal through aquaporin-4[133–136].

In addition to regulating neurotransmitters and ion levels within the retina, Müller cells also participate in the retinoid cycle with cone photoreceptors by taking up all-*trans* retinol from the subretinal space[137–140]. During the visual cycle, photons of light lead to isomerization of 11-*cis* retinal to all-*trans* retinal in the rod and cone photoreceptors. Once isomerized, all-*trans* retinal is expelled from the opsin protein to be reduced by retinol dehydrogenases to all-*trans* retinol[141]. The all-*trans* retinol from the cones is then released into the extracellular space where it is taken up by Müller cells, isomerized back to 11-*cis* retinol by all-*trans* retinol isomerase, and released back to the extracellular space to be taken up by the cone photoreceptors where it can finally be oxidized from 11-*cis* retinol back to original 11-*cis* retinal to restart the visual cycle[137–139,142].

Müller cells seem a primary site of nutrient storage for the retina. It has been shown that ATP production in Müller cells drastically declines when glycolysis is inhibited.

However, ATP levels remained equal in aerobic versus anaerobic conditions as long as glucose was provided, indicating that Müller cells live primarily from glycolysis rather than oxidative phosphorylation[143]. This is important as it spares oxygen for retinal neurons and other cell types that use oxidative phosphorylation for ATP production. Furthermore, Müller cells are the primary site of glycogen storage in the retina[143,144]. When nutrient supplies are low Müller cells can utilize this glycogen storage to provide metabolites for other cell types. Furthermore, the large amounts of lactate they produce via glycolysis and irreversible conversion of pyruvate to lactate due to a specific lactate dehydrogenase isoform can be transported to photoreceptors to be used as a potential alternative source of energy in case of need[143,145,146]. Interestingly, studies suggest that the metabolism of glucose and glycogen by Müller cells is regulated by light being absorbed by the photoreceptors[129]. This means that as photoreceptors absorb light, the Müller cells respond by metabolizing more glucose in order to provide more lactate for photoreceptors as needed, indicating that Müller cells and photoreceptors are tightly coupled in their respective functions by metabolism. In addition to providing lactate as a fuel source for photoreceptors, Müller cells can also regulate nutrient supplies to the retina via regulation of retinal blood flow. In a healthy retina, increased light stimulation results in increased retinal blood flow, which is required to supply the activated neurons with oxygen and other nutrients, a process termed neurovascular coupling. Müller cells play a crucial role in neurovascular coupling as they release metabolites controlling vasoconstriction and vasodilation of retinal blood vessels[147,148].

One of the most important functions of Müller cells is their regulation of retinal blood flow and contribution to the blood retinal barrier. The blood retinal barrier is essential for preventing leakage of blood and other potentially harmful stimuli such as pathogens from entering the retinal tissue. It has been shown that Müller cells induce blood-barrier properties in retinal endothelial cells[149,150]. Studies using conditional ablation of Müller cells showed severe blood retinal barrier breakdown[151]. The exact mechanism of how Müller cells maintain the blood retinal barrier is debated but includes the secretion of factors such as pigment epithelium-derived factor (PEDF) and thrombospondin-1 which are anti-angiogenic and increase the tightness of the endothelial barrier[152,153].

It is clear that Müller cells are an integral part of a healthy and well-functioning retina. Any disturbance to these cells certainly affects cellular cross-talk within the retina and its proper function. However, despite their importance Müller cells are still an under-studied cell type in the context of diseases such as diabetic retinopathy. The following aims to provide an overview about the effects of diabetes on Müller cells and the role Müller cells play in pathological events in the diabetic retina.

1.3.3 Influence of diabetes on neurotransmitter and potassium regulation in Müller cells

Functional changes that have been determined in Müller cells begin early in the disease, with significant decreases in glutamate transport via GLAST beginning after just 4 weeks of diabetes in rats[154]. This is consistent with reports showing significantly increased glutamate accumulation in the retinas of diabetic rats[155,156]. Furthermore, these studies have shown that there is decreased glutamine synthetase activity and a

subsequent decrease in the conversion of glutamate to glutamine necessary for neurotransmitter regeneration[155,156]. These results are in line with reports demonstrating glutamate increases to a potentially neurotoxic level in the vitreous of diabetic patients[157]. However, in neurological diseases such as stroke, therapies targeting glutamate increase have been ineffective indicating that increased glutamate levels might not play a pathophysiological role[158,159]. Whether increased glutamate levels actually cause neurotoxicity over time in diabetic retinopathy has yet to be determined.

It seems that Müller cells not only contribute to glutamate toxicity directly by decreased glutamate uptake, but Müller cells also contribute indirectly via decreased K^+ uptake during the progression of diabetic retinopathy. There is decreased K^+ conductance on the plasma membrane of Müller cells isolated from rat retinas after 4 months of experimental diabetes[160]. Redistribution of the Kir4.1 K^+ channel has been identified as the mechanism of decreased K^+ conductance[160]. This decrease in K^+ conductance was also observed in Müller cells of patients with proliferative diabetic retinopathy[161]. Alteration of the Kir4.1 K^+ channel localization in Müller cells in the diabetic retina has been attributed to the accumulation of advanced glycation endproducts (AGEs)[162]. Together, this can lead to an imbalance in K^+ concentrations and altered K^+ homeostasis leading to neuronal excitation and subsequent glutamate toxicity.

In diabetes and diabetic macular edema, Müller cells have been shown to downregulate the Kir4.1 channels, but not Kir2.1, leading to continued potassium uptake with no release into the microvasculature[160,163,164]. This leads to subsequent swelling of

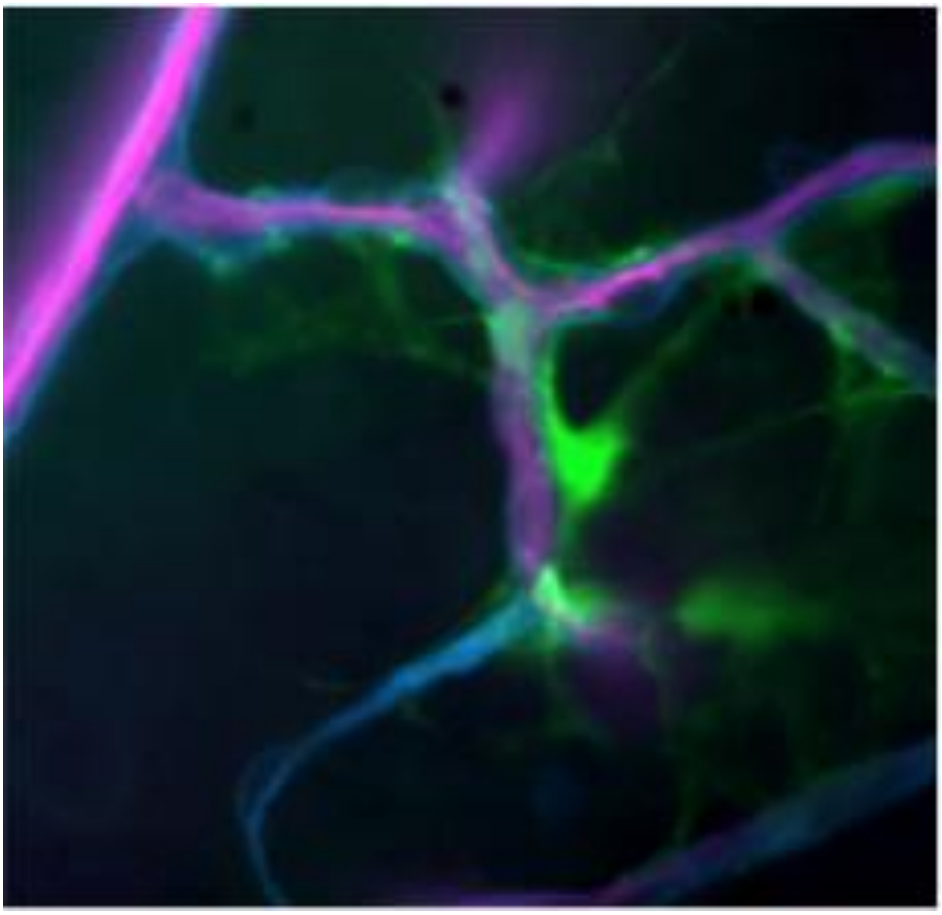
Müller cells contributing to Müller cell dysfunction and decreased fluid removal contributing to diabetic macular edema. Diabetic macular edema leads to thickening of the macula due to fluid accumulation and can be observed by optical coherence tomography (OCT). The thickening of the macula due to fluid accumulation typically leads to disruption of the retinal structure and changes in visual acuity.

1.3.4 Release of growth factors and pro-/anti-inflammatory cytokines from Müller cells in response to hyperglycemia – the bad and the potentially good

As already stated above, Müller cell have contact with every cell in the retina. Müller cell ablation leads to photoreceptor degeneration, vascular leak, and intraretinal neovascularization demonstrating that Müller cells are necessary for both neuronal and vascular function and viability[151,165]. Changes to their environment by hyperglycemia alters functional interaction with pericytes[166]. Deletion of the dystrophin-Dp71 protein within Müller cells caused extensive vascular leakage and edema in the mouse retina. It was suggested that breakdown of the blood retinal barrier was initiated by improper localization of proteins in the endfeet of Müller cells that are necessary for establishing barrier function[167]. Other studies have shown that Müller cells participate in regulation of vascular tone in a process of neurovascular coupling[147,148]. They are also seemingly involved in lactate exchange with neurons, glia, and vascular cells[168]. Given the intricate contact Müller cells have with other retinal cell types it is easy to see that any disturbance to Müller cells will certainly affect proper function and viability of neurons as well as cell of the microvasculature.

In diabetes, it has been well established that Müller cells become activated[169–172]. One of the most prominent signs that Müller cells are activated in diabetic retinopathy is the increased expression of glial fibrillary acidic protein (GFAP), a common marker of reactive gliosis[155,170,173]. In healthy conditions, Müller cells generally do not express GFAP[116,169]. Interestingly, while Müller cells upregulate GFAP expression in the diabetic retina astrocytes seemingly downregulate GFAP expression[174]. Figure 9 demonstrates the high level of GFAP expression in Müller cells in the diabetic retina. It also highlights the extensive contact that Müller cells have with the retinal microvasculature making it easy to comprehend the influence activated Müller cells have on proper function of the microvasculature.

Figure 9. Müller cell interaction with the retinal vasculature. Picture (40x) taken of a retinal flat mount obtained from a STZ (streptozotocin) diabetic mouse that expressed a GFP (green fluorescence protein) tagged GFAP specifically in Müller cells. Duration of diabetes: 10 weeks. *Purple*: microvasculature; *Green*: Müller cells.



Despite GFAP several other markers might be more useful to determine early glial activation such as phospho-ERK (extracellular signal-regulated kinase)[175].Although elevated GFAP expression happens early and persists throughout the disease, no study to date has been able to connect increased levels of GFAP to any functional outcome. However, hyperglycemia-induced gliosis goes hand in hand with stimulation of growth factor, cytokine, and chemokine release by Müller cells at least *in vitro*. Hyperglycemia promotes release of (1) growth factors, such as vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF), and (2) cytokines and chemokines including interleukin-1β (IL-β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and chemokine ligand-2 (CCL2)[116,176–182,183–185]. *In vitro* studies have provided ample evidence that Müller cells are a potential source for growth factors and cytokines when stimulated with elevated glucose levels. Considering that most of the growth factors, cytokines, and chemokines released by Müller cells have been identified in the vitreous of diabetic patients it is fair to assume that Müller cells contribute to the overall synthesis of these factors *in vivo*[88,102,186,187].

Growth factors – the bad: How much Müller cell derived growth factors really contribute to the pathology of diabetic retinopathy *in vivo* is still not fully understood. The first studies to understand the contribution and effect of Müller cell derived VEGF to the development and progression of diabetic retinopathy were done by the group of Y.Z. Le. This group disrupted VEGF in Müller cells with an inducible Cre/*lox* system and examined diabetes-induced retinal inflammation and vascular leakage in these conditional VEGF knockout (KO) mice. The diabetic conditional VEGF KO mice exhibited an overall

decrease in parameters associated with the pathology of diabetic retinopathy such as leukostasis, expression of inflammatory biomarkers, depletion of tight junction proteins, numbers of acellular capillaries, and vascular leakage compared to diabetic control mice[180,188,189]. Additional studies focusing on altering known regulators of VEGF production such as HIF-1 (hypoxia inducible factor 1)[190] and the Wnt signaling pathway[191] specifically in Müller cells have supported the notion that Müller cell derived VEGF is actually a major component in the process of retinal angiogenesis and pathology in diabetic retinopathy. Besides VEGF, Müller cell derived PEDF has also been suggested to have its part in diabetes-induced retinal angiogenesis[152]. Taken together, it seems that Müller cell derived growth factors contribute heavily to pathological vascular events in diabetic retinopathy.

Growth factors – the potentially good: Although Müller cell derived VEGF contributes to detrimental effects on the microvasculature in the diabetic retina, the intent of such growth factor production by Müller cells in the first place might have been to protect itself and the retinal neurons from a diabetic insult. This idea is supported by a study using mice that carry a disrupted VEGFR2 specifically in Müller cells. Loss of VEGFR2 caused a gradual reduction in Müller glial density, decreased of scotopic and photopic electroretinography amplitudes, and accelerated loss of photoreceptors, ganglion cells, and inner nuclear layer neurons in the diabetic retina[100]. More studies are needed to fully explore and understand the beneficial effects of Müller cell derived growth factors on Müller cells itself and retinal neurons in the context of disease. This is especially important

since long-term anti-VEGF treatment might hamper functional integrity of Müller cells and neurons causing unexpected additional problems in treating diabetic retinopathy.

Cytokines – the bad: Besides growth factors, Müller cells release a variety of cytokines and chemokines under hyperglycemic conditions. For example, Müller cells are a major source of retinal interleukin-1beta (IL-1β) production[112,116,183–185]. Caspase-1, originally named interleukin-1β converting enzyme (ICE), produces the active cytokines IL-1β and IL-18 by cleavage of their inactive proform[192–195]. In Müller cells, hyperglycemia strongly induces the activation of the caspase-1/IL-1β signaling pathway as we have previously shown[116,184]. Increased caspase-1 activation and elevated IL-1β levels have also been identified in the retinas of diabetic mice and retinal tissue and vitreous fluid of diabetic patients[112,184,186,196,197]. We have identified that targeting this pathway by knocking down caspase-1 or the IL-1 receptor (IL-1R1) or by pharmacological intervention protects against the development of diabetic retinopathy in diabetic rats and mice[183,198]. Prolonged IL-1β production by Müller cells has been shown to affect endothelial cell viability in a paracrine fashion[112]. Endothelial cells are extremely susceptible to IL-1β and rapidly progress to cell death in response to this pro-inflammatory cytokine[112]. Endothelial cell death is detectable in the retinal microvasculature of diabetic animals and isolated retinal blood vessels of diabetic donors and has been associated with the formation of acellular capillaries, a hallmark of retinal pathology in diabetic retinopathy[199]. Besides IL-1β, Müller cells produce other well-known pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6)[116,183,198,200–203]. Anti-TNFα therapy has been proposed as a strategy to treat diabetic retinopathy in diabetic animals[115,204–

[206]. Detrimental effects of IL-6 have been associated with vascular dysfunction and promotion of angiogenesis[207–209] which is why IL-6 recently has become a new therapeutic target of interest to prevent diabetes-induced vascular damage. The production and release of pro-inflammatory cytokines by Müller cells strongly contributes to the chronic inflammatory environment detected in the diabetic retina that over time promotes drop-out of retinal cells.

Cytokines – the potentially good: From a vascular perspective, IL-6 has been solely associated with detrimental effects[207–209]. However, we have previously shown that IL-6 prevents hyperglycemia-induced Müller cell dysfunction and loss clearly supporting a beneficial and protective nature of IL-6[116]. This observation is well in line with reports that in the retina IL-6 is an important cytokine responsible for maintaining proper neuronal function as well as stimulating neuroprotective effects[116,210–213]. Treatment with IL-6 has been shown to protect retinal ganglion cells from pressure-induced cell death[210]. Additionally, in an experimental model of retinal detachment, genetic ablation or neutralization of IL-6 led to a significant increase in photoreceptor cell death. However, treatment with exogenous IL-6 resulted in a significant increase in photoreceptor density in the outer nuclear layer[213]. These different effects of IL-6 can potentially be attributed to the two distinct signaling pathways IL-6 acts through. Classical IL-6 signaling - thought to be the anti-inflammatory and protective pathway - is mediated by the membrane-bound form of the IL-6 receptor (IL-6R) and the ubiquitously expressed glycoprotein 130 (gp130). Only cells such as Müller cells (but not endothelial cells) that express IL-6R are able to signal through classical IL-6 signaling. Conversely, IL-6 trans-signaling, which is mediated

by binding of IL-6 to the soluble form of the IL-6 receptor (sIL-6R) and gp130, is thought to be the more pro-inflammatory and pro-angiogenic pathway[116,207,208,211,214–223]. In diabetic patients, correlations between increased levels of IL-6 and the development of complications in the eye have been made[224–229]. However, whether IL-6 levels are increased in diabetes as an attempt to protect from a pro-inflammatory environment or whether high levels of IL-6 synergistically exaggerate diabetes-induced inflammation has yet to be determined.

1.3.5 Müller cell loss in diabetic retinopathy

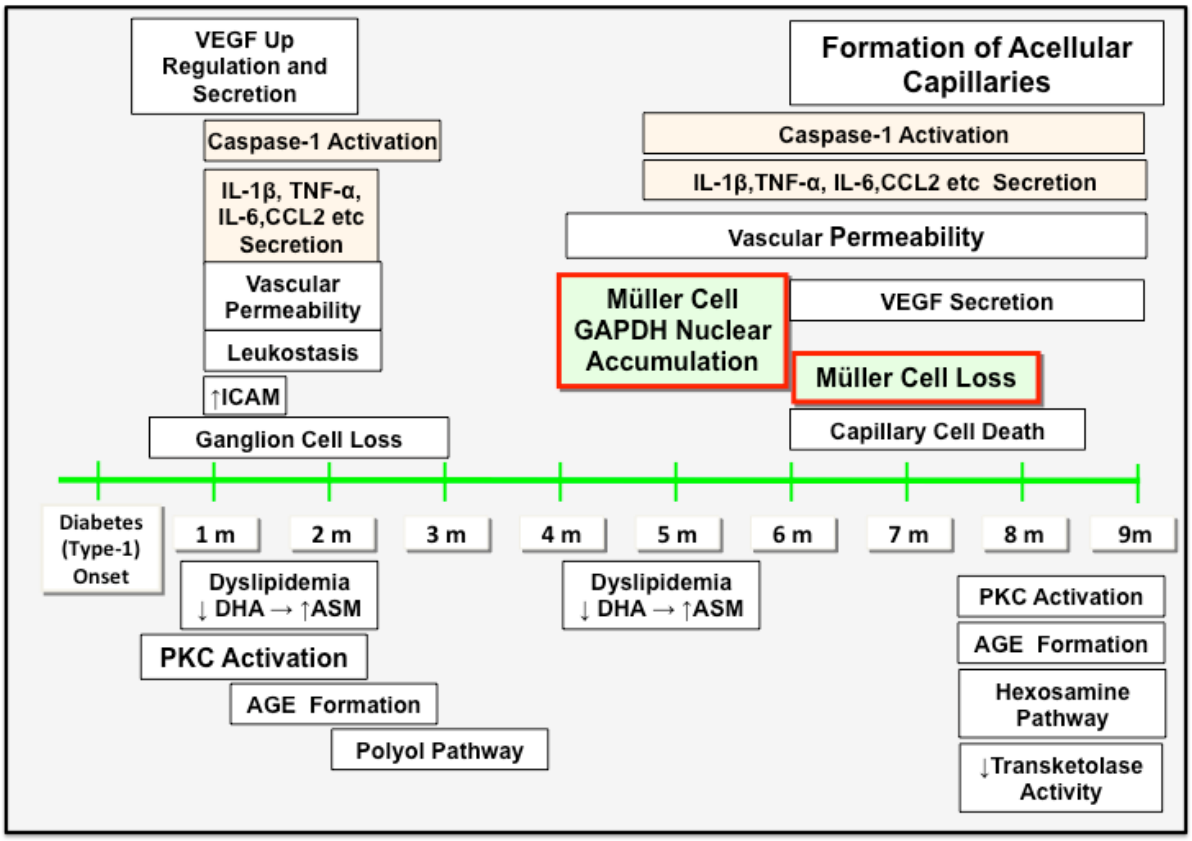
Whether Müller cells die in diabetic retinopathy has long been a matter of debate. It is easy to see that Müller cells are “sturdy” cells taking into account how well equipped these cells are to produce fair amounts of protective factors that shield them at least in the beginning from a chronic diabetic insult as discussed above. However, newer studies indicate that over time Müller cells actually do begin to die the longer diabetic retinopathy progresses. Frequency of Müller cell death in the diabetic retina rapidly accelerates when protective growth factors are blunted[100].

Better understanding of types of cell deaths has furthered studies to look for mechanisms other than apoptosis by which Müller cells can die in a diabetic environment. We have identified one particular mechanism of cell death that stands out and can explain histological features described for Müller cells in the diabetic retina. Pyroptosis is an inflammatory driven type of cell death that depends on caspase-1 activation[230–232]. Müller cells show increased caspase-1 activity and IL-1β production following exposure to

hyperglycemic conditions and cells die as a consequence[202,233]. While it is known that initiation of pyroptosis is caspase-1 and IL-1β driven, the execution phase of pyroptosis is not yet completely understood. Execution of pyroptosis shares traits with both apoptosis and necrosis[234,235]. Since execution of pyropototic cell death lacks specific marker, identifying retinal cells dying by pyroptosis *in vivo* is a difficult task. Markers such as TUNEL staining used to detect apoptotic cell death may not adequately detect pyroptosis. Therefore, we have performed a study actually counting Müller cells in the healthy and diabetic retina and determined roughly 15% cell death at 7 months of diabetes[201]. Even more important, inhibition of the caspase-1/IL-1β pathway inhibited diabetes-induced Müller cell death *in vivo* as we had previously shown *in vitro*[116,183,201]. Several other studies are in line with our observation that Müller cells die in a hyperglycemic environment. The first study to describe dying Müller cells in diabetic retinopathy was done using EM analysis[236]. Dying Müller cells are described as being hypertrophic consistent with the notion that during pyroptosis, cells swell rather than shrink as observed in apoptotic cell death[170]. To collect more evidence for Müller cells death in the diabetic retina we looked at earlier markers of cell death and we have identified that GAPDH (glyceraldehyde-3-phosphate dehydrogenase) accumulates in the nucleus of Müller cells in the retinas of diabetic rats[172]. Nuclear accumulation of GAPDH has been closely associated with cell death induction[237–239]. Consistent with our finding that Müller cells die by pyroptotic cell death, hyperglycemia-induced nuclear accumulation of GAPDH depends on the activation of the caspase-1/IL-1β pathway[116,240]. The consequences of dying Müller cells are multi-faceted. On the bad side – Müller cell death will promote loss of retinal blood barrier integrity, increased vascular permeability, and loss of

neuroprotection affecting both neurons and vascular cells. Loss of Müller cells in diabetes has also been associated with aneurysm formation, a clinical characteristic of diabetic retinopathy[236]. However, one can also argue that on the good side – removal of activated and pro-inflammatory Müller cells might be a “shut off” mechanism to deal with an increasing inflammatory environment in the diabetic retina. A lot more studies are needed to determine the full pathway of Müller cells death and to identify whether all Müller cells are equally affected by hyperglycemia (Figure 10).

Figure 10. STZ diabetic mice and rat DR timeline. Timeline of caspase-1 activation, cytokine secretion, Müller cell death initiation and execution in comparison to other prominent events associated with diabetic retinopathy in retinas of STZ diabetic mice and rats.



1.3.6 Conclusion

Müller cells are a major component of a healthy retinal environment. Once chronic hyperglycemia disturbs their environment, Müller cells become dysfunctional and start activating pathways to counter-regulate and “repair” the environment.

In order to do so, Müller cells release a large variety of growth factors and cytokines in a diabetic environment. Most of the research to date has focused on the detrimental effects the release of these growth factors and cytokines causes to the retina. When taking a closer look most of these effects are associated with vascular dysfunction and angiogenesis. On the other hand, it seems that production of these growth factors and cytokines by Müller cells are primarily intended to protect Müller cells and consequently retinal neurons from diabetic insult and might only secondarily turn into the damaging components observed in diabetic retinopathy. Very few studies have started to consider the protective nature of Müller cell derived growth factors and cytokines in regards to the integrity of glia cells and neurons. A lot more studies are needed to understand the nature of Müller cells derived growth factors and cytokines. For a successful development of a new therapy targeting these factors both detrimental as well as beneficial effects need to be considered.

Understanding Müller cell functions within the retina and restoring such function in diabetic retinopathy should become a cornerstone for developing effective therapies to treat diabetic retinopathy. Some approaches have been tested to increase Müller cell

function by stimulating the beta-adrenergic pathway[241,242]. Whether these studies materialize into effective therapy strategies has to be seen in the future.

1.4 Interleukin-6 (IL-6) Biology

1.4.1 Introduction

IL-6 is an inflammatory cytokine released by multiple retinal cell types, such as Müller cells, and due to its pleiotropic capabilities it is one of the most highly debated cytokines in the field of DR. IL-6 is known to be elevated in the vitreous and aqueous humor of patients with DR, however, its role in disease progression remains unclear[105,243]. Even though very little is known about IL-6 and IL-6 signaling in the healthy or diabetic retina, progress is being made towards developing targeted therapies against IL-6. Therefore, it is crucial to gain a better understanding of IL-6 signaling and its potential role in disease development in order to determine if targeting IL-6 and IL-6 signaling would be efficacious for the treatment of DR.

1.4.2 IL-6 family, receptors, and structure

The IL-6 family is a group of cytokines that includes: IL-6, interleukin-11 (IL-11), leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-like cytokine (CLC), and cardiotrophin-1 (CT-1). In order to promote their desired response, each of these IL-6 family members signals through a membrane-bound co-receptor known as glycoprotein 130 (gp130), which is ubiquitously expressed on most cell types. In addition, what makes each family member unique is the primary receptor

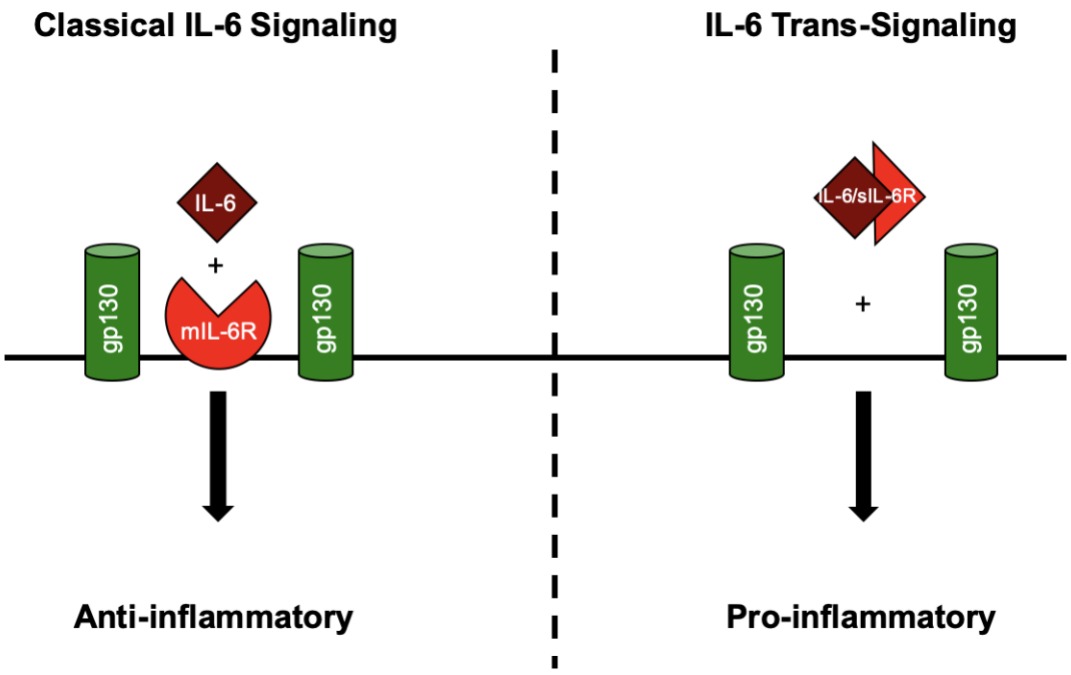
each cytokine binds with in order to interact with gp130. For example, in order for IL-6 to promote downstream signaling it needs to bind to an IL-6 receptor, either the membrane-bound IL-6 receptor (mIL-6R) or the soluble IL-6 receptor (sIL-6R), and then the co-receptor gp130. IL-6 is a 21-28 kilo-Dalton protein made up of four alpha helices containing 185 amino acids and three distinct binding sites. Binding site one facilitates the interaction between IL-6 and mIL-6R or sIL-6R, while binding sites two and three interact with gp130[214,244–248]. IL-6 is a pleiotropic cytokine whose function is dependent on microenvironment, cell type, and receptor expression.

1.4.3 IL-6 signaling: classical IL-6 versus IL-6 trans-signaling

Depending on the microenvironment, cell type, and receptor expression patterns, IL-6 can either act as an anti-inflammatory or pro-inflammatory cytokine. The diverse actions of IL-6 are due to IL-6 having two main signaling pathways: classical IL-6 signaling and IL-6 trans-signaling[214,249]. Typically, classical IL-6 signaling has been associated with anti-inflammatory or regenerative effects, while IL-6 trans-signaling is mostly considered to be pro-inflammatory or pathogenic in nature[222,250]. In classical IL-6 signaling, IL-6 binds to the mIL-6R found only on select cells types, such as immune cells, certain epithelial cells, and select neuronal cell types. Once bound, the IL-6/mIL-6R complex can then interact with two molecules of gp130, which is expressed by most cell types. This four component complex will then activate multiple downstream signaling pathways including JAK/STAT3, PI3K/AKT, and MAP kinase pathways (Figure 11)[251–253].

On the other hand, IL-6 trans-signaling occurs in the absence of the mIL-6R and in the presence of the sIL-6R. The sIL-6R can be generated by two main processes: proteolytic cleavage of the mIL-6R by a disintegrin and metalloproteinase domain-contain protein 10 or 17 (ADAM 10 or 17) or alternative splicing of the mIL-6R mRNA[254,255]. Once bound to the sIL-6R, the IL-6/sIL-6R complex can bind and promote dimerization of two gp130 molecules and activate downstream signaling[256–258]. Generation of the sIL-6R through proteolytic cleavage by ADAM 10 or 17 and subsequent formation of the IL-6/sIL-6R complex is considered necessary for pathogenic IL-6 trans-signaling[259]. Furthermore, the body contains a natural inhibitor of the IL-6 trans-signaling pathway, soluble glycoprotein 130 (sgp130). Similar to sIL-6R, sgp130 can be generated by alternative splicing[221,260]. Once generated, the sgp130 can bind to the IL-6/sIL-6R complex and prevent this complex from binding to gp130 and ultimately prevent IL-6 trans-signaling. In a variety of inflammatory-based diseases, such as arthritis and Crohn's disease, IL-6 trans-signaling has been shown to play a role in exacerbating disease pathology, and blockade of IL-6 trans-signaling can attenuate pathogenesis[221,261]. Both classical IL-6 and IL-6 trans-signaling have important physiological roles, but it is when these pathways lack regulation that we see disease-associated pathology.

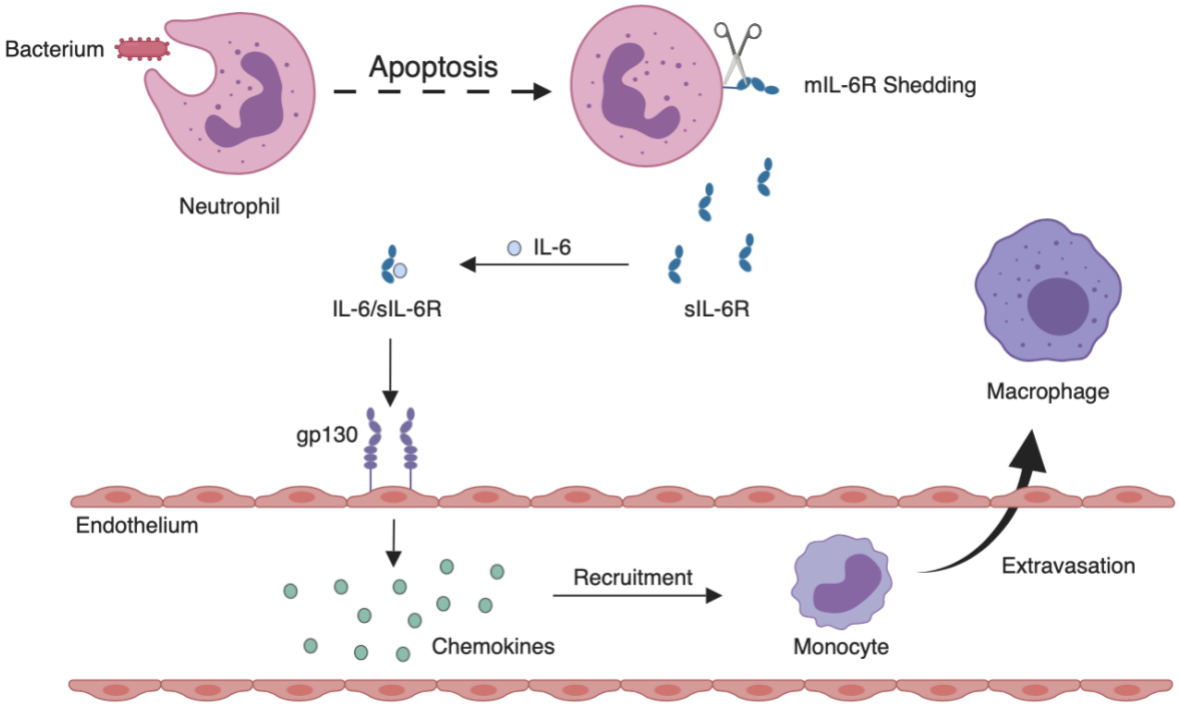
Figure 11. Classical IL-6 signaling vs. IL-6 trans-signaling. Classical IL-6 signaling mediated through the mIL-6R is thought to be more anti-inflammatory in nature, while IL-6 trans-signaling mediated by the sIL-6R is associated with more pro-inflammatory events.



1.4.4 Relationship of IL-6 systemic function and receptor expression

The potentially harmful pro-inflammatory effects of IL-6 trans-signaling have been associated with a wide variety of chronic inflammatory diseases, and one most commonly linked with IL-6 trans-signaling is rheumatoid arthritis[261–265]. In order to test the effects of IL-6 trans-signaling, IL-6 knockout mice have been used in designing experimental models of arthritis. In these experimental models, administration of IL-6 was not sufficient to induce arthritis associated pathology. However, the use of an IL-6/sIL-6R fusion protein (HYPER-IL-6), an agonist of IL-6 trans-signaling, was able to promote pathogenic activity: increased chemokine secretion, leukocyte recruitment and adhesion, and thickening of the synovial lining[266–268]. Additionally, it has also been reported that IL-6 trans-signaling prevents certain immune cells from undergoing apoptosis through recruitment of Bcl-2 and Bcl-x_L[269,270]. Furthermore, blocking IL-6 trans-signaling through sgp130 slowed disease progression and ameliorated experimental arthritis associated pathology[266,271]. Although IL-6 trans-signaling has long been associated with harmful pro-inflammatory properties, it also plays a crucial role in initiating a physiological acute inflammatory response[272]. For example, it has been demonstrated that apoptosis of neutrophils promotes the activation of ADAM 17 and subsequent shedding of the mIL-6R. Once generated, the IL-6/sIL-6R complex interacts with gp130 on endothelial cells and leads to the release of mononuclear phagocytic chemokines[273,274]. These chemokines recruit monocytes to the site of inflammation and take part in the clearance of neutrophils. This acute inflammatory process is non-functional in IL-6 knockout mice, which further establishes an important physiological role for IL-6 trans-signaling (Figure 12)[275].

Figure 12. Physiological role for IL-6 trans-signaling in an acute inflammatory response. Apoptosis of neutrophils in a physiological acute inflammatory response leads to the shedding of the mIL-6R and the formation of the IL-6/sIL-6R complex. The IL-6/sIL-6R then binds with gp130 on endothelial cells to promote chemokine production and recruitment of mononuclear phagocytic cells, such as monocytes. *Created with Biorender.com



While IL-6 trans-signaling is mostly associated with pro-inflammatory processes and progression of disease pathology, classical IL-6 signaling is thought to be more anti-inflammatory and regenerative. The anti-inflammatory properties of IL-6 have largely been associated with the brain and peripheral nervous tissue by protecting against neuronal cell loss and inducing nerve regeneration[276,277]. Previous studies have shown regeneration of the hypoglossal nerve after ligation injury was dependent on both IL-6 and mIL-6R. Interestingly, treatment with an anti-mIL-6R antibody significantly stunted the regenerative properties of the severed hypoglossal nerve, while transgenic overexpression of both IL-6 and mIL-6R accelerated nerve regeneration[278]. Another tissue linked with the regenerative properties of IL-6 is the intestinal epithelium. Studies using the cecum puncture ligation model to study sepsis have shown that blockage of IL-6 trans signaling using sgp130 compared to global IL-6 knockout was more sufficient to protect mice from death and allow for regeneration of the gut epithelium. This indicates that left over IL-6 not bound to the sIL-6R is responsible for the regeneration of the gut epithelium. Due to the fact that intestinal epithelial cells have the mIL-6R, it can be concluded that the regenerative properties of IL-6 likely depend on classical IL-6 signaling[279,280].

1.4.5 IL-6 function in the eye

Recently, more emphasis has been placed on IL-6 and the role it may play in different ocular diseases. Vitreous and aqueous humor levels of IL-6 have been elevated in multiple diseases such as glaucoma and diabetic retinopathy[281–284]. However, whether or not IL-6 is present as an anti- or pro-inflammatory cytokine has yet to be determined and

the actions of IL-6 are most likely different on a case by case basis. One such example is the ability of IL-6 to protect against retinal ganglion and photoreceptor cell loss in response to experimental models of glaucoma and retinal detachment, respectively. Previous *in vitro* studies using primary retinal ganglion cells showed increased retinal ganglion cell death in response to elevated pressure (70mmHg); however, treatment with media containing IL-6 or recombinant IL-6 significantly prevented retinal ganglion cell loss. IL-6 treatment was able to reduce pro-apoptotic genes, such as c-jun and jun-B, and reduce the frequency of TUNEL labeling[210]. Similarly, IL-6 is also crucial for the survival of photoreceptors in response to retinal detachment *in vivo.* More specifically, in an experimental model of retinal detachment, IL-6 knockout mice or injections of an IL-6 neutralizing antibody into the subretinal space at the time of detachment significantly increased TUNEL positive staining and thinning of the outer nuclear layer. When treated with exogenous IL-6 at the time of detachment the outer nuclear layer was preserved[213]. One of the most well-known functions associated with IL-6 is neuroprotection, but IL-6 has also been implicated in promoting epithelial cell migration as well as inducing angiogenesis[285,286]. The link between IL-6 and angiogenesis is of great interest, especially due to the fact that uncontrolled angiogenesis is largely associated with retinal pathology in DR.

1.4.6 IL-6 and diabetic retinopathy

Many inflammatory cytokines and growth factors have been implicated in the initiation and development of diabetic retinopathy, and one cytokine currently in the spotlight is IL-6. IL-6 has been shown to be elevated in the vitreous and aqueous humor of diabetic

patients, however, very few studies have looked into the effects of IL-6 and IL-6 signaling in the diabetic retina[104–106,224,284,287,288]. The few studies that have been done tend to associate adverse effects of IL-6 with the retinal vasculature and the beneficial effects with the neuroretina. For instance, IL-6 has been implicated in promoting vascular inflammation and retinal endothelial cell barrier disruption. Previous studies have shown that streptozotocin-induced diabetes leads to a greater than two-fold increase in the number of leukocytes adhered to retinal vessels and greater than five-fold increase in albumin extravasation. Yet, mice deficient of IL-6 had a reduction in leukocyte adhesion and albumin extravasation by 75%[289]. More specifically, IL-6 trans-signaling has been shown to promote vascular inflammation and barrier disruption *in vitro*, and selective inhibition of IL-6 trans-signaling by sgp130 attenuates this response[290,291]. On the other hand, the beneficial effects of IL-6 on the neuroretina are linked to neuroprotection.

As previously discussed, retinal Müller cells play an essential role in maintaining the blood retinal barrier. Previous studies have shown hyperglycemia to promote Müller cell death, and exogenous treatment of IL-6 *in vitro* prevents Müller cells from hyperglycemia-induced cell death. Additionally, treatment with IL-6 prevents IL-1β induced nuclear accumulation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), considered a precursor to Müller cell death[116]. Nevertheless, the exact mechanism behind the beneficial effects of IL-6 are unknown. Therefore, future studies are needed in order to determine the role of IL-6 in the diabetic retina, and to determine if targeting IL-6 and IL-6 signaling may be effective for the treatment of DR.

1.5 Dissertation Objectives

To date, the role of IL-6 in the initiation and progression of diabetic retinopathy remains unclear. Nevertheless, advances are being made towards developing new therapeutic strategies targeting IL-6, such as a neutralizing IL-6 antibody, for the treatment of DR without having a complete understanding of IL-6 and IL-6 signaling in the diabetic retina. Therefore, the working hypothesis examined in this dissertation is as follows:

Hypothesis: We hypothesize that IL-6 protects Müller cells via classical IL-6 signaling against hyperglycemic insults. We postulate that increased IL-6 trans-signaling negatively affects proper vascular function. We propose that targeting IL-6 trans-signaling rather than IL-6 direction will provide a feasible therapeutic strategy to prevent hyperglycemia-induced injury of retinal cells.

The objective of this study was to identify the effects of IL-6 and its diverse signaling pathways on human Müller and retinal endothelial cells in order to determine the feasibility of targeting IL-6 trans-signaling rather than IL-6 itself.

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Chapter 2. Interleukin-6 (IL-6) Mediates Protection against Glucose Toxicity in Human Müller Cells via Activation of VEGF-A Signaling

Authors: Brandon A. Coughlin, Brett T. Trombley, and Susanne Mohr

This chapter is a modified version of a manuscript currently under review

2.1 Abstract

Interleukin-6 (IL-6) has become a target of interest for drug development aiming to treat diabetic retinopathy. Since IL-6 signaling can promote beneficial as well as detrimental effects via two different signaling pathways, the objective of the present study was to investigate the effects of classical IL-6 and IL-6 trans-signaling on human Müller cells (HMC), which are important for the development of diabetic retinopathy. HMCs were cultured in normal (5mmol/L) and high (25 mmol/L) glucose plus or minus IL-6 or IL-6/sIL-6R. IL-6 receptor expression using immunohistochemistry and flow cytometry and cytokine release using magnetic bead assays were determined. HMCs express the membrane bound form of the IL-6 receptor (mIL-6R), gp130, and can release the soluble forms sIL-6R and sgp130 demonstrating that HMCs are capable of responding to classical IL-6 and IL-6 trans-signaling. IL-6 protected HMCs from glucose toxicity via VEGF-A signaling. IL-6/sIL-6R caused only modest protection, which was not mediated by VEGF-A. Our data show for the first time that classical IL-6 signaling exerts its beneficial effects through VEGF-A action contrary to IL-6 trans-signaling, which was

VEGF-A independent. These results have clinical implications for drug development targeting IL-6 since strict anti-IL-6 therapies might further decrease neuroretinal functions in the diabetic retina.

2.2 Introduction

Neovascularization and retinal edema are two vision threatening complications for patients with diabetic retinopathy (DR). The current treatment strategy of choice for these patients is anti-VEGF therapy; however, this therapy is not reliable and is only effective for a small portion of the patient population[1]. Since the diabetic population is steadily rising, there is a pressing need for additional and more reliable therapies.

Recently, interleukin-6 has risen to the forefront as a target for drug development either as an anti-IL-6 therapy alone or in combination with anti-VEGF-A therapy. IL-6 is a pleiotropic cytokine known to participate in both acute and chronic inflammatory events. Its effects are dependent on microenvironment, cell type, and receptor expression[2]. Depending on which receptors are being expressed IL-6 can have beneficial or detrimental actions. The diverse effects of IL-6 are potentially due to activation of different IL-6 signaling pathways: classical IL-6 signaling and IL-6 trans-signaling. Classical IL-6 signaling, originally thought to be a protective pathway, is mediated by the binding of IL-6 to the membrane-bound IL-6 receptor (mIL-6R) and its association with membrane-bound glycoprotein 130 (gp130). On the other hand, IL-6 trans-signaling, which is has been suggested to be more pro-inflammatory in nature, is mediated by IL-6 binding to the

soluble form of the IL-6 receptor (sIL-6R) and forming the IL-6/sIL-6R complex. This complex then interacts with gp130 to promote downstream signaling[3–5].

In the retina, IL-6 has been shown to be protective with regards to the neuroretina. Previous studies have shown that IL-6 is a key player in protecting photoreceptors from retinal detachment-induced cell death, retinal ganglion cells from pressure-induced cell death, and retinal Müller cells from high glucose-induced cell death[6–8]. In contrast, detrimental outcomes linked with IL-6, such as retinal barrier disruption, have been associated with the retinal vasculature[9–12]. However, the full outcomes of the diverse actions of IL-6 in the retina have yet to be determined. Nevertheless, new treatment strategies are being developed towards targeting IL-6 as a potential treatment for diabetic retinopathy.

In the present study, we focused on characterizing expression and regulation of receptors that are associated with IL-6 signaling (mIL-6R, sIL-6R, gp130, and sgp130) and gaining more insight into the effects of IL-6 signaling on human Müller cells under hyperglycemic conditions. Human Müller cells play an important role in the development of diabetic retinopathy[13–16]. They maintain the inner blood retinal barrier and are the only other retinal cell type besides photoreceptors found within the macula making them a strong candidate for promoting neovascularization as well as edema in the diabetic retina. They are also a well-known source for pro-inflammatory cytokines and VEGF-A production[13,17,18]. Therefore, the purpose of this study was to evaluate the ability of human Müller cells to signal through either classical IL-6 or IL-6 trans-signaling and to identify

potential mechanisms of action for the protective effects of IL-6 on neuroretinal cells, such as Müller cells.

2.3 Materials and Methods

2.3.1 Antibodies and recombinant proteins

IL-6, IL-6/sIL-6R, VEGF-A, VEGFR inhibitor (Axitinib), human BD Fc Block, mouse anti-mIL-6R-APC antibody, and mouse anti-gp130-PE antibody were purchased from R&D Systems (Minneapolis, MN). Rabbit anti-mIL-6R and rabbit anti-gp130 antibodies were obtained from Abcam (Cambridge, MA). Anti-rabbit Texas Red antibody was purchased from Invitrogen (Carlsbad, CA). Human Milliplex MAP magnetic bead panels were purchased from MilliporeSigma (Burlington, MA).

2.3.2 Tissue culture of human retinal Müller cells

Handling of human tissue conformed to the tenets of the Declaration of Helsinki for research involving-human tissue. Human retinal Müller cells (HMCs) were isolated from retinal tissue of healthy donors with no history of diabetes or chronic inflammatory diseases. Following isolation, HMCs were purified by trypsin splits (0.25% trypsin), and cultured in Dulbecco's Modified Eagle's Medium (DMEM)/HAM F12 (1:1 ratio) media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) containing normal (7.8 mmol/L) glucose at 37°C and 5% CO_2 in a humidified incubator, as previously described[19]. After the third trypsin split, cell cultures were >95% pure.

Criteria for pure HMCs were positive staining for vimentin and CRALBP and negative staining for GFAP.

2.3.3 Cytokine treatment

HMCs (1 x 10^5) were incubated in FBS free DMEM/HAM F12 media containing 1% P/S and normal (7.8 mM) glucose in the presence or absence of IL-6 (5 ng/mL), IL-6/sIL-6R chimera (10 ng/mL), VEGF-A (10 ng/mL), or VEGFR inhibitor (10nM) for one hour. Following pre-treatment, HMCs were transferred to DMEM/HAM F12 supplemented with 2% FBS and 1% P/S containing either normal (7.8 mmol/L) or high (25 mmol/L) glucose. After 48 hours a medium change including fresh pre-treatments was performed.

2.3.4 Flow cytometry

HMCs (1 x 10^6) were treated as described above, washed with phosphate buffered saline (PBS), and collected following a 10 min accutase treatment. HMCs were washed with FACS buffer (PBS, 0.5% BSA, 7.5mM sodium azide, and 5mM EDTA), blocked with 50μL of human Fc block (4 μg/mL BD Fc Block in FACS buffer) for 15 minutes at 4°C, and incubated with 25 μL of FACS buffer containing antibodies for mIL-6R (mouse anti-mIL-6R conjugated to APC; 1μL per 1 x 10^6 cells) and gp130 (mouse anti-gp130 conjugated to PE; 1μL per 1 x 10^6 cells) for 30 minutes at 4°C. After staining with antibody solution, cells were washed with FACS buffer, and fixed with 10% formalin. HMCs were then analyzed with an LSR II flow cytometer using BD FACSDiva Software. Data was quantified using FlowJo software.

2.3.5 Immunofluorescence.

HMCs (5 x 10^4) were plated on cover slips and treated as described above. After treatment, HMCs were fixed in 4% paraformaldehyde, permeabilized with ice-cold acetone for 10 minutes, blocked with 1% BSA in PBS, and incubated overnight at 4°C with antibodies against mIL-6R (mouse anti-mIL-6R; 1:200 dilution) or gp130 (mouse anti-gp130; 1:200 dilution). Following antibody incubation, HMCs were blocked in 5% goat serum, followed by a 1-hour incubation with secondary antibody (anti-mouse secondary antibody conjugated to Texas Red; 1:200 dilution) at room temperature. Cover slips were mounted on glass slides using antifade fluorescence mounting medium containing DAPI. mIL-6R and gp130 expression was determined using fluorescence microscopy (Nikon Eclipse TE 2000-U; 40x magnification) and MetaMorph Microscopy Automation and Image Analysis Software. As a control, cells were incubated with either primary or secondary antibody only to exclude effects from autofluorescence.

2.3.6 Measurement of cytokines.

HMCs (1 x 10^5) were treated as described above. After treatment, medium was removed and retained, cells were lysed, and protein content was determined. Cytokine concentrations in retained medium were determined using human Milliplex MAP magnetic bead panels (IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-17A, IL-18, IP-10, TNFα, IFNγ, RANTES, MCP-1, sgp130, sIL-6R, sIL-1R1, sIL-1R2, sRAGE, VEGF-A) according to manufacturer's instructions. Briefly, 25 μL of medium was incubated with 25 μL of magnetic beads coated with antibodies for individual cytokines in a 96-well plate overnight at 4°C. Plates were washed, incubated in secondary antibody solution (1 hour at RT),

developed using streptavidin-phycoerythrin solution (30 min at RT), and analyzed using the Luminex200. Levels of specific cytokines were calculated from internal standard curves for each individual cytokine, normalized to protein concentrations, and expressed as mean ± SDEV (pg/mL/mg protein).

2.3.7 Cell Death Assay.

HMCs (1 x 10^5) were treated as described above. At 96 hours, samples were assessed for trypan blue inclusion. Cell death was quantified as the number of blue cells per total cell number (%) and expressed as a mean ± SDEV.

2.3.8 Statistical Analysis

Non-parametric data were analyzed using Mann Whitney U or Kruskal-Wallis test followed by a Dunn's post-hoc analysis. Parametric data were analyzed using one-way ANOVA followed by a post-hoc Tukey analysis. For all data, we decided to present exact p-values[20]. Findings were labeled as being significant (Asterisk) if the p-values was less than 0.05. For details in statistical analysis, see the GraphPad Prism 7 statistics guide (https://www.graphpad.com/guides/prism/7/statistics/).

2.4 Results

2.4.1 Determination of mIL-6R and gp130 levels on human Müller cells

Human Müller cells (HMC) express the membrane bound form of the IL-6 receptor (mIL-6R) and the co-receptor gp130 under normal conditions. Figure 1 shows that HMCs

express low levels of mIL-6R (Figure 13A and B) as demonstrated by immunofluorescence staining and flow cytometry. In addition, HMCs express high levels of gp130 (Figure 13C and D). Some of the gp130 seems to be located around or within the nucleus.

2.4.2 Effects of hyperglycemia and IL-6 treatment on expression levels of mIL-6R, sIL-6R, gp130, and sgp130 in HMCs

Hyperglycemia increased mIL-6R levels by 2.63±1.81 fold (p=0.047) and gp130 levels by 1.53±0.73 fold (p=0.050) (Figure 14A and D). IL-6 treatment itself had no effects on mIL-6R and gp130 expression levels, neither under normal nor hyperglycemic conditions (Figure 14B and E). Since HMCs express mIL-6R, they have the ability of potentially releasing the soluble form of the IL-6 receptor (sIL-6R). Under normal glucose conditions there was a slight release of sIL-6R (8.9±1.2 pg/mL/mg protein). While hyperglycemia had no further effect on sIL-6R release, IL-6 treatment increased the release of sIL-6R by 23.0±11.5% (p=0.027) (Figure 14C). Similarly, HMCs release the soluble form of gp130 (sgp130) under normal conditions (1009±170.5pg/mL/mg protein). However, IL-6 treatment only increased the release of sgp130 under hyperglycemic conditions (37.7±25.4%; p=0.005). Hyperglycemia itself had no effect on sgp130 release (Figure 14F).

2.4.3 IL-6-mediated protection against hyperglycemic insult via activation of VEGF-A signaling

IL-6 treatment induced an increase in VEGF-A production and release from 1418±162.8 to 2340±768.1 pg/mL/mg protein (p=0.137) under normal and from 1435±190.5 to 2691±1071pg/mL/mg (p=0.036) under hyperglycemic conditions (Figure 15A). VEGF-A itself decreased hyperglycemia-induced cell death by 81.6±14.4% (p<0.001) (Figure 15B). To test whether IL-6 protective effects were mediated by VEGF-A, cells were pre-treated with a VEGF receptor blocker (VEGFR). Results show that pre-treatment with VEGFR completely prevented IL-6 mediated protection (Figure 15C). In addition to VEGF-A, IL-6 also induced a slight increase in IFNγ and IP-10. Levels of MCP-1 (CCL2) increased by 11.5±2.78 fold (p=0.024) following IL-6 treatment. These effects were independent of glucose conditions and had no effects on cell viability.

2.4.4 Effects of IL-6 trans-signaling on HMC survival under hyperglycemic conditions

IL-6/sIL-6R treatment reduced hyperglycemia-induced cell death from 7.5±1.2% to 4.7±1.8% (p=0.054) indicating slight protective properties (Figure 16A). Since data were not as consistent as for IL-6 treatment, VEGF-A production was determined. IL-6/sIL-6R treatment did not cause any VEGF-A release from HMCs in stark contrast to IL-6 treatment (Figure 16B). Interestingly though, IL-6/sIL-6R treatment corrected hyperglycemia-induced IL-10 loss by upregulating IL-10 production by 79.8±36.1% (Figure 16C) under hyperglycemic conditions. This effect was most pronounced at 48 hours and diminished over time. At 72 hours of treatment, there was a 55.5±8.5% increase in pro-inflammatory IL-1β release detectable (Figure 16D). At the same time

point, IL-6/sIL-6R treatment also caused a late release of IL-6 (7110±1769.0 pg/mL/mg protein) compared to normal (2657±103.8pg/mL/mg protein) (p=0.002).

2.5 Discussion

The primary objective of the present study was to determine the effects of IL-6 on human retinal Müller cells. This is the first study looking in detail at consequences of the activation of the two distinct IL-6 signaling pathways, classical IL-6 signaling versus IL-6 trans-signaling, under normal and hyperglycemic conditions in human Müller cells, which are hugely important for the development of diabetic retinopathy. The major findings of our study are that human Müller cells strongly respond to classical IL-6 signaling which upon activation protects human Müller cells from glucose toxicity by inducing VEGF-A signaling. Müller cells' response to IL-6 trans-signaling is modest and although slightly protective in the beginning it seems that longer exposure to IL-6 trans-signaling might turn towards more detrimental outcomes.

Our results have shown that human Müller cells express mIL-6R and gp130. Of interest, gp130 is distributed throughout the cells but some of the gp130 seems to be located around or within the nucleus. This phenomenon is not observed in other human retinal cells such as human retinal endothelial cells, which also express high levels of gp130. Identifying whether this effect is real or unique to human Müller cells and what the purpose of a potential translocation of gp130 might serve needs to be determined by future studies that go beyond the scope of this study. The presence of both receptors,

mIL-6R and gp130, make human Müller cells perfectly capable of responding to classical IL-6 signaling. Surprisingly, the activation of classical signaling by IL-6 leads to production of VEGF-A. IL-6-induced VEGF-A production has never been shown for human Müller cells before but is of clinical significance. It seems under healthy conditions a low-level IL-6/mIL-6R/VEGF-A/VEGFR cycle maintains the well-being of the cells. The protective nature of VEGF-A on Müller cells has been demonstrated before[17,18,21] and has been confirmed in our study. Under hyperglycemic conditions, Müller cells seem to upregulate this protective cycle by increasing IL-6 production and mIL-6R expression leading to a pronounced release of VEGF-A in line with suggestions that Müller cells are an important source for VEGF-A in the diabetic retina[17,18]. The hyperglycemia-induced release of IL-6 by human Müller cells is modest but consistent. In the retinal tissue, the cycle can be additionally fed by microglia-derived IL-6. Microglia are known to produce massive amounts of IL-6 in the diabetic retina[22]. Strong VEGF-A production probably serves a protective purpose initially. In the long run, however, these large amounts of VEGF-A will certainly reach the vasculature due to the close proximity of Müller cells with the retinal blood vessels. Ultimately, this will cause breakdown in vascular integrity and leakage turning a good effect into more and more detrimental effects.

In addition to classical IL-6 signaling, human Müller cells can respond to IL-6 trans-signaling. Whether Müller cells themselves are capable of initiating IL-6 trans-signaling on their own is questionable. They release small amounts of sIL-6R which seem to be countered by the simultaneous release of sgp130, the natural inhibitor of IL-6 trans-signaling. However, in retinal tissue microglia shed sIL-6R and given the rich IL-6

environment in the diabetic retina, Müller cells might become exposed to IL-6/sIL-6R. As our data demonstrate, IL-6 trans-signaling has a modest protective effect against glucose toxicity potentially mediated by an increased IL-10 production that is normally decreased under hyperglycemic conditions. It has long been suggested that IL-10 furthers cellular survival[23]. Although the effect of IL-6/sIL-6R on IL-10 production is quite strong after short time exposure it fades the longer human Müller cells are exposed to IL-6 trans-signaling. Interestingly, IL-6 trans-signaling does not induce VEGF-A production in stark contrast to classical IL-6 signaling. Extended IL-6/sIL-6R treatment drives detrimental effects like the production of IL-1β. Hyperglycemia-induced IL-1β production causes Müller cells death *in vitro* and *in vivo* as previously shown by us and others[8,24]. IL-6 trans-signaling might exacerbate IL-1β production from Müller cells contributing to the chronic inflammatory environment that diabetes initiates in the retinal tissue. The effect of IL-6 trans-signaling on human Müller cells seems to be a balance between protective and detrimental effects. At the end, human Müller cells seem to increase IL-6 production maybe in an attempt to initiate VEGF-A production, which is clearly not made under IL-6 trans-signaling conditions. Although the release of IL-6 and sIL-6R from human Müller cells under hyperglycemic condition is modest as stated above, it can not be excluded that they play a role in a potential shift of the retinal environment from classical IL-6 signaling to IL-6 trans-signaling. While the increased VEGF-A triggers vascular breakdown and leakage, IL-6/sIL-6R might be responsible for induction of angiogenesis, all characteristics that define diabetic retinopathy.

Decreasing excessive VEGF-A levels using anti-VEGF-A drugs has shown beneficial effects for some patients with diabetic retinopathy. Although targeting IL-6 to prevent IL-6 trans-signaling might seem at first glance like a valid treatment strategy, it disregards the protective effect IL-6 has on the neuroretina. Since IL-6 and VEGF-A production are intricately linked as our data have demonstrated, combination therapies especially might lead to adverse outcomes by lowering VEGF-A levels below a threshold needed for the well-being of neuroretinal cells. Based on our data, drug development targeting IL-6 directly should be cautiously approached. On the other hand, strategies targeting IL-6 trans-signaling using sgp130 could provide a feasible option that would prevent vascular effects while leaving IL-6 levels untouched. Therefore, designing therapies that interfere in detrimental IL-6 signaling might lead to new and additional treatment options for patients with diabetic retinopathy.

APPENDIX

Figure 13. Human Müller cells express mIL-6R and gp130. HMCs were stained for mIL-6R (A) or gp130 (C) using immunocytochemistry (left panels: nucleus stained with DAPI (blue); middle panels: stained for mIL-6R or gp130 (red); right panels: overlay). Expression levels of mIL-6R and gp130 using Flow cytometry are shown as the median fluorescence intensity of cells stained with an APC-conjugated antibody against mIL-6R (B) or a PE-conjugated antibody against gp130 (D) compared to unstained controls. Results are expressed as mean ± SDEV (n=3-9). Exact p-values are presented – (*) $p<0.05$ (Mann-Whitney U test).

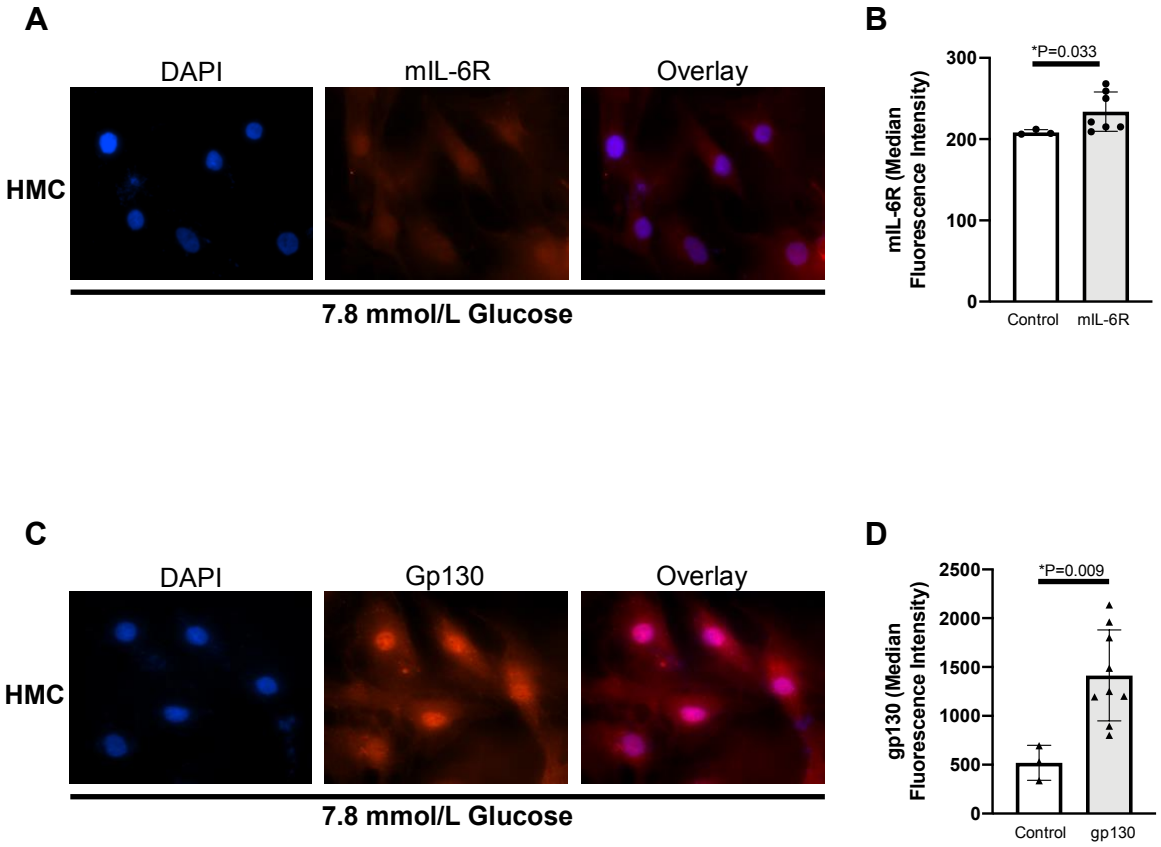


Figure 14. The effects of IL-6 treatment on IL-6 receptors. HMCs were treated in normal (7.8 mmol/L) or high (25 mmol/L) glucose in the presence or absence of IL-6 (5 ng/mL) for up to 96 hours. At 48 hours, HMCs were incubated with an APC-conjugated antibody against mIL-6R (A and B) and a PE-conjugated antibody against gp130 (D and E). Using flow cytometry, the median fluorescence intensity was measured, and the shift of the median fluorescence intensity between HMCs treated in 7.7 mmol/L glucose vs 25 mmol/L was determined. Results are expressed mean ± SDEV of either median fluorescence intensity (A and D) or fold change (B and E) (n=7-9). At 72 hours, media samples were analyzed for sIL-6R (C) and sgp130 (F) levels using magnetic bead assays. Results are expressed as mean ± SDEV (pg/mL/mg protein) (n=5). Exact p-values are presented – (*) p<0.05 (Kruskal-Wallis followed by Dunn's post hoc (A, B, D, E) and one-way ANOVA followed by Tukey post hoc (C, F)).

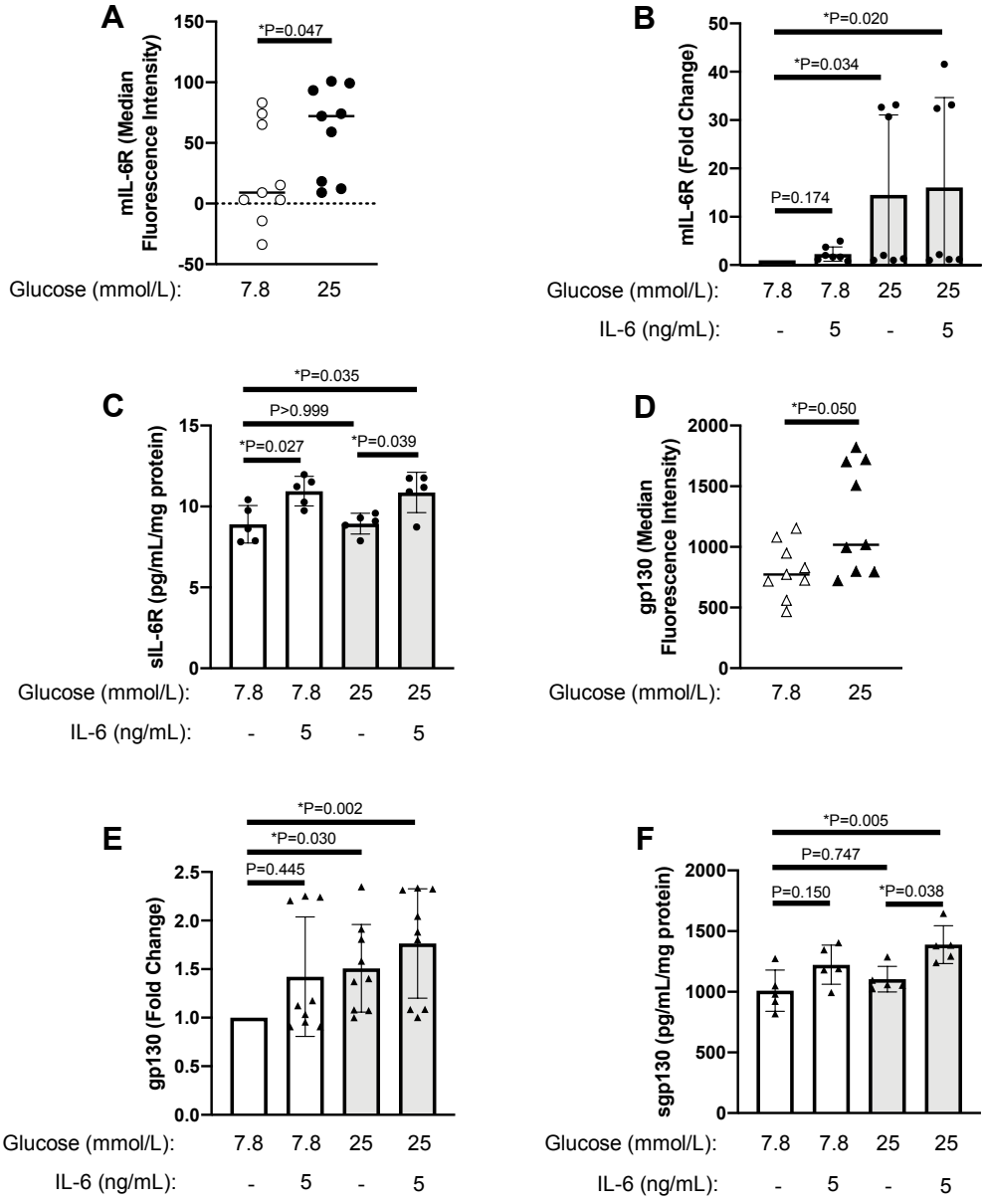
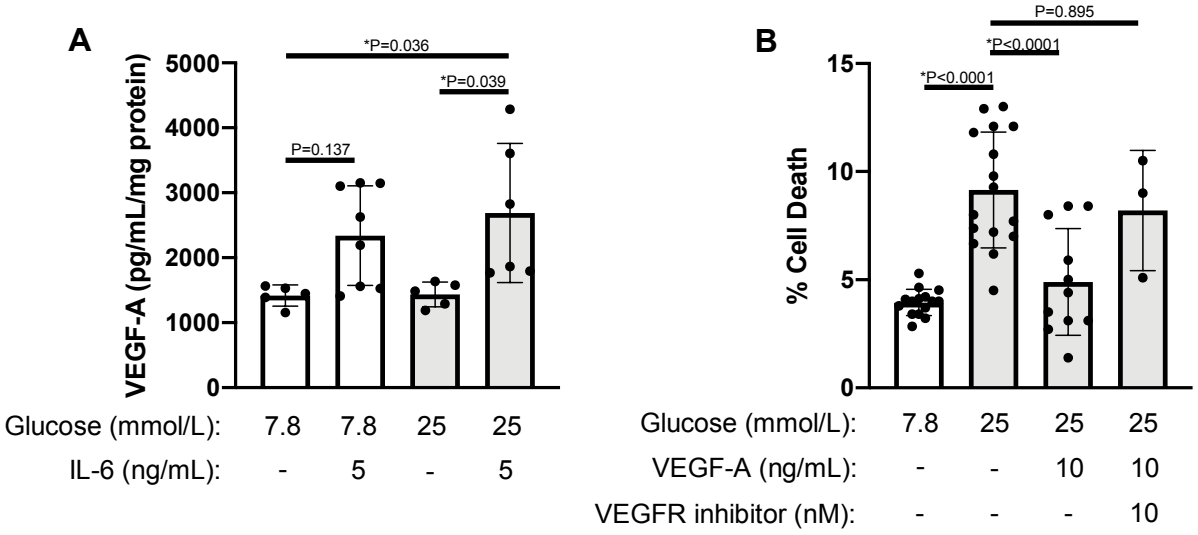


Figure 15. The protective effects of IL-6 on human Müller cells are VEGF-A dependent. HMCs were treated in normal (7.8 mmol/L) or high (25 mmol/L) glucose in the presence or absence of IL-6 (5 ng/mL), VEGF-A (10 ng/mL), or a VEGF receptor inhibitor (10 nM) for up to 96 hours. At 72 hours, media samples were analyzed for VEGF-A using magnetic bead assays (A). Results are expressed as mean ± SDEV (pg/mL/mg protein) (n=5-7). At 96 hours, HMCs were assessed for viability using trypan blue exclusion (B and C). HMC viability was quantified as the number of blue cells per total cell number and expressed as mean ± SDEV (%) (n=3-16). Exact p-values are presented – (*) p<0.05 (Kruskal-Wallis followed by Dunn's post hoc (B, C) and one-way ANOVA followed by Tukey post hoc (A)).



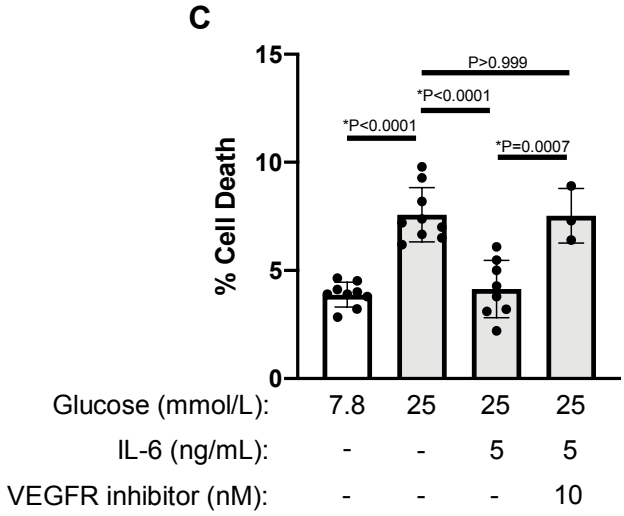


Figure 16. The effect of IL-6 trans-signaling on human Müller cells. HMCs were treated in normal (7.8 mmol/L) or high (25 mmol/L) glucose in the presence or absence IL-6/sIL-6R (10 ng/mL) or a VEGF receptor inhibitor (10 nM) for up to 96 hours. Media samples were assessed for cytokine levels at 48 and 72 hours of treatment using magnetic bead assays – IL-10 (C; 48h), VEGF-A (B; 72h), and IL-6 (D; 72h). Results are expressed as mean ± SDEV (pg/mL/mg protein) (n=3-6). At 96 hours, HMCs were assessed for viability using trypan blue exclusion (A). HMC viability was quantified as the number of blue cells per total cell number and expressed as mean ± SDEV (%) (n=3-10). Exact p-values are presented – (*) $p<0.05$ (Kruskal-Wallis followed by Dunn's post hoc (A) and one-way ANOVA followed by Tukey post hoc (B, C, D)).

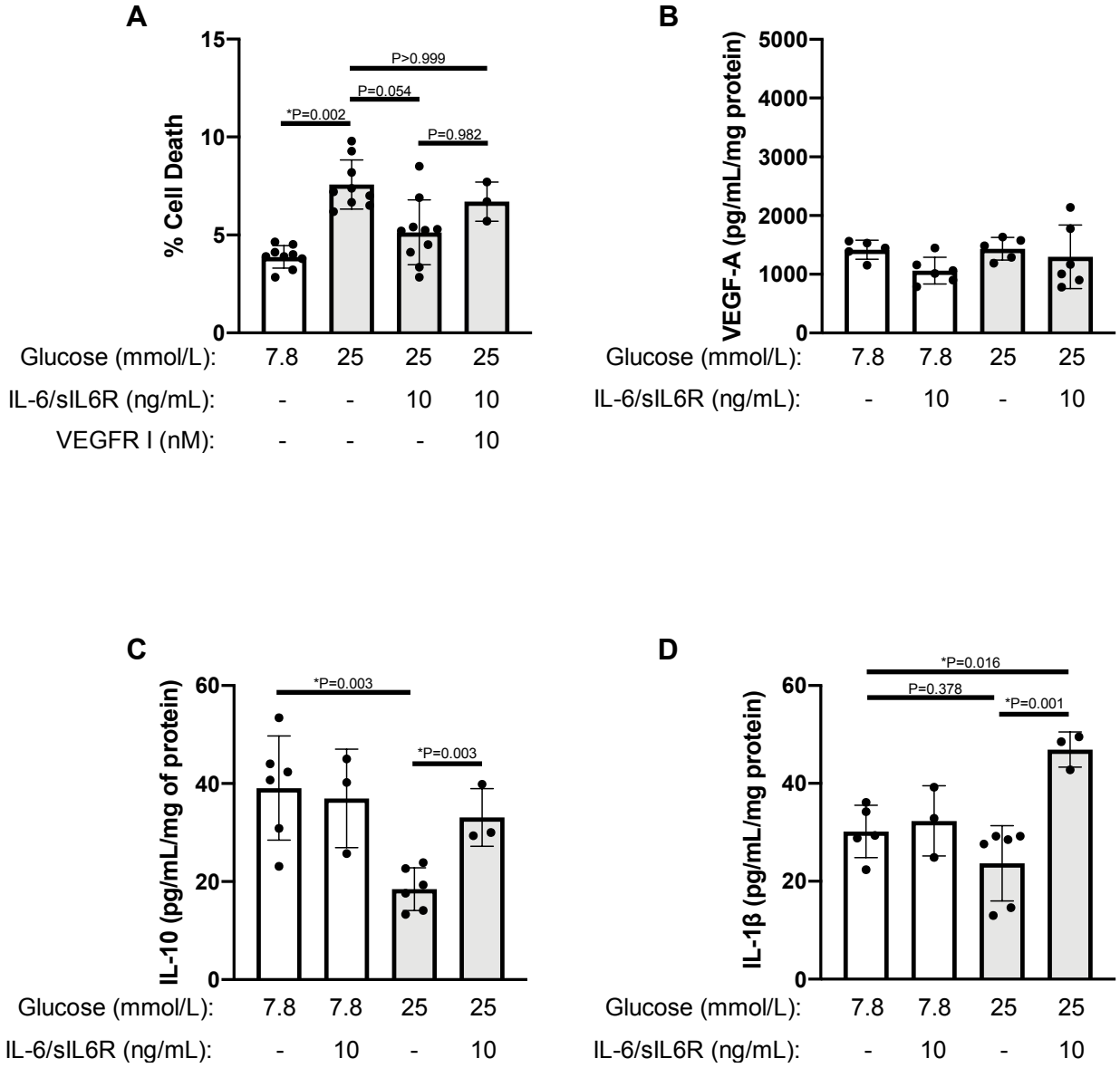
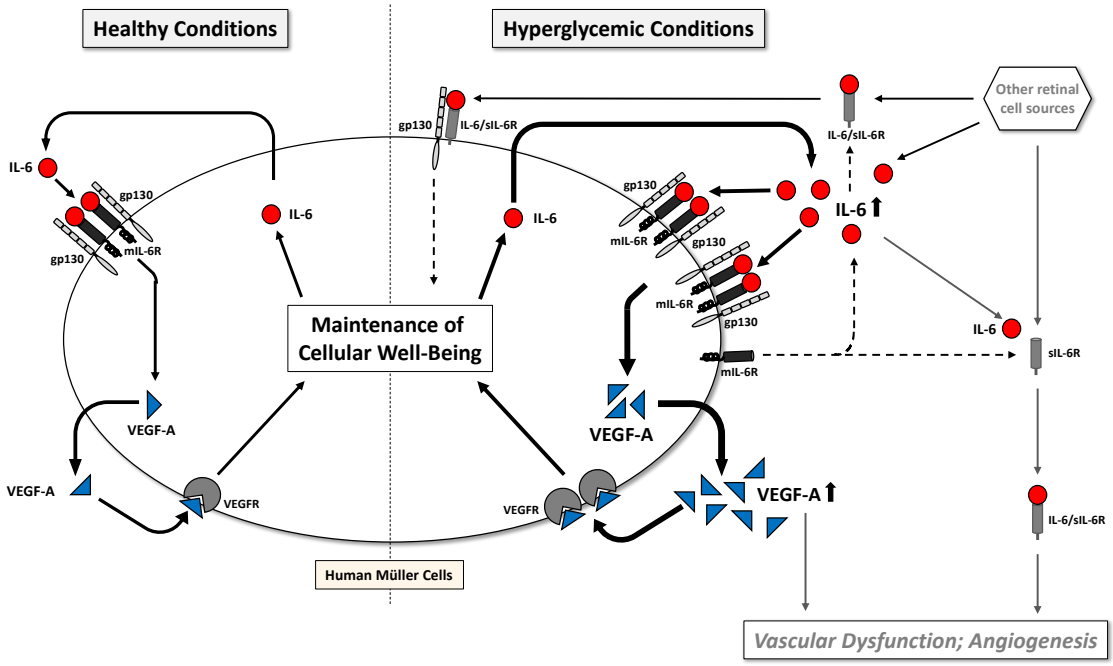


Figure 17. Summary of IL-6 signaling on human Müller cells.



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Chapter 3. The Effect of Interleukin-6 Trans-Signaling on Human Retinal Endothelial Cells under Hyperglycemic conditions

3.1 Abstract

Purpose: Diabetic retinopathy is a vision threatening complication of diabetes that is initiated in response to hyperglycemia-induced chronic inflammation. Over time, unresolved inflammation within the retina can have detrimental consequences on the retinal vasculature. In other inflammatory based diseases, interleukin-6 (IL-6) signaling, and more specifically IL-6 trans-signaling, is known to play a prominent role in disease progression. However, its role in diabetic retinopathy remains unclear. Therefore, this studied aimed at determining the effect of IL-6 trans-signaling on induction of inflammation and angiogenesis on human retinal endothelial cell.

Methods: Primary human retinal endothelial cells (HRECs) were treated with normal (7.8 mmol/L) or high (25 mmol/L) glucose in the presence or absence of IL-6 (5 ng/mL) or IL-6/sIL-6R (10-50 ng/mL) for up to 120 hours. Expression of IL-6 receptors were determined using immunocytochemistry and flow cytometry. Furthermore, release of soluble receptors, inflammatory cytokines, and growth factors were measured using multiplex magnetic bead assays. Angiogenesis was measured through tube formation assays. Finally, HREC viability was determined using trypan blue exclusion.

Results: HRECs do not express mIL-6R under normal or hyperglycemic conditions. As expected, HRECs express gp130 under normal glucose conditions and its expression is downregulated by 1.19±0.06 fold under hyperglycemic conditions. Treatment of HRECs with IL-6/sIL-6R (10 ng/mL) increased production of inflammatory cytokines such as IL-1β (2.2±1.3 fold), IL-6 (1.9±1.3 fold), TNF-α (3.1±1.2 fold), and IFN-γ (2.3±2.3 fold) under hyperglycemic conditions compared to normal glucose controls. Higher concentrations of IL-6/sIL-6R (50ng/mL) dramatically increased VEGF-A production by 5.9±1.4 fold. In addition, IL-6/sIL-6R (50ng/mL) increased tube length from 567.5±207.7μM to 3236.0±1724μM under hyperglycemic conditions compared to normal glucose controls. Intriguingly, the effects of IL-6/sIL-6R were independent of glucose concentrations.

Conclusion: Our study demonstrates that activation of IL-6 trans-signaling on HRECs leads to a pronounced inflammatory response. More importantly, for the first time we were able to demonstrate that IL-6-trans-signaling indeed induces angiogenesis of human retinal endothelial cells. Our study indicates that targeted therapies towards IL-6 trans-signaling may be efficacious for the treatment diabetic retinopathy since uncontrolled angiogenesis is one of the major complications in the proliferative stage of diabetic retinopathy.

3.2 Introduction

Neovascularization and subsequent hemorrhaging in the retina and vitreous body are hallmarks of the proliferative stage of diabetic retinopathy[1]. Historically, the belief was that

low oxygen levels due to the formation of acellular capillaries caused the production of VEGF which in turn was thought to be responsible for vascular leakage and angiogenesis. However, newer studies suggested that the initiation of neovascularization is also a consequence of chronic low-grade inflammation in response to hyperglycemia[2]. Inflammatory mediators such as cytokines and growth factors play a crucial role in driving retinal inflammation and ensuing retinal pathology in diabetes. Interleukin-6 (IL-6) is a prominent inflammatory cytokine whose role in retinal inflammation and neovascularization still remains unclear. The few studies that have been done looking at the effects of IL-6 within the retina, have suggested that IL-6 has strong neuroprotective capabilities[3–6]. In other tissues of the eye, such as the cornea and choroid, it has been clearly demonstrated that IL-6 plays a role in inflammation and neovascularization[7–9]. What makes IL-6 unique is that it can promote pro-inflammatory and anti-inflammatory responses within the same microenvironment. These diverse outcomes are dependent on receptor expression. Both classical IL-6 (anti-inflammatory) and IL-6 trans-signaling (pro-inflammatory) utilize the same co-receptor, glycoprotein 130 (gp130). However, each pathway signals through a different variation of the IL-6 receptor. Classical IL-6 signaling uses the membrane-bound form of the IL-6 receptor (mIL-6R), while IL-6 trans-signaling uses the soluble IL-6 receptor (sIL-6R). The expression patterns of these IL-6 receptors ultimately determine the fate of IL-6 signaling, and the induction of IL-6 trans-signaling is commonly associated with vascular dysfunction and angiogenesis[10–13]. Due to the lack of knowledge regarding the expression patterns of IL-6 receptors in the healthy or diabetic retina, very little is known about IL-6 and IL-6 signaling.

Very few studies have looked into the effect of classical IL-6 and IL-6 trans-signaling on the retinal vasculature. Previous studies using IL-6 knock out mice have shown that IL-6 signaling is necessary for retinal VEGF production, leukostasis, and vascular remodeling[14,15]. However, it was not determined if classical IL-6 and/or IL-6 trans-signaling was responsible for these IL-6 dependent effects. More recently, the effects of IL-6 trans-signaling were investigated on primary human retinal endothelial cells (HRECs), a major cellular component of the retinal vasculature. The study was able to show that treatment of HRECs with IL-6 and the sIL-6R led to increased expression of adhesion molecules, reactive oxygen species production, apoptosis, and HREC barrier disruption[16].

To date, no studies have been done to determine if IL-6 trans-signaling can initiate retinal inflammation by HRECs through the production of inflammatory cytokines and growth factors. Furthermore, whether IL-6 trans-signaling is able to stimulate HREC angiogenesis has yet to be determined. Therefore, this studied aimed to determine if activating IL-6 trans-signaling on HRECs can promote pro-inflammatory and/or pro-angiogenic responses.

3.3 Materials and Methods

Materials

Recombinant human IL-6, IL-6/sIL-6R, BD Fc Block, and mouse anti-mIL-6R-APC and anti-gp130-PE antibodies were purchased from R&D Systems (Minneapolis, MN).

Rabbit anti-mIL-6R and anti-gp130 antibodies were obtained from Abcam (Cambridge, MA). Anti-rabbit Texas Red antibody was from Invitrogen (Carlsbad, CA). Antifade fluorescence mounting medium acquired from Vectashield (Vector Laboratories, Burlingame, CA) Human Milliplex MAP magnetic bead panels were purchased from Millipore (Billerica, MA). Matrigel was obtained from Corning (Corning, NY).

Methods

Tissue Culture of Human Retinal Endothelial Cells

Handling of human tissue conformed to the tenets of the Declaration of Helsinki for research involving human tissue. Human retinal endothelial cells (HRECs) were isolated and purified from retinal tissue of healthy donors with no history of diabetes or chronic inflammatory diseases as previously described[17,18]. HRECs were characterized using acetylated LDL uptake. HRECs were cultured in DMEM/HAM F12 (1:1 ratio) media supplemented with 10% FBS, 5% endothelial cell growth supplement (ECGS), 1% penicillin-streptomycin (P/S), and 1x insulin transferrin selenium mix (ITS) containing normal (7.8 mmol/L) glucose in 75 cm^2 flasks coated 0.2% gelatin at 37°C and 5% CO_2 in a humidified incubator. Only HRECs from passages 3 to 10 were used for experiments.

Cytokine Treatment

HRECs (1 x 10^5) were incubated in FBS free DMEM/HAM F12 containing 1% P/S, 5% ECGS, ITS, normal (7.8 mmol/L) glucose in the presence or absence of IL-6 (5 ng/mL) or IL-6/sIL-6R (10-50 ng/mL). After one hour incubation, media was switched to

DMEM/HAM F12 media containing 2% FBS, 1% P/S, 5% ECGS, ITS, and either normal (7.8 mmol/L) or high (25 mmol/L) glucose. Every 48 hours, media samples were collected and retained for analysis, and a media change including pre-treatment was performed as decribed above. Treatment of HRECs continued up to 120 hours.

Flow Cytometry

HRECs (1 x 10^6) were treated as described above. HRECs were washed with phosphate buffered saline (PBS) and lifted from treatment plates using an accutase solution for 10 minutes at 37°C. HRECs were then washed with fluorescence activated cell sorting (FACS) buffer (PBS, 0.5% BSA, 7.5mM sodium azide, and 5mM EDTA) and incubated with 50μL of human Fc block (4 μg/mL BD Fc Block in FACS buffer; BD Pharmigen) for 15 minutes at 4°C. Next, HRECs were incubated with 25 μL of FACS buffer containing antibodies for mIL-6R (mouse anti-mIL-6R conjugated to APC; 1μL per 1 x 10^6 cells) and gp130 (mouse anti-gp130 conjugated to PE; 1μL per 1 x 10^6 cells) for 30 minutes at 4°C. After staining, HRECs were washed with FACS buffer, and fixed with 10% formalin. Cells were then analyzed with an LSR II flow cytometer using BD FACSDiva Software. Data was further gated, analyzed, and quantified using FlowJo software.

Immunofluorescence

HRECs (5 x 10^4) were plated on cover slips and treated as described above, fixed in 4% paraformaldehyde, and permeabilized with ice-cold acetone for 10 minutes. HRECs were then blocked with 1% BSA in PBS and incubated overnight at 4°C with antibodies

against mIL-6R (mouse anti-mIL-6R; 1:200 dilution) and gp130 (mouse anti-gp130; 1:200 dilution). After incubation with primary antibodies, HRECs were blocked in 5% goat serum, followed by a 1-hour incubation with secondary antibody (anti-mouse secondary antibody conjugated to Texas Red; 1:200 dilution) at room temperature. Cover slips were mounted on glass slides using antifade fluorescence mounting medium. mIL-6R and gp130 expression was detected with a fluorescence microscope (Nikon Eclipse TE 2000-U; 40x magnification) and digital images were acquired and analyzed using MetaMorph Microscopy Automation and Image Analysis Software.

Measurement of Cytokines

HRECs (1×10^5) were treated as described above. After treatment, medium was removed and retained, cells were lysed, and protein content was determined with a Bradford assay. Cytokine concentrations in retained medium were determined using a human Milliplex MAP magnetic bead panel according to manufacturer's instructions. Briefly, 25 μL of medium was incubated with 25 μL of magnetic beads coated with antibodies against individual cytokines (IL-6, IL-1β, TNF-α, IFN-γ, and VEGF-A) in a 96-well plate overnight at 4°C. Plates were washed, incubated using detection antibodies (1 hour at room temperature), and developed with a streptavidin-phycoerythrin solution (30 minutes at room temperature). Beads were analyzed using the Luminex200. Cytokine levels were calculated from internal standard curves for each individual cytokine, normalized to protein concentrations, and expressed as mean $\pm$ SDEV (pg/mL/mg protein).

Cell Death Assay

HRECs (1 x 10^5) were treated as described above. At 72 hours, HRECs were assessed for blue inclusion indicating cell death. Cell death was quantified as the number of blue cells per total cell number (%) and expressed as mean ± SDEV.

Tube Formation Assay

HRECs (4 x 10^4) were plated in 96-well plates containing 75 μL of matrigel. HRECs were allowed to settle into the matrigel for one week prior to treatment. After one week, HRECs were treated as described above. After 120 hours, each individual well was examined for tube formation using microscope (Bausch & Lomb BT 123). The length of the tubes were measured and calculated using ImageJ software and expressed as mean ± SDEV (μM).

Statistical Analysis

Parametric and non-parametric data were analyzed by Student's t-test and one-way ANOVA followed by Tukey post-hoc analysis (parametric) or Mann-Whitney U and Kruskal-Wallis rank test followed by a Dunn's post-hoc analysis (non-parametric) to determine statistical significance among groups respectively (95% confidence intervals). Data shown are presented with exact p-values[19]. Data were considered significant if the p-value was less than 0.05 and were marked with an asterisk (*). For details in statistical analysis, see the GraphPad Prism 8 statistics guide (https://www.graphpad.com/guides/prism/8/statistics/index.htm).

3.4 Results

3.4.1 No mIL-6R but gp130 expression on Human retinal endothelial cells

HRECs do not express mIL-6R under normal and hyperglycemic conditions (Figure 18A and B). As expected, gp130 was expressed under normal glucose conditions. Under hyperglycemic conditions, its expression was decreased by 1.19±0.06 fold (Figure 19A and B) (p=0.007). These data suggest that due to the lack of mIL-6R, HRECs are only capable of responding to IL-6 trans-signaling.

3.4.2 Release of soluble forms of the IL-6 receptor and gp130 from HRECs

Although HRECs lack mIL-6R, HRECs might still be able to release the soluble form of the IL-6 receptor via *de novo* synthesis as described for other cell systems[20–23]. Under normal glucose conditions, HRECs release a small amount of sIL-6R (20.9±1.9 pg/mL/mg protein). In addition, HRECs also release sgp130, the natural antagonist of IL-6 trans-signaling, under normal conditions (1995.0±206.8 pg/mL/mg protein) potentially neutralizing the effect of sIL-6R. However, hyperglycemia decreased the release of sIL-6R by 14.2±1.0% (p=0.053) and sgp130 by 16.9±3.6% (p=0.054) (Figure 20A and B).

3.4.3 Activation of IL-6 trans-signaling and HREC viability

There was no change in viability of HRECs treated under hyperglycemic conditions (13.7±3.1%) compared to normal glucose (13.0±2.6%) conditions as previously observed[17]. However, when HRECSs were treated with IL-6/sIL-6R (10 ng/mL) cell death increased from 13.0±2.6% to 25.3±9.4% under normal glucose conditions (p=0.005).

Similarly, under hyperglycemic conditions cell death increased from 13.7±3.1% to 24.8±4.8% (p=0.004) in the presence of IL-6/sIL-6R indicating that induction of cell death by IL-6 trans-signaling is independent of glucose concentration (Figure 21A). To confirm that classical signaling has no effect on HREC viability, HRECs were treated with IL-6 alone. IL-6 itself did not lead to increased cell death, neither under normal nor hyperglycemic conditions (Figure 21B).

3.4.4 Pro-inflammatory cytokine release from HRECs in response to IL-6 trans-signaling

Studies in other systems have shown that IL-6 trans-signaling can promote production of inflammatory cytokines[24–26]. Most prominent was production and release of interleukin-1β (IL-1β) following treatment of HRECs with IL-6/sIL-6R. Activation of IL-6 trans-signaling caused increased release of IL-1β (2.1±1.0 fold; p=0.016), IL-6 (2.2±1.1 fold; p=0.034), TNFα (2.0±0.3 fold; p=0.316), and IFNγ (2.0±0.7 fold; p=0.032) under normal conditions and increased release of IL-1β (2.2±1.3 fold; p=0.011), IL-6 (1.9±1.3 fold; p=0.127), TNFα (3.1±1.2 fold; p=0.020), and IFNγ (2.3±2.3 fold; p=0.038) under hyperglycemic conditions when compared to normal glucose controls (Figure22A-D). Based on our data, IL-6 trans-signaling seems to induce the triumvirate of first responder cytokines (IL-1β, TNFα, and IL-6) causing a strong inflammatory response that is further enhanced by the release of IFNγ.

3.4.5 The dose-dependent release of VEGF-A in response to IL-6/sIL-6R

HRECs released VEGF-A in response to IL-6/sIL-6R treatment. VEGF-A release occurred in a dose dependent manner under both normal and hyperglycemic conditions

following IL-6/sIL-6R treatment. VEGF-A release (2360.0±523.3 pg/mL/mg protein) was maximal at 50 ng/mL of IL-6/sIL-6R compared to normal glucose controls (503.6±163.1 pg/mL/mg protein) (p=0.001) (Figure 23).

3.4.6 IL-6 trans-signaling-induced angiogenesis of HRECs

IL-6 trans-signaling has been associated with angiogenesis[8,9,27]. HRECs did not form tubes under normal or hyperglycemic conditions. However, when treated with IL-6/sIL-6R (50ng/mL) HRECs formed long tubular structures. When treated with IL-6/sIL-6R under normal conditions tube length increased from 567.5±207.7 to 2013.0±1739 μM compared to normal glucose controls (P=0.028). Furthermore, when treated with IL-6/sIL-6R under hyperglycemic conditions HREC tube length increased from 430.0±81.2 to 3236.0±1724 μM compared to hyperglycemic controls (P=0.005). The pro-angiogenic response to IL-6 trans-signaling was independent of glucose concentration (Figure 24A and B).

3.5 Discussion

Our study confirmed that HRECs do not express mIL-6R but do express gp130, indicating that HRECs are only capable of signaling through IL-6 trans-signaling. Additionally, our data show that HRECs are able to synthesize and secrete the soluble forms of the IL-6 receptors, sIL-6R and sgp130, which supports the idea that HRECs are capable of expressing all the necessary components to activate and regulate IL-6 trans signaling. Furthermore, activation of IL-6 trans-signaling decreased HREC viability and led to the release of pro-inflammatory cytokines and growth factors: IL-1β, IL-6, TNFα,

IFNγ, and VEGF-A. Finally, for the first time our study revealed that activation of IL-6 trans-signaling was able to stimulate HREC angiogenesis.

One of the major consequences associated with the proliferative stage of diabetic retinopathy is the increased accumulation of fluid within the retinal cell layers. Previous studies have shown that IL-6 trans-signaling leads to HREC barrier disruption, however, the mechanism of action remains unclear[16]. One possible mechanism could be HREC drop out in response to an IL-6 trans-signaling-induced pro-inflammatory microenvironment. Activation of IL-6 trans-signaling with low levels of IL-6/sIL-6R (10 ng/mL) led to the release of pro-inflammatory cytokines from HRECs, which include the inflammatory triumvirate: IL-1β, TNFα, and IL-6. All of these inflammatory cytokines have previously been shown to decrease HREC viability[17,28–30]. Additionally, other retinal cell types that make up the blood retinal barrier, such as Müller cells and pericytes, are also negatively affected by these pro-inflammatory cytokines[4,31–33]. Taken together, it can be implied that the pro-inflammatory microenvironment generated by IL-6 trans-signaling on HRECs can have a negative impact on the cellular components that make up the blood retinal barrier. As a consequence, this may cause disruption of the barrier and leakage of fluid into the retina.

In addition to retinal edema, uncontrolled angiogenesis is also a vision threatening complication for patients with proliferative diabetic retinopathy. VEGF-A is the most commonly studied and pharmacologically targeted growth factors for the treatment of proliferative diabetic retinopathy. Current advancements towards treating

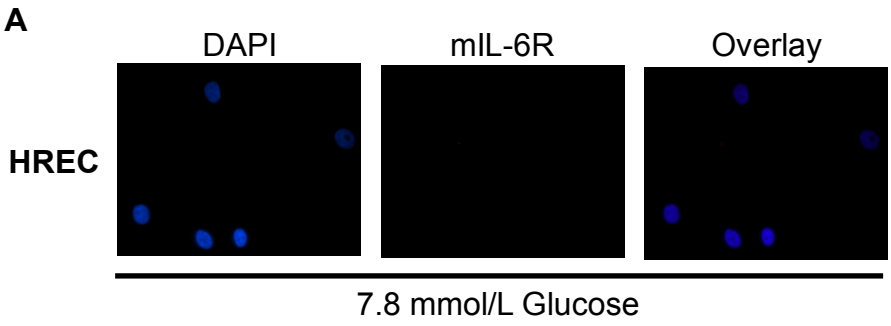
neovascularization have been narrowly focused on making improvements to the existing anti-VEGF therapies. However, anti-VEGF therapy is only effective for a small portion of the patient population and it is crucial that newly targeted therapies are developed[34]. To the best of our knowledge, this is the first time that IL-6 trans-signaling has been shown to have pro-angiogenic effects on human retinal endothelial cells. Within other tissues such as the peritoneal membrane, IL-6 trans-signaling is linked with neovascularization[27]. Within the eye, IL-6 leads to neovascularization in a VEGF-A dependent manner. Specifically, these effects have been associated with corneal and choroidal neovascularization[8,9]. Our data demonstrate that IL-6 trans-signaling on HRECs leads to a dose-dependent release of VEGF-A. Furthermore, activation of the IL-6 trans-signaling pathway led to increased HREC tube formation. Whether or not this response is VEGF-A mediated, compared to the cornea and choroid, has yet to be determined and is the focus of future experiments. Besides IL-6, other prominent inflammatory cytokines can lead to the production of VEGF-A within the retina[35,36]. Therefore, it may be more effective to target the source (inflammation) rather than VEGF-A which seems to be downstream of chronic retinal inflammation. Interestingly, it seems that treatment of HRECs with lower concentrations of IL-6/sIL-6R (10 ng/mL) led to inflammatory cytokine production while higher concentrations (50 ng/mL) promoted the release of VEGF-A. One possible explanation is once HRECs are overwhelmed by IL-6 trans-signaling induced pro-inflammatory cytokines they make an attempt to counteract reduced viability by secreting VEGF-A. Classically, VEGF-A is considered to be pro-survival[37–39]. However, when trans-signaling is activated for an extended period of time the levels of VEGF-A become too great and can potential become a contributing factor to neovascularization.

One question that still remains is the origin of the sIL-6R that is found to be elevated in the vitreous and aqueous humor of patients with diabetic retinopathy[40–42]. Interestingly, our study reveals that HRECs seem to release low levels of the sIL-6R (low picograms) compared to sgp130 (low nanograms). This suggests that the concentration of sIL-6R might not be sufficient to overcome blockage of IL-6 trans-signaling by sgp130, and, therefore, might be unable promote the observed pro-inflammatory and pro-angiogenic effects. Consequently, in order to effectively activate IL-6 trans-signaling, sIL-6R must be coming from an alternative source. One possible source may be microglia, a resident tissue immune cell known to secrete large amounts of IL-6 and express mIL-6R. Furthermore, microglia are also known to play a major role diabetic retinopathy[43–48]. Additionally, migrating immune cells, such as neutrophils, are known to shed sIL-6R and may be an alternative source. However, whether or not microglia, neutrophils, or any other retinal cell types shed the sIL-6R in the retina has yet to be determined and needs to be the focus of future studies.

In summary, our data provide novel insight into the role of IL-6 trans-signaling on human retinal endothelial cells. We have shown that activation of IL-6 trans-signaling leads to endothelial cell death, pro-inflammatory cytokine production, VEGF-A release, and tubular network formation which demonstrate its inflammatory and angiogenic capabilities. Therefore, targeting IL-6 trans-signaling by way of sgp130 may be an effective therapeutic strategy for the treatment of diabetic retinopathy.

APPENDIX

Figure 18. HRECs do not express the membrane-bound IL-6R. HRECs were treated with either 7.8 mmol/L or 25 mmol/L glucose. After 48 hours, HRECs were A) stained for mIL-6R using immunofluorescence. Left panel: nucleus stained with DAPI (blue); middle panel: mIL-6R (red); right panel: overlay. Additionally, B) mIL-6R expression was measured using flow cytometry. The shift in the median fluorescence intensity peak of unstained HREC controls (gray) was compared to HRECs stained with an APC-conjugated antibody against mIL-6R (red) when treated 7.8 mmol/L (left panel) or 25 mmol/L (right panel) for 48 hours.



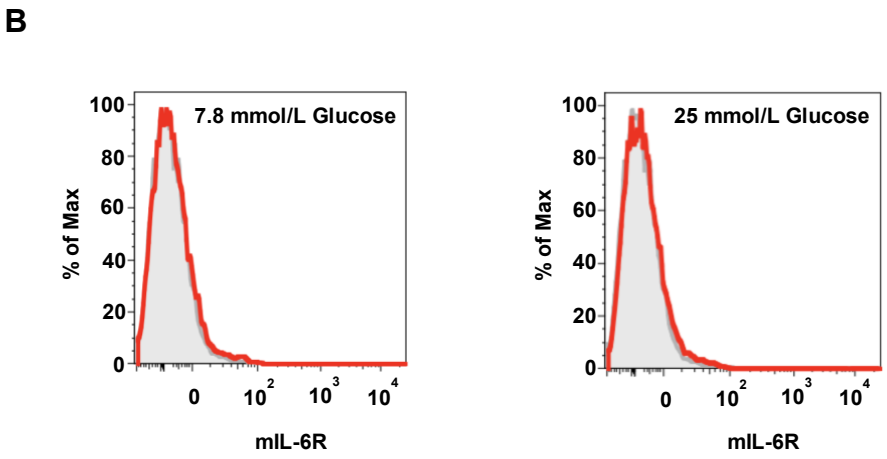


Figure 19. gp130 on HRECs is downregulated under hyperglycemic conditions. HRECs were treated with either 7.8 mmol/L or 25 mmol/L glucose. After 48 hours, HRECs were A) stained for gp130 using immunofluorescence. Left panel: nucleus stained with DAPI (blue); middle panel: gp130 (red); right panel: overlay. Additionally, B) gp130 expression was measured using flow cytometry. The shift in the median fluorescence intensity peak of unstained HREC controls (gray) was compared to HRECs stained with a PE-conjugated antibody against gp130 (blue) when treated 7.8 mmol/L (left panel) or 25 mmol/L (middle panel) for 48 hours. Right panel: results of the median fluorescence shift are expressed as mean fold change ± SDEV (n=5). Exact p-values are given (Mann-Whitney U test).

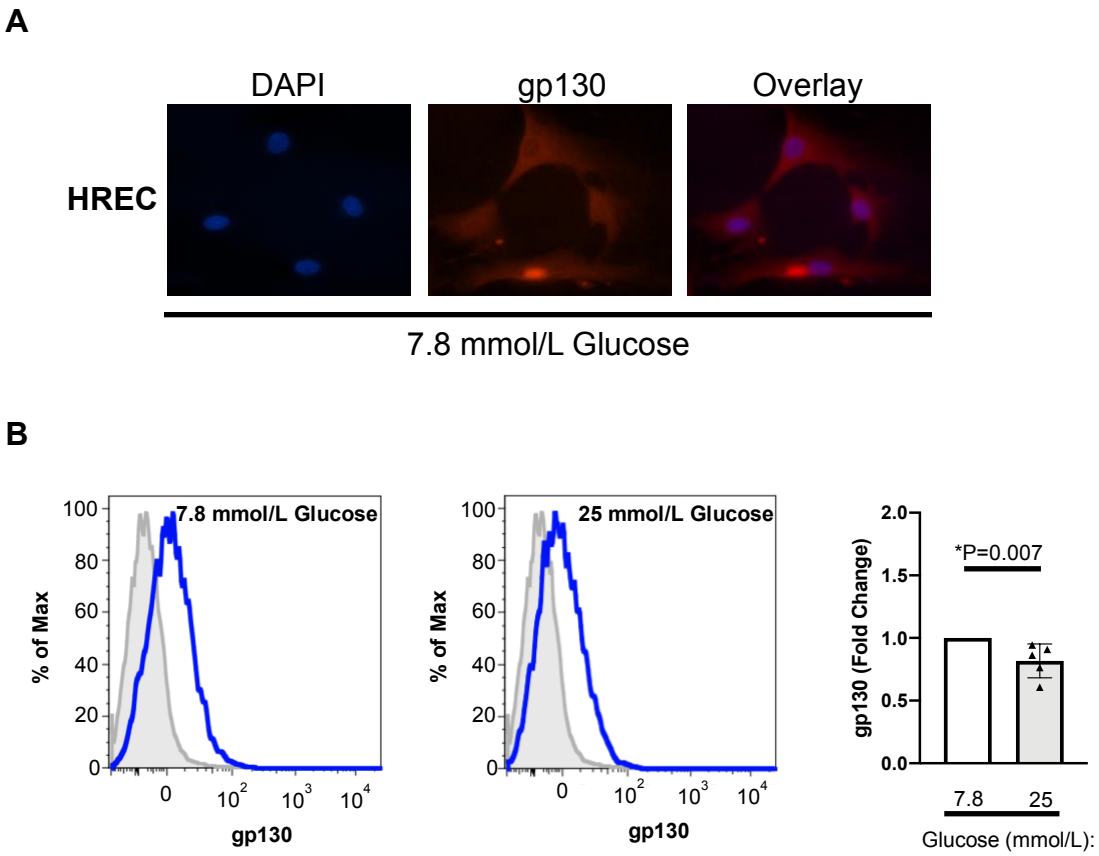


Figure 20. Release of sIL-6R and sgp130 from HRECs. HRECs were treated with 7.8 mmol/L or 25 mmol/L glucose. After 72 hours media samples were taken and A) sIL-6R and B) sgp130 release was measured using a multiplex magnetic bead assay. Results are expressed mean pg/mL/mg protein ± SDEV (n=3). Exact p-values are given (Student's t-test).

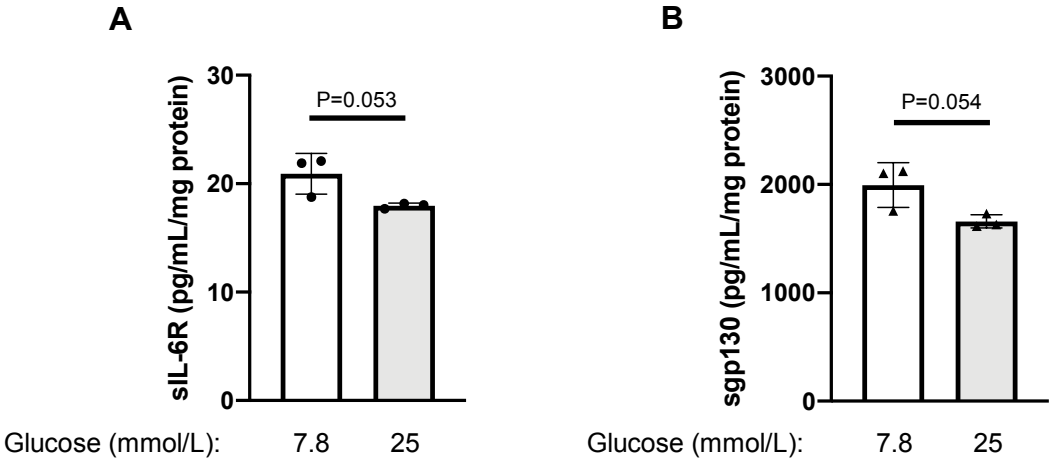


Figure 21. Decreased HREC viability in response to IL-6 trans-signaling. HRECs were treated in 7.8 mmol/L or 25 mmol/L glucose in the presence or absence of A) IL-6/sIL-6R (10 ng/mL) or B) IL-6 (5 ng/mL). After 72 hours HREC viability was measured using trypan blue exclusion. Results are expressed as mean number of blue cells per total cells (% cell death) ± SDEV (n=5-11). Exact p-values are given (Kruskal-Wallis followed by Dunn's post-hoc analysis).

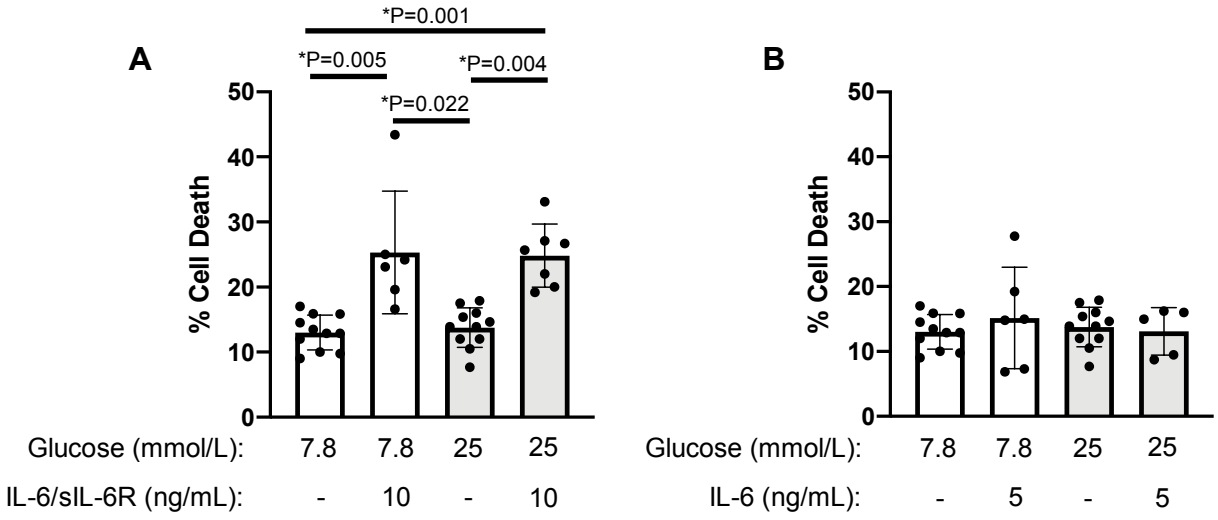


Figure 22. IL-6 trans-signaling induces inflammatory cytokine production from HRECs. HRECs were treated in either 7.8 mmol/L or 25 mmol/L glucose in the presence or absence of IL-6/sIL-6R (10 ng/mL). After 72 media samples were taken and levels of A) IL-1β, B) IL-6, C) TNFα, and D) IFNγ were measured using a multiplex magnetic bead assay. Results are expressed as mean pg/mL/mg protein ± SDEV (n=5). Exact p-values are given (A-C: One-way ANOVA followed by Tukey post hoc analysis; D: Kruskal-Wallis followed by Dunn's post hoc analysis) .

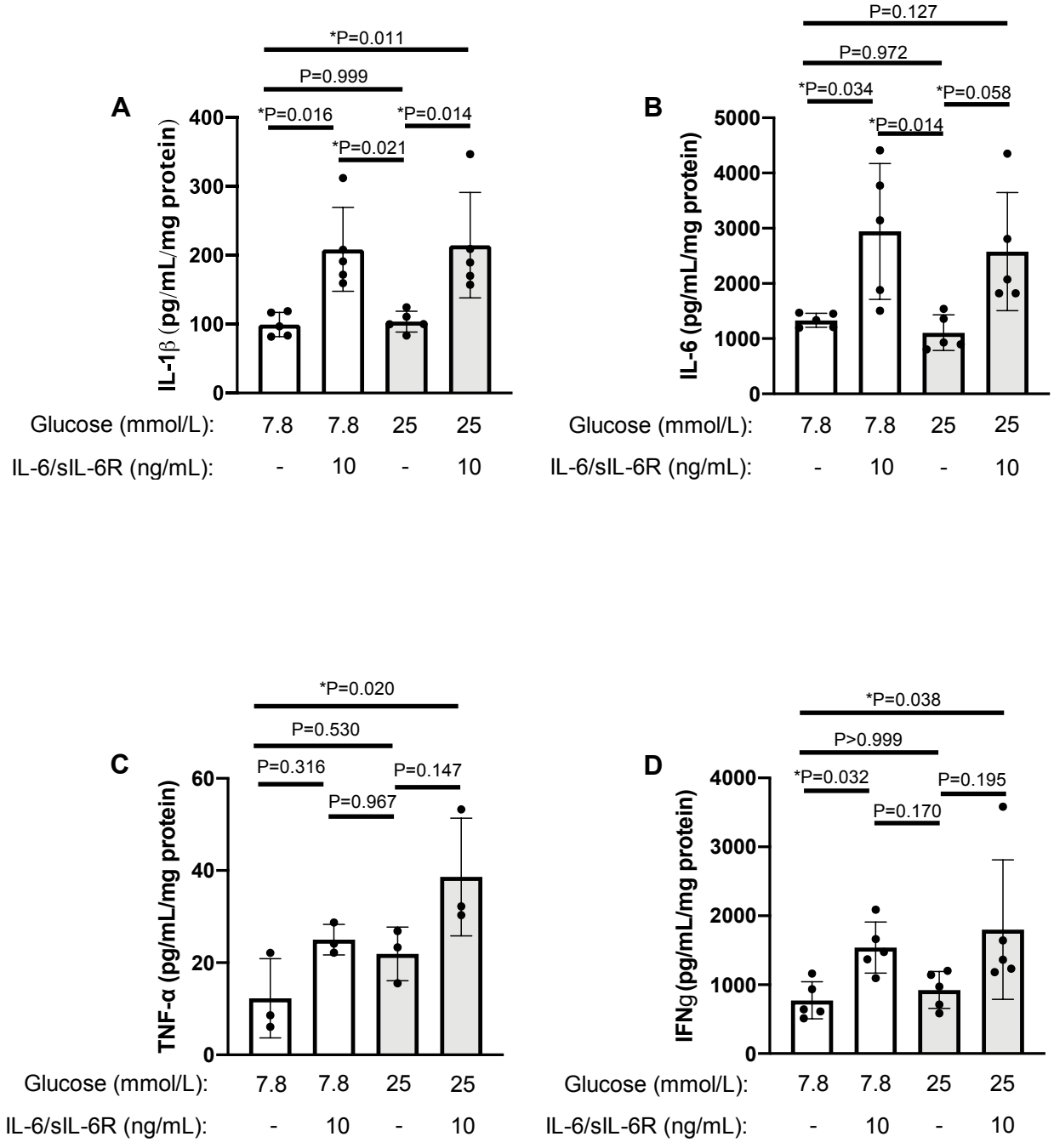


Figure 23. VEGF-A production as a result of IL-6 trans-signaling is dose-dependent. HRECs were treated with 7.8 mmol/L glucose or 25 mmol/L glucose in the presence of IL-6/sIL-6R (10 ng/mL). After 72 hours media samples were taken and levels of VEGF-A were measured using a multiplex magnetic bead assay. Results are expressed as mean pg/mL/mg protein ± SDEV (n=5). Exact p-values are given (One-way ANOVA followed by Tukey post-hoc analysis).

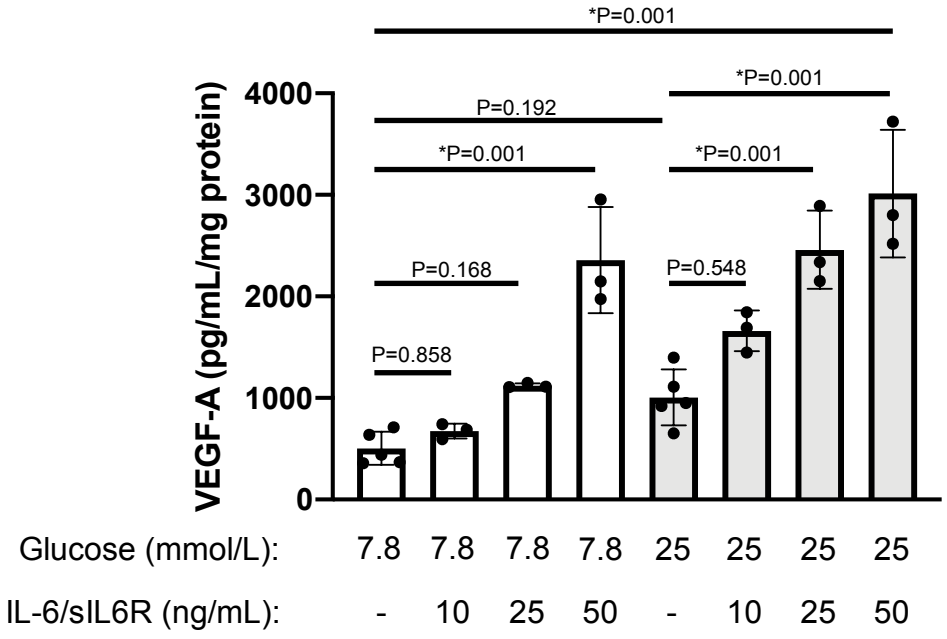
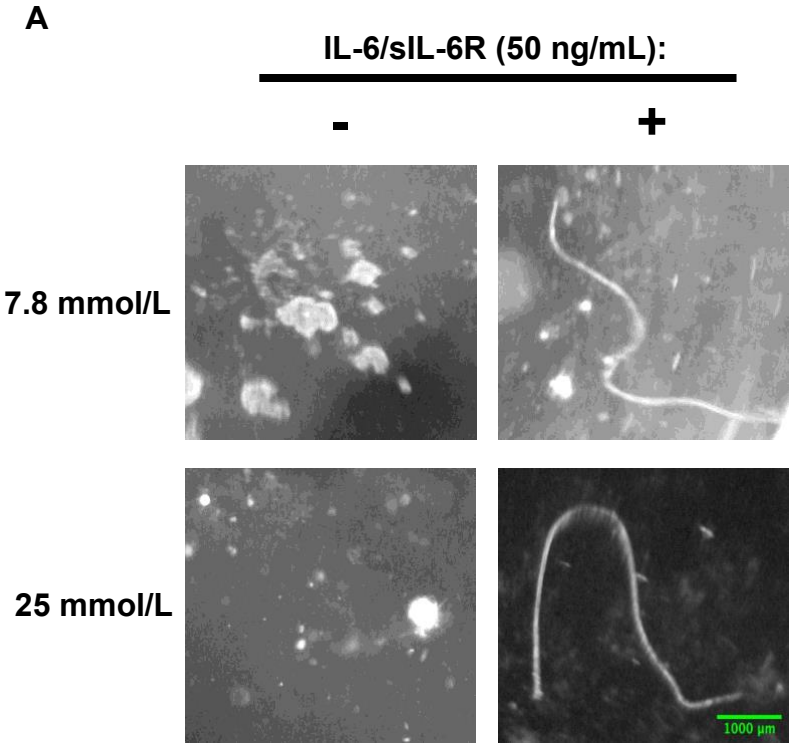


Figure 24. Pro-angiogenic effects of IL-6 trans-signaling. HREC were treated with 7.8 mmol/L glucose or 25 mmol/L glucose in the presence of IL-6/sIL-6R (50 ng/mL). After 5 days, A) the formation of tubes are determined using a dissecting microscope and B) further quantified using ImageJ software. Results are expressed as mean μM ± SDEV (n=4-7). Exact p-values are given (Kruskal-Wallis followed by Dunn's post-hoc analysis).



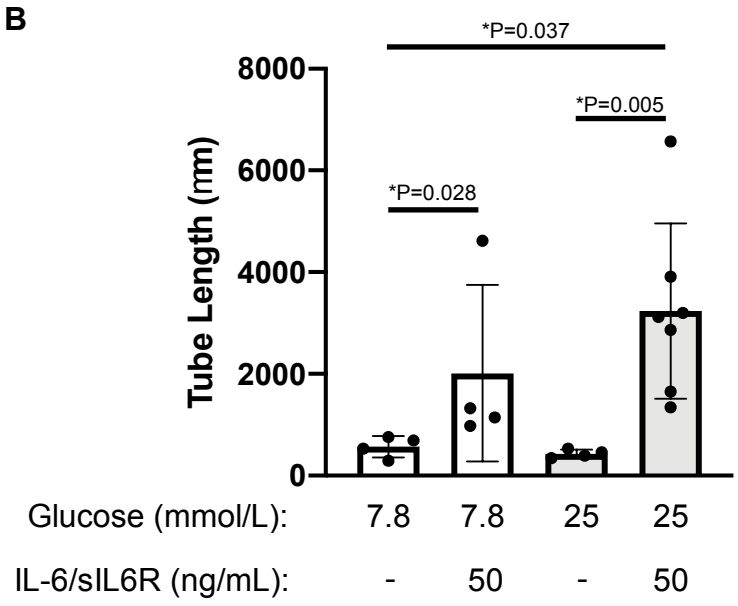
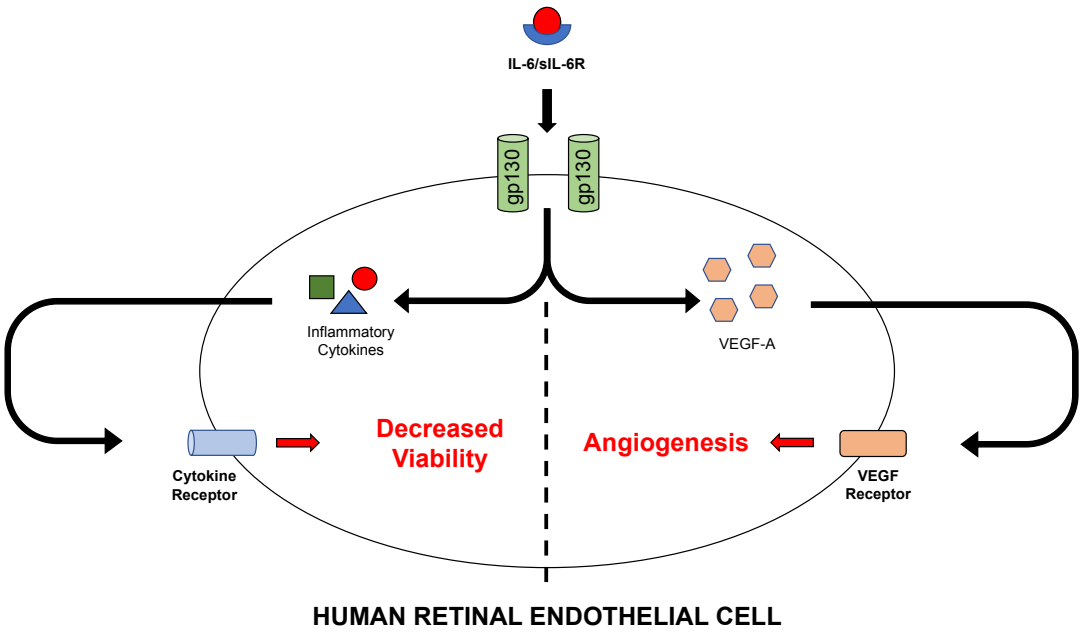


Figure 25. Summary of IL-6 trans-signaling on human retinal endothelial cells. Treatment of HRECs with lower concentrations of IL-6/sIL-6R (10ng/mL; Left) leads to inflammatory cytokine production and decreased viability. On the other hand, treatment with higher concentrations (50 ng/mL; Right) leads to VEGF-A production and pro-angiogenic effects.



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Chapter 4. Ranibizumab Alters Levels of Soluble Cytokine Receptors in Patients with Diabetic Macular Edema

Authors: Brandon A. Coughlin, Pratim Guha-Niyogi, Alla Sikorskii, Louis C. Glazer, and Susanne Mohr

This chapter is a modified version of a manuscript currently under review

4.1 Abstract

Purpose: Ranibizumab, an anti-VEGF-A (vascular endothelial cell growth factor-A) monoclonal antibody fragment, is a well-established treatment for diabetic patients with macular edema. However, very little is known about the effect of ranibizumab on regulation of pro- and anti-inflammatory signaling pathways and their regulation of VEGF family members, which was the aim of this study.

Materials and Methods: Diabetic patients (n=10) aged ≥18 years with central diabetic macular edema, BCVA >24 and <78, and central macular thickness (CMT) greater than 250 μm were enrolled in this study. Following a full eye exam, imaging, and an aqueous tap, patients received ranibizumab (0.3mg/0.05mL) injections at day one and weeks four and eight. At week 12, a full eye exam, imaging, and a second aqueous tap was obtained prior to the last injection of ranibizumab. Pre- and post-treatment aqueous humor samples were then analyzed using Milliplex MAP magnetic bead assays.

Results: As expected, ranibizumab lowered levels of VEGF-A, decreased CMT, and improved VA (visual acuity). In addition, it significantly lowered aqueous levels of IL-10, IFNγ, sIL-1R1, sIL-1R2, sRAGE, and VEGF-D. Changes in levels of VEGF-A and VEGF-C strongly correlated with changes in soluble receptors, sgp130 and sIL-6R, associated with IL-6 signaling pathways. In contrast, changes in VEGF-D correlated with sIL-1R1 and sIL-1R2, soluble receptors participating in IL-1 signaling. Changes in CMT and VA did not correlate with changes in levels of VEGF family members. However, post-treatment values of CMT correlated with post-treatment levels of VEGF-C. Post-treatment VA values correlated with a wide variety of potential biomarkers linked to inflammation.

Conclusions: Ranibizumab treatment had strong effects on regulating levels of soluble receptors closely linked to IL-1 and IL-6 signaling pathways. Therefore, a complete understanding of the actions of ranibizumab will further the development of additional therapies to support treatment of diabetic macular edema.

4.2 Introduction

Diabetic retinopathy (DR), a major complication of diabetes mellitus, is the leading cause of blindness among working-aged adults. Among patients with DR, approximately 7.4% have sight-threatening DR in the form of diabetic macular edema (DME)[1]. DME is characterized by the accumulation of fluid within the macula, a structure of the central retina specializing in high visual acuity, which leads to retinal dysfunction and blurred vision. One of the metabolic abnormalities associated with the initiation and progression of DR and DME is hyperglycemia[2]. For example, chronic hyperglycemia has been shown

to play a key role in promoting the disruption of the inner blood-retinal barriers[3,4]. More specifically, chronic hyperglycemia seems to alter cell to cell tight junctions and lead to the loss of vascular pericytes, endothelial cells, and retinal Müller cells[5–8]. The loss of the inner blood-retinal barrier's integrity allows for the leakage and accumulation of solutes and fluid into the retinal extracellular spaces[9,10]. As a consequence, the retinal layers begin to thicken and cause distorted or blurred vision.

One of several growth factors known to be produced in response to hyperglycemia and play a key role in the development of DME is vascular endothelial growth factor (VEGF). Members of the VEGF family include: VEGF-A, placental growth factor (PLGF), VEGF-B, VEGF-C, VEGF-D, and viral VEGF-E[11]. In the retina, multiple cell types are known to produce VEGF in response to hyperglycemia, such as Müller cells, retinal pigmented epithelial cells, pericytes, and endothelial cells[12,13]. Looking at retinal capillary damage, the release of VEGF promotes the process of generating new blood vessels known as angiogenesis. However, chronic and uncontrolled release of VEGF encourages the formation of immature and leaky retinal capillaries which further promote the progression of DME[14,15]. Historically, the cause for increased VEGF-A production was thought to be the activation of hypoxia inducible factor 1 (HIF1) in response to low oxygen levels due to the formation of acellular capillaries[16,17]. More recently however, there are speculations that chronic inflammatory events underlying the progression of DR directly promote continuous production of growth factors such as VEGF, which over time can lead to macular edema and neovascularization. Prominent inflammatory cytokines that have been linked with the progression of DR and DME include interleukin-1β (IL-1β) and tumor

necrosis factor-α (TNF-α)[18–20]. IL-1β has been shown to play a critical role in hyperglycemia-induced Müller cell death as well as the formation of acellular capillaries through the activation of the caspase-1/interleukin-1β signaling pathway[21,22]. Furthermore, IL-1β and TNF-α have been implicated in increasing the permeability of retinal endothelial cells by altering tight junction proteins[23,24]. In addition, interleukin-6 (IL-6) has been associated with vascular damage[25] and increased levels of IL-6 have been found in vitreous and aqueous humor samples of patients with diabetic retinopathy[26,27].

To date, very few treatment strategies are available for patients with DME, and of these strategies none are efficacious for all DME patients. Current therapeutic strategies include pars plana vitrectomy, laser photocoagulation, corticosteroids, and intravitreal injections of drugs targeting VEGF. Currently, anti-VEGF drugs, such as ranibizumab, are the treatment strategy of choice for patients with DME. Previous studies have shown the effects of ranibizumab on aqueous humor levels of VEGF-A and additional growth factors and how it alters central macular thickness (CMT) and visual acuity (VA)[28–36]. However, no study to date has looked at the effect of ranibizumab on additional VEGF family members beyond VEGF-A and their effect on inflammatory signaling pathways. Therefore, this study was aimed at determining the effect of ranibizumab on VEGFs, such as VEGF-C and VEGF-D, and pro- and anti-inflammatory cytokines and their respective soluble receptors. Specific focus was given to cytokines and soluble receptors that belong to either the IL-1, TNFα, or IL-6 signaling pathways.

4.3 Materials and Methods

Materials: Human Milliplex MAP magnetic bead assays (HCYTOMAG-60K; HSCRMAG-32k; HAGP1MAG-12k) were obtained from MilliporeSigma (Burlington, MA).

Patients: Diabetic patients (n=10) with central diabetic macular edema were enrolled from the practice of Vitreoretinal Associates in West Michigan between March 2017 and September of 2017. Research adhered to the declaration of Helsinki and conduct of the research was in accordance with the principles of ethics described in the Belmont report. IRB oversight was obtained from Western Institutional Review Board. At baseline entry into the study, all patients signed a written informed consent, reviewed and approved by the IRB. All patients received a complete eye exam along with optical coherence tomography (OCT), fluorescein-angiography (FA), and fundus photography. Visual acuity (VA) was obtained using ETDRS charts. All OCT scanning and FAs were obtained with the Heidelberg Spectralis OCT imaging system. All ten enrolled patients exited the study without complications.

Major enrollment criteria for the protocol were: 1) diagnosis of type 1 or type 2 diabetes; 2) Best corrected ETDRS visual acuity >24 and <78; 3) clinical exam demonstrating definite macular thickening due to diabetic macular edema; 4) central subfield thickness on OCT greater than 250 microns with associated signs of diabetic macular edema including hard exudate, intraretinal cysts and/or central edema noted on fundus contact exam; 5) Media clarity, pupillary dilation, and individual cooperation

sufficient for adequate fundus photographs; 6) age greater than or equal to 18 years; and 7) all women of child bearing age had to consent to a pregnancy test during the study.

Key exclusion criteria were: 1) individuals in poor glycemic treatment within the last four months; 2) systemic anti-VEGF or pro-VEGF treatment within four months prior to randomization; 3) an ocular condition present (other than diabetic macular edema and retinopathy) that in the opinion of the investigator might affect the macular edema or alter visual acuity during the course of the study such as vein occlusion, uveitis, or other ocular inflammatory diseases; 4) history of anti-VEGF treatment for diabetic macular edema within the past 12 months; 5) history of pan-retinal laser within four months prior to randomization or anticipated need for pan-retinal laser six months following randomization; 6) history of major ocular surgery within four months prior to randomization; and 7) history of YAG capsulotomy performed within two months prior to randomization.

Visit and Treatment Schedule: On day one, patients completed the eye exam and imaging. An aqueous tap was obtained prior to the injection of ranibizumab. The intravitreal injection of ranibizumab (6mg/mL – prefilled syringe 0.3mg/0.05mL) was performed using sterile technique with injection site 3.5 mm from the limbus. At weeks four and eight, a follow up exam consisting of a complete eye exam, IOP check, and OCT was performed and followed by an injection of ranibizumab (0.3mg/0.05mL). At week 12 (end of study), another complete follow up exam was performed, and a second aqueous humor sample was obtained. Following the aqueous tap, a final injection of ranibizumab

was administered. All patients were exited from the protocol at week 12 and continued to be followed up in the clinic within one month. No complications were reported from the aqueous taps or the ranibizumab injections.

Aqueous Humor Sampling: Undiluted aqueous humor samples (~0.1 mL) were collected from study participants before the first treatment (baseline). At week 12 after completion of monthly intravitreal injections of ranibizumab (a total of three treatments) and before the fourth and final injection, a final aqueous humor sample was taken. Aqueous humor samples were immediately frozen and stored at -20°C until further analysis.

Measurement of Aqueous Pro- and Anti-Inflammatory Mediators: Cytokine concentrations in retained aqueous humor were determined using a custom made, human Milliplex MAP magnetic bead panel (Millipore) according to manufacturer's instructions. Briefly, 25 μL of undiluted aqueous humor sample was incubated with 25 μL of magnetic beads coated with antibodies against individual cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-17A, IL-18, IP-10, TNFα, IFNγ, RANTES, MCP-1, leptin, sgp130, sIL-6R, sIL-1R1, sIL-1R2, sRAGE, VEGF-A, VEGF-C, and VEGF-D) in a 96-well plate overnight at 4°C. Plates were washed and incubated with detection antibodies (1 hour at room temperature), developed with streptavidin-phycoerythrin solution (30 minutes at room temperature), and analyzed using the Luminex200. Each pre- and post-treatment sample was run in triplicates. Levels of specific cytokines were calculated from internal standard curves for each individual cytokine and expressed as mean ± SDEV picograms per

milliliter (pg/mL). All samples were run simultaneously to avoid errors due to multiple freeze/thaw cycles and to ensure intra-assay consistency.

Statistical Analysis: Since the shapes of the distributions are difficult to be tested with n=10, both non-parametric (Wilcoxon signed-ranked test) and parametric (Student's t-test) tests were used to examine the associations of changes in VEGFs, CMT, and VA from pre- to post-treatment with changes in pro- and anti-inflammatory biomarkers. For correlation analyses, both non-parametric Spearman and parametric Pearson correlation coefficients were evaluated. Correlations above 0.5 are presented. A correlation coefficient of 0.5 is considered a moderate positive (negative) correlation and was chosen as a cut off value[37]. For all data, we decided to present exact p-values[38]. Findings were labeled as being significant (bold label) if the p-values for either one of the tests were less than 0.05. Statistical analysis was performed in SAS 9.4.

4.4 Results

4.4.1 Patients characteristics

To study the effects of ranibizumab on the changes in pro- and anti-inflammatory markers in diabetic patients with diagnosed macular edema, 10 patients were recruited. Aqueous humor samples (n=10) were collected at the start of the small clinical trial and at the end of the study. Baseline characteristics are outlined in Table 1. The mean patient age was 62.1 $\pm$ 9.96 years. Of the 10 patients, five (50%) patients had non-proliferative (mild, moderate, or severe) DR and five (50%) patients had proliferative DR. All patients

had clinically significant OCT-confirmed diabetic macular edema as defined in the study protocol and confirmed by the recruiting physician's clinical exam. Seven patients (70%) had intravitreal anti-VEGF injections, and eight patients (80%) had focal or panretinal laser before entry into the study. Three patients (30%) had received intravitreal corticosteroid injections.

4.4.2 Changes in visual parameters and pro- and anti-inflammatory markers before and after ranibizumab treatment

The median and interquartile ranges (IQR) of all pro- and anti-inflammatory markers measured from aqueous humor samples pre- and post-injections are summarized in Table 2. Cytokines that had significant differences between pre and post levels are in bold. Due to the small sample size of 10 patients, two different tests were chosen to determine statistics: the Wilcoxon signed-rank test (WT; non-parametric test) and the Student's t-test (ST; parametric test). Out of the 21 markers measured, levels of seven of them (IL-10, IFNγ, sIL-1R1, sIL-1R2, sRAGE, VEGF-A, and VEGF-D) were statistical different between pre- and post-injections by either one or both tests. After three intravitreal injections of ranibizumab (0.3mg/0.05mL) a significant change in visual acuity +10.3 letters was observed [pre-injections: 59.4$\pm$12.94; post-injections: 68.7$\pm$11.75 (mean letters$\pm$SDEV); WT: p=0.019; ST: p=0.027]. In addition, a significant decrease in CMT of -137.6μm [pre-injections: 518.1$\pm$215.4μm; post-injections: 380.5$\pm$111.9μm (mean μm$\pm$SDEV); WT: p=0.048; ST: p=0.052] was determined (Table 2).

Figure 26 presents the IQR for pre- and post-levels as well as the IQR of the change between post- minus pre-levels of the pro- and anti-inflammatory markers with significant differences. Aqueous humor levels of VEGF-A [median change: -46.43; IQR changes: -61.74, -2.89; WT: p=0.02; ST: p=0.01] and VEGF-D [median change: -3.92; IQR changes: -10.65, 0.09; WT: p=0.065; ST: p=0.051] decreased over the three months treatment period. Aqueous humor levels of VEGF-C were not affected by the treatment. Significant changes of pro- and anti-inflammatory cytokines such as IL-10 [median change: -0.21; IQR changes: -0.37, -0.05; WT: p=0.049; ST: p=0.046] and IFNγ [median change: -0.68; IQR changes: -1.14, 0.10; WT: p=0.027; ST: p=0.019] were observed. Interestingly, treatment with ranibizumab had its most prominent effect on aqueous humor levels of soluble receptors, which control signaling of pro- and anti-inflammatory cytokines. Aqueous humor levels of sIL-1R1 [median change: -2.81; IQR changes: -6.27, -0.57; WT: p=0.02; ST: p=0.014], sIL-1R2 [median change: -101.8; IQR changes: -138.4, 3.65; WT: p=0.02; ST: p=0.007], and sRAGE [median change: -12.53; IQR changes: -16.05, -2.95; WT: p=0.004; ST: p=0.002] were significantly reduced. Values of VA [median change: 8.00; IQR changes: 3.75, 12.00; WT: p=0.019; ST: p=0.027] and CMT [median change: -123.5; IQR changes: -218.00, -23.50; WT: p=0.048; ST: p=0.052] significantly improved following ranibizumab treatment.

4.4.3 Correlations of changes in pro- and anti-inflammatory mediators with changes in the VEGF family members

Since ranibizumab targets VEGF levels we were interested in how the changes in VEGF-A, VEGF-C, or VEGF-D levels between pre- and post-treatment correlate with

changes observed in levels of pro- and anti-inflammatory markers. The changes of five pro-/anti-inflammatory mediators correlated with changes in VEGF-A (Table 3). Changes in sgp130 [correlation coefficient factors Pearson (P): r=0.594 (p=0.069); Spearman (S) r=0.551 (p=0.098)], sIL-6R [P: r=0.812 (p=0.004); S: r=0.854 (p=0.001)], TNFα [P: r=0.595 (p=0.069); S: r=0.648 (p=0.042)], and VEGF-C [P: r=0.752 (p=0.012); S: r=0.745 (p=0.013)] correlated positively with changes in VEGF-A whereas changes in IL-10 [P: r= -0.803 (p=0.005); S: r= -0.757 (p=0.011)] correlated negatively. Changes in VEGF-C significantly correlated with sgp130 [P: r=0.801 (p=0.005); S: r=0.878 (p=0.001)], sIL-6R [P: r=0.791 (p=0.006); S: r=0.769 (p=0.009)], IL-8 [P: r=0.653 (p=0.040); S: r=0.318 (p=0.369)], IP-10 [P: r=0.641 (p=0.045); S: r=0.612 (p=0.060)], MCP-1 [P: r=0.840 (p=0.002); S: r=0.684 (p=0.028)], and VEGF-A [P: r=0.752 (p=0.012); S: r=0.745 (p=0.013)]. Changes in VEGF-D levels correlated with changes in sIL-1R2 levels [P: r= -0.722 (p=0.018); S: r= -0.636 (p=0.047)]. Again, two tests were chosen to determine correlations, the Pearson and the Spearman correlation test. All correlations that had a correlation coefficient factor above 0.5 are listed. Correlations between changes in pro- and anti-inflammatory markers versus changes in VEGF-A, VEGF-C, and VEGD-D levels that were significant are bolded. Interestingly, changes in VEGF levels did not correlated with changes in either VA or CMT (Table 3). Changes in CMT significantly correlated with changes in TNFα and VA whereas changes in VA significantly correlated with changes in CMT (Supplemental Table S1).

4.4.4 Correlations of changes in pro- and anti-inflammatory mediators with changes in soluble receptors

Treatment with ranibizumab seems to have a significant influence on levels of soluble receptors. Therefore, we determined correlations of changes in soluble receptor levels with changes in pro-and anti-inflammatory mediators. Changes in sgp130 positively correlated with changes in sIL-6R [P: r=0.616 (p=0.057); S: r=0.551 (p=0.098)], IL-6 [P: r=0.443 (p=0.199); S: r=0.624 (p=0.053)], IP-10 [P: r=0.629 (p=0.051); S: r=0.709 (p=0.021)], and VEGF-C [P: r=0.801 (p=0.005); S: r=0.878 (p=0.001)]. MCP-1 and VEGF-A were trending towards significance (Table 4). Another soluble receptor besides sgp130 involved in IL-6 signaling is the sIL-6R. Changes in sIL-6R correlated positively with changes in sgp130 [P: r=0.616 (p=0.057); S: r=0.551 (p=0.098)], MCP-1 [P: r=0.623 (p=0.054); S: r=0.563 (p=0.089)], VEGF-A [P: r=0.812 (p=0.004); S: r=0.854 (p=0.001)], and VEGF-C [P: r=0.791 (p=0.006); S: r=0.769 (p=0.009)] (Table 4). No correlation was observed with changes in CMT and VA. In addition, we determined correlations between changes in soluble receptors (sIL-1R1 and sIL-1R2) regulating the IL-1 signaling pathway and sRAGE with changes in pro- and anti-inflammatory mediators. Changes in sIL-1R1 correlated with changes sIL-1R2, sRAGE, and IL-17A. changes in sIL-1R2 correlated with changes in sIL-1R2, IL-17A, and VEGF-D (Supplemental Table S2).

Interestingly, correlation calculations demonstrate that changes in soluble receptors linked to IL-6 signaling (sgp130 and sIL-6R) closely correlate with changes in VEGF-A and VEGF-C levels. However, changes in VEGF-D levels were correlated more closely

with changes in soluble receptors belonging to IL-1 signaling (sIL-1R1 and sIL-1R2) (Figure 27).

4.4.5 Post-treatment correlations of pro- and anti-inflammatory markers with CMT and VA

Changes in CMT and VA did not seem to correlate with changes of most of the pro- and anti-inflammatory mediators that we measured although ranibizumab treatment significantly improved these vision parameters. Therefore, we explored how post-treatment CMT or VA correlated with post-treatment levels of pro- and anti-inflammatory mediators. Post-treatment CMT values significantly correlated with post-treatment levels of VEGF-C [P: r=0.614 (p=0.058); S: r=0.709 (p=0.021)]. CMT was also trending to correlated with post-treatment levels of IP-10, IL-18, and VA. In contrast to CMT, post-treatment values of VA significantly correlated with post-treatment levels of a variety of pro- and anti-inflammatory markers such as sgp130 [P: r= -0.805 (p=0.004); S: r= -0.769 (p=0.009)], sIL-1R2 [P: r= -0.716 (p=0.019); S: r= -0.615 (p=0.058)], sIL-6R [P: r= -0.768 (p=0.009); S: r= -0.733 (p=0.015)], IL-10 [P: r= -0.727 (p=0.017); S: r= -0.589 (p=0.072)], leptin [P: r= -0.852 (p=0.001); S: r= -0.915 (p=0.001)], IL-17A [P: r=0.744 (p=0.013); S: r=0.612 (p=0.060)], IL-1α [P: r= -0.642 (p=0.045); S: r= -0.793 (p=0.006)], IL-6 [P: r= -0.512 (p=0.129); S: r= -0.733 (p=0.015)], IP-10 [P: r= -0.702 (p=0.023); S: r= -0.854 (p=0.001)], and IL-18 [P: r= -0.782 (p=0.007); S: r= -0.684 (p=0.028)]. Post-treatment values of VA were also trending to correlate with post-treatment levels of VEGF-C and values of CMT (Table 5).

4.4.6 Correlations of combined pre- and post- values of mediators belonging to the IL-1 and IL-6 signaling pathways

All correlation calculations so far indicated that ranibizumab treatment influenced mediators associated with two important signaling pathways, the IL-1 and IL-6 signaling pathways. The activation of both pathways has been suggested to play a role in the development of diabetic retinopathy[19,22,39–44]. For these pathways to play a significant role, the different mediators that regulate the specific pathway need to correlate with each other. To determine the strength of correlations between mediators belonging to one pathway we combined the pre- and post-treatment values of individual mediators and determined paired correlations. We also aimed to identify whether the two different pathways are correlated with each other and how they link to levels of the different VEGF family members. Figure 28 shows that mediators of the IL-6 pathway significantly correlate with each other. IL-6 correlates with sgp130: [P: r= 0.338 (p=0.144); S: r= 0.445 (p=0.049)] and the sIL-6R: [P: r= 0.170 (p=0.473); S: r= 0.509 (p=0.021)]. Sgp130 and sIL-6R, both known to influence IL-6 trans-signaling strongly correlated with each other [P: r= 0.618 (p=0.003); S: r= 0.701 (p=0.001)] (Figure 28, A-C). As already indicated above, VEGF-A and VEGF-C levels seem to be closed linked to the IL-6 pathway. Two examples are shown in Figure 28 (D and E) with IL-6 correlating with VEGF-A [P: r= 0.451 (p=0.045); S: r= 0.537 (p=0.014)] and sgp130 correlating with VEGF-C [P: r= 0.534 (p=0.015); S: r= 0.523 (p=0.017)]. Interestingly, the IL-6 and the IL-1 pathway seem to influence each other since IL-6 strongly correlated with IL-1β [P: r= 0.586 (p=0.006); S: r= 0.598 (p=0.005)] (Figure 28 F). Looking at correlations among mediators of the IL-1 signaling pathway, IL-1β significantly correlated with sIL-1R1 [P: r= 0.628 (p=0.003); S:

r= 0.562 (p=0.009)]. IL-1α strongly correlated with sIL-1R1 [P: r= 0.799 (p=0.001); S: r= 0.764 (p=0.001)] and sIL-1R2 [P: r= 0.833 (p=0.001); S: r= 0.791 (p=0.001)] (Figure 28, G - H). Both IL-1β and IL-1α also correlated with each other [P: r= 0.534 (p=0.015); S: r= 0.525 (p=0.017)]. IL-1β and IL-1α did not correlated with VEGF-C but did with VEGF-A [IL-1β/VEGF-A: P: r= 0.541 (p=0.013); S: r= 0.605 (p=0.004)] and VEGF-D [IL-1β/VEGF-D: P: r= 0.595 (p=0.005); S: r= 0.473 (p=0.034) and IL-1α/VEGF-D: P: r= 0.753 (p=0.001); S: r= 0.671 (p=0.001)] (Figure 28, I – K). In addition, IL-1β and IL-6 correlated with TNFα [IL-1β/TNFα: P: r= 0.902 (p=0.0001); S: r= 0.875 (p=0.0001) and IL-6/TNFα: P: r= 0.534 (p=0.015); S: r= 0.726 (p=0.0003)] (Figure 28L) indicating that the three most prominent pro-inflammatory cytokines associated with DME to date are also linked to each other.

4.5 Discussion

Ranibizumab (0.3mg/0.05mL) significantly lowered levels of VEGF-A, decreased fluid within the macula, and improved visual acuity within this small study group of 10 patients, as expected. The effectiveness of the drug was pronounced considering that the study group included two patients that did not respond well to ranibizumab treatment in regard to lowering VEGF-A levels. Exclusion of these patients decreased post-treatment VEGF-A levels by an additional 44%. Interestingly, both patients still showed improved CMT and VA values despite not showing reduced VEGF-A levels. Therefore, we decided to analyze data of all 10 patients without excluding patients based on post-treatment VEGF-A levels. Although ranibizumab was designed to predominantly target VEGF-A it also altered VEGF-D levels. Whether this is due to direct actions of the drug with VEGF-

D or indirectly as a consequence of changes in other parameters has yet to be determined. VEGF-C levels were not affected by ranibizumab which was not surprising since it preferentially targets VEGF-A. Only levels of VEGF-A, -C, and -D were measured since these were VEGF family members for which well-established antibodies are available. In the future, it would be valuable to understand how all known VEGF family members are regulated by ranibizumab treatment to potentially identify pathways regulating VEGF production.

One of the major goals of the study was to understand the effects of monthly treatments with ranibizumab on aqueous humor levels of pro- and anti-inflammatory markers identified as potentially important in the development of diabetic retinopathy[18,26,43,45–53]. Following ranibizumab treatment, aqueous humor levels of IL-10, IFNγ, sIL-1R1, sIL-1R2, sRAGE, VEGF-A, and VEGF-D differed between pre- and post-treatment. Results regarding cytokine levels were consistent with previous studies looking at cytokine levels in aqueous humor samples of diabetic patients[26,28,29,36,46,48]. However, a full comparison with these studies is difficult since all studies measured different outcome parameters in response to different drug treatment protocols or no drug treatment at all. Our study focused on effects of ranibizumab in relation to regulation of different VEGF family members and their relationship to inflammatory signaling pathways, which included analysis of cytokines and respective soluble receptors, therefore, setting it apart from previous studies. These correlation studies suggest that VEGF family members seem to be linked to specific signaling pathways, such as the IL-6 and the IL-1 signaling pathways. Changes in VEGF-A and VEGF-C levels strongly correlated with

changes in mediators of IL-6 signaling. Activation of IL-6 signaling has been shown to induce VEGF-A production and proliferation especially in cancer[54–56]. In regards to the eye, IL-6 has been shown to promote VEGF-A production in the cornea but there have been no studies done so far examining the proliferative action of IL-6 signaling in the retina[57]. Surprisingly, ranibizumab strongly targeted soluble receptors levels of sgp130 and sIL-6R, both closely associated with IL-6 signaling, rather than affecting levels of the cytokine IL-6 itself. Understanding the link between IL-6 signaling and VEGF production in the retina would open up new venues for potential strategies to treat retinal diseases, such as diabetic retinopathy. Based on our data, targeting soluble receptors could be a promising strategy. In contrast to VEGF-A and -C, changes in VEGF-D levels correlated with changes in mediators controlling IL-1 actions. Ranibizumab treatment affected levels of soluble receptors sIL-1R1 and sIL-1R2, which regulate IL-1 signaling. IL-1 has long been associated with the development and progression of diabetic retinopathy[19,22,41,44]. Elevated levels of active caspase-1 and IL-1β have been shown in retinal tissue of diabetic patients[41,58]. Inhibition of the IL-1 pathway by either using the drug minocycline or by knock down of components of the IL-1 signaling pathway prevents formation of acellular capillaries in animal models of diabetic retinopathy[22,59]. Further, minocycline has shown promising results treating macular edema in diabetic patients[60]. Despite the increasing awareness that the IL-1 pathway plays an important role in the development of diabetic retinopathy, there is only a vague understanding how this pathway potentially influences VEGF levels in the retina. Both, the IL-1 and the IL-6 pathway also seem to link with TNFα, another prominent cytokine associated with vascular damage. TNFα correlated well with levels of VEGF-A.

Our study suggests that prominent pro-inflammatory cytokines link to different VEGF family members. To date, there are surprisingly few studies aiming to understand the functions of the different VEGF family members in the retina. On one hand, there is an abundance of literature demonstrating the effects of VEGF-A on retinal function. The detrimental effects of VEGF-A overproduction in retinal diseases, such as diabetic retinopathy, are well-established. This discovery has driven the development of drugs like ranibizumab and aflibercept targeting VEGF-A signaling, the current drugs of choice to treat diabetic macular edema. On the other hand, we know almost nothing about the role of other VEGF family members in retinal diseases. Studies have shown VEGF-C and VEGF-D to be key players in the development and maintenance of lymphatic vessels in various tissues, such as the colon, lungs, and skin. Furthermore, transgenic mice overexpression of VEGF-C in the skin leads to lymphatic vessel hyperplasia[61–63]. In the retina, it has been suggested that VEGF-C may play a role in the development of retinal lymphatic vessels and protection of retinal endothelial cells[64,65]. Variations in the VEGF-C gene are associated with diabetic retinopathy and diabetic macular edema[66]. There are some suggestions in the literature that VEGF-A and VEGF-C regulate each other although this seems to be tissue and stimulus specific[67,68]. Results of our study also strengthen the idea that VEGF-A and VEGF-C relate to each other.

Although changes in the levels of the different VEGF family members correlated with different signaling pathway, surprisingly, none of the changes in VEGF levels correlated with reduction in macular fluid and vision improvement. Therefore, the effect of

ranibizumab on fluid levels in the macular has to be an indirect effect rather than a direct effect by lowering VEGF-A levels. However, when focusing on correlations of post-treatment levels alone, CMT correlated with VEGF-C. Considering that changes in VEGF-A correlated with changes in VEGF-C one could speculate that ranibizumab exerts its effect on improving macular edema via a VEGF-A regulating VEGF-C mechanism, but this has to be positively confirmed by future studies. Visual acuity closely correlated with post-treatment levels of a variety of pro- and anti-inflammatory cytokines indicating that regulations of these potential biomarkers are more important for visual improvement than regulation of VEGF-A or any of the other VEGF family members.

In summary, ranibizumab treatment had strong effects on regulating levels of soluble receptors closely linked to pro- and anti-inflammatory signaling pathways, such as the IL-1 and IL-6 pathway. Further, the IL-6 and IL-1 pathways seem to correlate with changes in levels of different VEGF family members, VEGF-A and VEGF-C versus VEGF-D, respectively. Understanding the full actions of ranibizumab is crucial for the development of new target-driven treatment strategies for diabetic macular edema in addition to anti-VEGF treatment.

APPENDIX

Table 1. Baseline characteristics of patients with DME.

Characteristics		Values
	Number of Participants	10
	Sex, male/female (%)	6/4 (60% Male)
	Mean age of patients, y, mean (+/- SDEV)	62.1 (+/-9.96)
	Glycated hemoglobin,%, mean (+/- SDEV)	7.76 (+/-1.51)
	Treatment	
	Oral hypoglycemic agent, n (%)	8 (80)
	Insulin, n (%)	8 (80)
	Oral hypoglycemic agent plus insulin, n (%)	6 (60)
	Hypertension, n (%)	6 (60)
	Dyslipidemia, n (%)	3 (30)
	Duration of diabetes, n (%)	
	<5 years	2 (20)
	5-10 years	2 (20)
	11-15 years	2 (20)
	>15 years	4 (40)
Per-eye characteristics		
	Number of study eyes	10
	CMT, um, mean (+/- SDEV)	518.1 (+/-257.19)
	BCVA, letters, mean (+/- SDEV)	54 (+/-12.94)
	Phakic lens status, n (%)	8 (80)
	Posterior Vitreous detachment, n (%)	0 (0)
	Stage of DR, n (%)	
	Mild NPDR	0 (0)
	Moderate NPDR	4 (40)
	Severe NPDR	1 (10)
	Profliferative DR	5 (50)
	History of focal photocoagulation, n (%)	4 (40)
	History of panretinal photocoagulation, n (%)	4 (40)
	History of intravitreal anti-VEGF, n (%)	7 (70)
	History of Intravitreal corticosteroids, (%)	3 (30)

Table 2. Aqueous humor pro- and anti-inflammatory markers. Changes of pro- and anti-inflammatory markers (pg/mL) in aqueous humor samples of patients with diabetic macular edema at baseline and after treatment with Ranibizumab (0.3mg/0.05mL) are presented as interquartile ranges (IQR) [median (25%, 75%)].

Cytokine	Pre-Injection IQRs	Post-Injection IQRs	P-Value (WT)	P-Value (ST)
IL-1α	3.02 (2.10, 4.20)	2.01 (1.43, 4.06)	0.250	0.132
IL-1β	2.98 (2.27, 3.82)	2.40 (2.02, 3.43)	0.160	0.101
IL-6	3.49 (0.11, 14.35)	2.45 (1.42, 6.57)	0.492	0.182
IL-8	0.01 (0.00, 0.22)	0.06 (0.00, 0.33)	>0.999	0.389
IL-10	**0.71 (0.46, 1.36)**	**0.59 (0.46, 0.93)**	**0.049**	**0.046**
IL-17A	1.09 (0.87, 1.21)	0.92 (0.61, 1.20)	0.557	0.435
IL-18	0.52 (0.06, 1.39)	0.37 (0.24, 1.39)	0.922	0.926
IP-10	185.5 (156.30, 343.40)	263.8 (203.40, 393.30)	0.131	0.141
TNFα	3.18 (1.94, 4.08)	2.26 (1.12, 3.16)	0.131	0.123
IFNγ	**2.74 (2.148, 3.24)**	**1.81 (1.35, 2.35)**	**0.027**	**0.019**
RANTES	2.66 (2.30, 2.89)	2.61 (2.41, 2.98)	>0.999	0.745
MCP-1	1119 (893.30, 1618.00)	1182 (1028.00, 1480.00)	0.322	0.454
sgp130	9496.00 (8432.00, 10136.00)	9300.00 (7705.00, 11272.00)	0.922	0.876
sIL-1R1	**5.20 (2.49, 10.37)**	**1.48 (0.08, 7.43)**	**0.020**	**0.014**
sIL-1R2	**175.10 (138.70, 346.40)**	**78.76 (0.00, 247.20)**	**0.020**	**0.007**
sIL-6R	35.00 (23.69, 51.57)	43.10 (29.11, 75.40)	0.131	0.088
sRAGE	**25.06 (11.89, 35.00)**	**10.15 (5.04, 21.13)**	**0.004**	**0.002**
Leptin	739.40 (233.10, 2053.00)	353.20 (125.40, 2371.00)	0.084	0.441
VEGF-A	**93.43 (72.86, 155.70)**	**73.46 (40.06,112.30)**	**0.020**	**0.010**
VEGF-C	124.50 (109.00, 144.70)	127.90 (101.10, 151.20)	0.770	0.791
VEGF-D	**24.35 (22.54, 29.90)**	**22.15 (15.31, 26.23)**	**0.065**	**0.051**
Vision Parameters				
CMT	**518.1 (341.30, 616.50)**	**380.5 (305.30, 468.50)**	**0.048**	**0.052**
VA	**59.4 (50.75, 70.00)**	**68.70 (61.50, 76.25)**	**0.019**	**0.027**

Data that fulfill criteria (95% confidence interval; $p<0.05$) for either one or both of the statistical tests are highlighted in **bold** [Wilcoxon signed-rank test (WT) and Student's t-test (ST)].

Figure 26. IQRs of inflammatory markers and visual parameters pre- and post-treatment. Diabetic patients (n=10) with diagnosed diabetic macular edema were treated with ranibizumab (0.3mg/0.05mL) for 3 months. Aqueous humor samples at baseline and end of treatment were analyzed for pro- and anti-inflammatory markers using magnetic bead assays. Results are presented as IQRs for pre- and post-treatment levels (left bar graphs; $p<0.05$) and changes in post- minus pre-treatment levels (right bar graphs). IQRs of visual parameters (CMT and VA) are presented for pre- and post-treatment values and changes in post- minus pre- values.

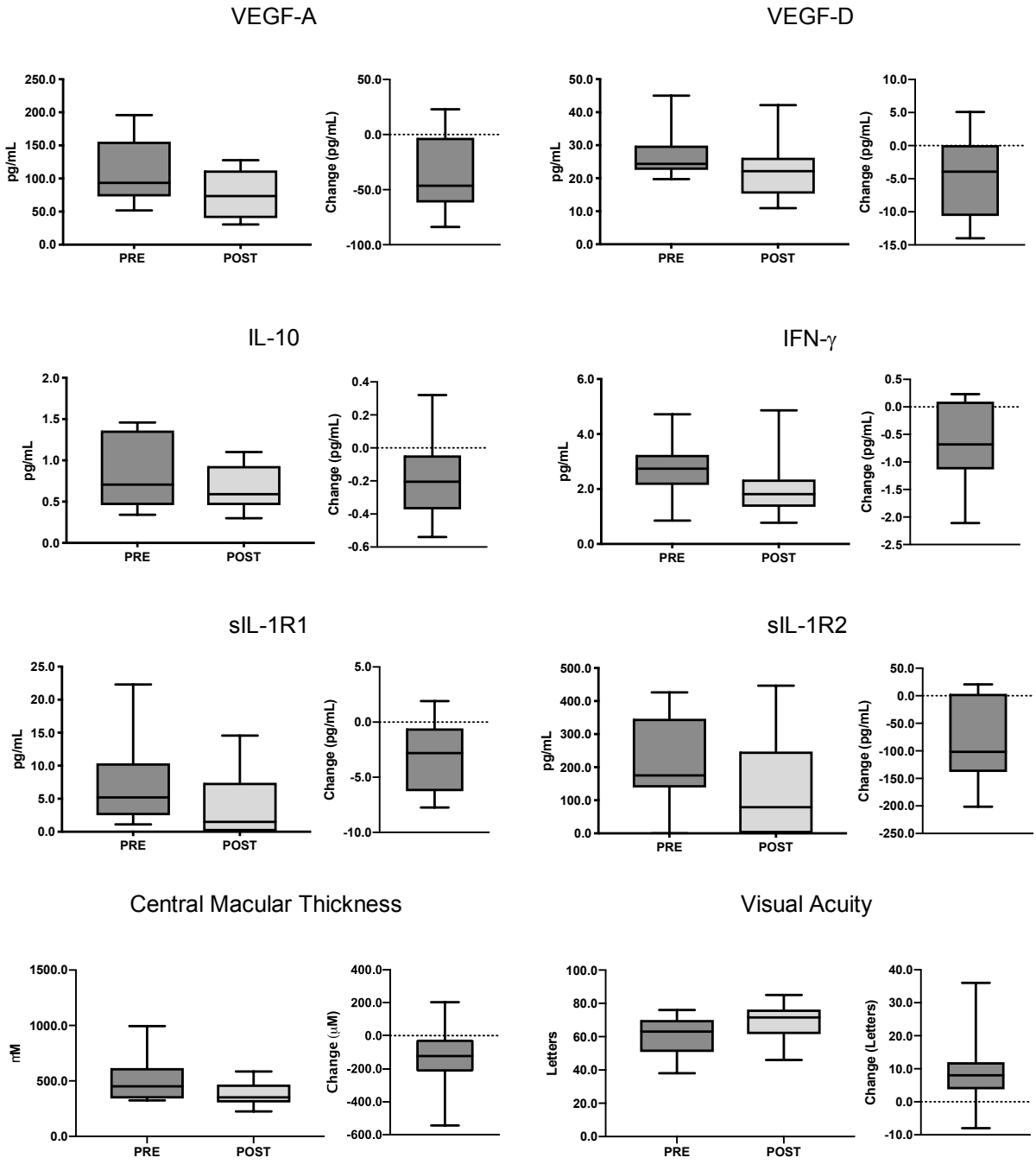


Table 3. Changes in VEGFs correlated with inflammatory markers and visual parameters. Changes (post- minus pre-treatment) in VEGF-A, VEGF-C, and VEGF-D levels were correlated with changes in levels of pro- and anti-inflammatory markers and values for visual parameters with correlation coefficient r>0.5 and p<0.05.

Change in VEGF-A Levels Correlated with		
Changes in Cytokines Levels	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
sgp130	0.594 (0.069)	0.551 (0.098)
sIL-6R	**0.812 (0.004)**	**0.854 (0.001)**
IL-10	**-0.803 (0.005)**	**-0.757 (0.011)**
VEGF-C	**0.752 (0.012)**	**0.745 (0.013)**
Changes in Vision Parameters	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
CMT	0.521 (0.122)	0.612 (0.060)
VA	0.349 (0.322)	0.458 (0.182)
Change in VEGF-C Levels Correlated with		
Changes in Cytokines Levels	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
sgp130	**0.801 (0.005)**	**0.878 (0.001)**
sIL-6R	**0.791 (0.006)**	**0.769 (0.009)**
sRAGE	-0.581 (0.077)	-0.527 (0.117)
IL-8	**0.653 (0.040)**	**0.318 (0.369)**
IP-10	**0.641 (0.045)**	**0.612 (0.060)**
MCP-1	**0.840 (0.002)**	**0.684 (0.028)**
RANTES	0.581 (0.078)	0.212 (0.556)
VEGF-A	**0.752 (0.012)**	**0.745 (0.013)**
Changes in Vision Parameters	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
CMT	0.110 (0.761)	0.212 (0.556)
VA	0.136 (0.707)	0.042 (0.907)
Change in VEGF-D Levels Correlated with		
Changes in Cytokines Levels	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
sIL-1R1	-0.572 (0.083)	-0.539 (0.107)
sIL-1R2	**-0.722 (0.018)**	**-0.636 (0.047)**
Changes in Vision Parameters	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
CMT	-0.097 (0.788)	0.042 (0.907)
VA	0.214 (0.551)	-0.151 (0.675)

Table 4. Change correlations for CMT and VA. Changes (post- minus pre-treatment) in CMT and VA were correlated with changes in levels of pro- and anti-inflammatory markers and values for visual parameters with correlation coefficient r>0.5 and p<0.05.

Changes in CMT Correlated with		
Changes in Cytokine Levels	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
sIL-6R	0.541 (0.106)	0.587 (0.073)
TNF-α	**0.528 (0.116)**	**0.709 (0.021)**
IL-10	-0.422 (0.223)	-0.575 (0.081)
VEGF-A	0.521 (0.122)	0.466 (0.060)
Changes in Vision Parameters	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
VA	**-0.777 (0.008)**	**-0.747 (0.012)**
Changes in VA Correlated with		
Changes in Cytokine Levels	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
IL-6	0.551 (0.098)	0.607 (0.062)
Changes in Vision Parameters	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
CMT	**-0.777 (0.008)**	**-0.747 (0.012)**

Table 5. Changes in sgp130 and sIL-6R correlated with inflammatory markers and visual parameters. Changes (post- minus pre-treatment) in sgp130 and sIL-6R levels were correlated with changes in levels of pro- and anti-inflammatory mediators and values for visual parameters with correlation coefficient r>0.5 and p<0.05.

Changes in sgp130 Correlated with		
Changes in Cytokine Levels	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
sIL-6R	**0.616 (0.057)**	**0.551 (0.098)**
IL-6	**0.443 (0.199)**	**0.624 (0.053)**
IP-10	**0.629 (0.051)**	**0.709 (0.021)**
MCP-1	0.595 (0.069)	0.515 (0.127)
VEGF-A	0.594 (0.069)	0.551 (0.098)
VEGF-C	**0.801 (0.005)**	**0.878 (0.001)**
Changes in Vision Parameters	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
CMT	-0.136 (0.706)	-0.103 (0.777)
VA	0.127 (0.726)	0.121 (0.738)
Changes in sIL-6R Correlated with		
Changes in Cytokine Levels	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
sgp130	**0.616 (0.057)**	**0.551 (0.098)**
MCP-1	**0.623 (0.054)**	**0.563 (0.089)**
VEGF-A	**0.812 (0.004)**	**0.854 (0.001)**
VEGF-C	**0.791 (0.006)**	**0.769 (0.009)**
Changes in Vision Parameters	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
CMT	0.541 (0.106)	0.587 (0.073)
VA	-0.312 (0.380)	-0.188 (0.602)

Table 6. Changes in sIL-1R1 and sIL-1R2 correlated with inflammatory markers and visual parameters. Changes (post- minus pre-treatment) in sIL-1R1 and sIL-1R2 were correlated with changes in levels of pro- and anti-inflammatory mediators and values for visual parameters with correlation coefficient r>0.5 and p<0.05.

Changes in sIL-1R1 Correlated with		
Changes in Cytokine Levels	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
sIL-1R2	**0.733 (0.015)**	**0.551 (0.098)**
sRAGE	**0.698 (0.024)**	**0.539 (0.107)**
IL-17A	**0.669 (0.034)**	**0.563 (0.089)**
IL-18	0.567 (0.087)	0.515 (0.127)
VEGF-D	-0.572 (0.083)	-0.539 (0.107)
Changes in Vision Parameters	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
CMT	0.236 (0.511)	0.054 (0.881)
VA	-0.178 (0.622)	-0.139 (0.700)
Changes in sIL-1R2 Correlated with		
Changes in Cytokine Levels	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
sIL-1R1	**0.733 (0.015)**	**0.551 (0.098)**
IL-17A	**0.733 (0.015)**	**0.781 (0.007)**
VEGF-D	**-0.722 (0.018)**	**-0.636 (0.047)**
Changes in Vision Parameters	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
CMT	0.191 (0.595)	0.224 (0.533)
VA	0.078 (0.829)	0.194 (0.590)

Figure 27. Change correlations of soluble receptors and VEGFs. Changes (post-minus pre-treatment) of soluble receptors within aqueous humor samples were calculated and correlated to changes in VEGF-A (A, B), VEGF-C (C, D), and VEGF-D (E, F) levels. Data are presented as scatter plots with $r>0.5$ and $p<0.05$ (Spearman correlation).

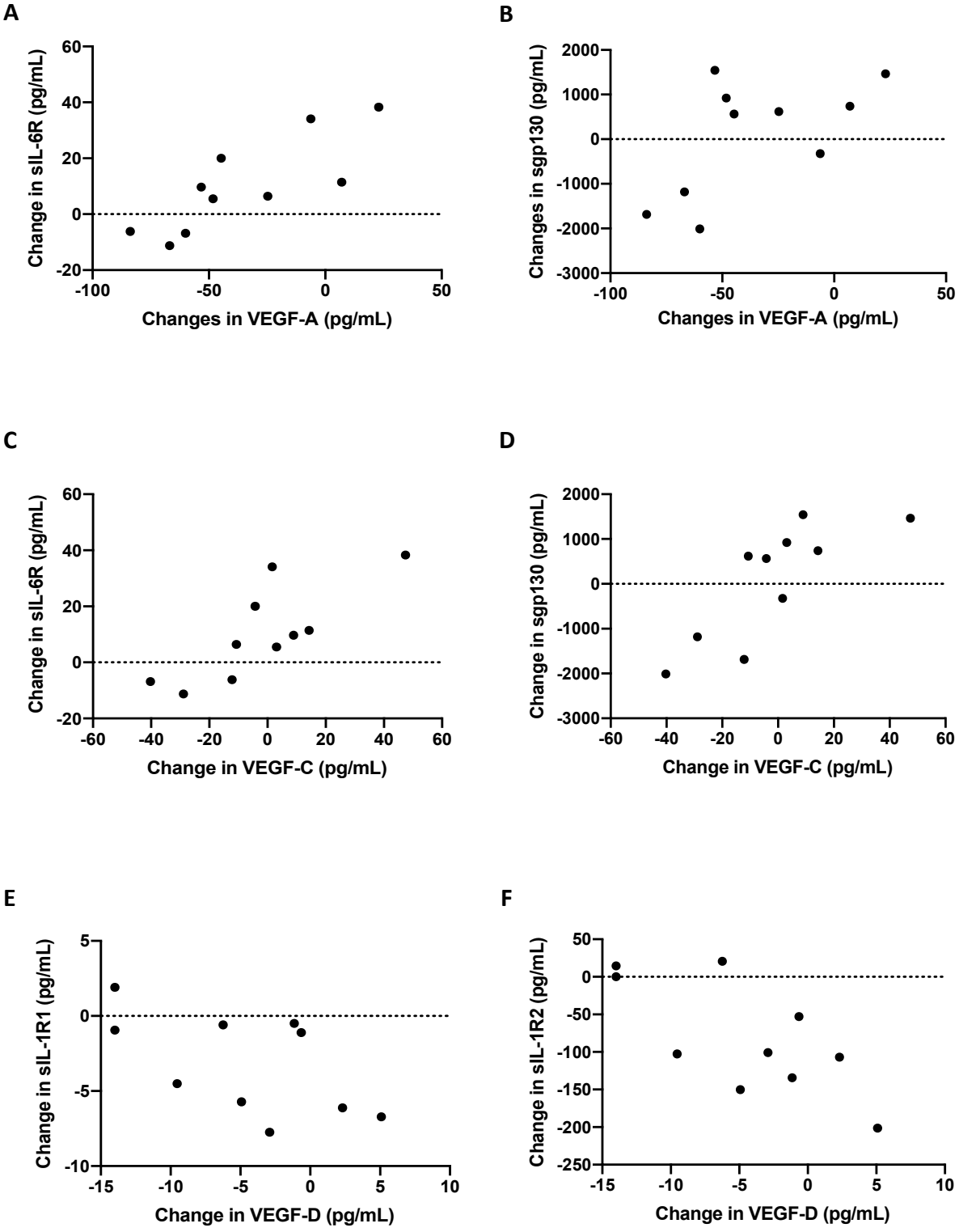
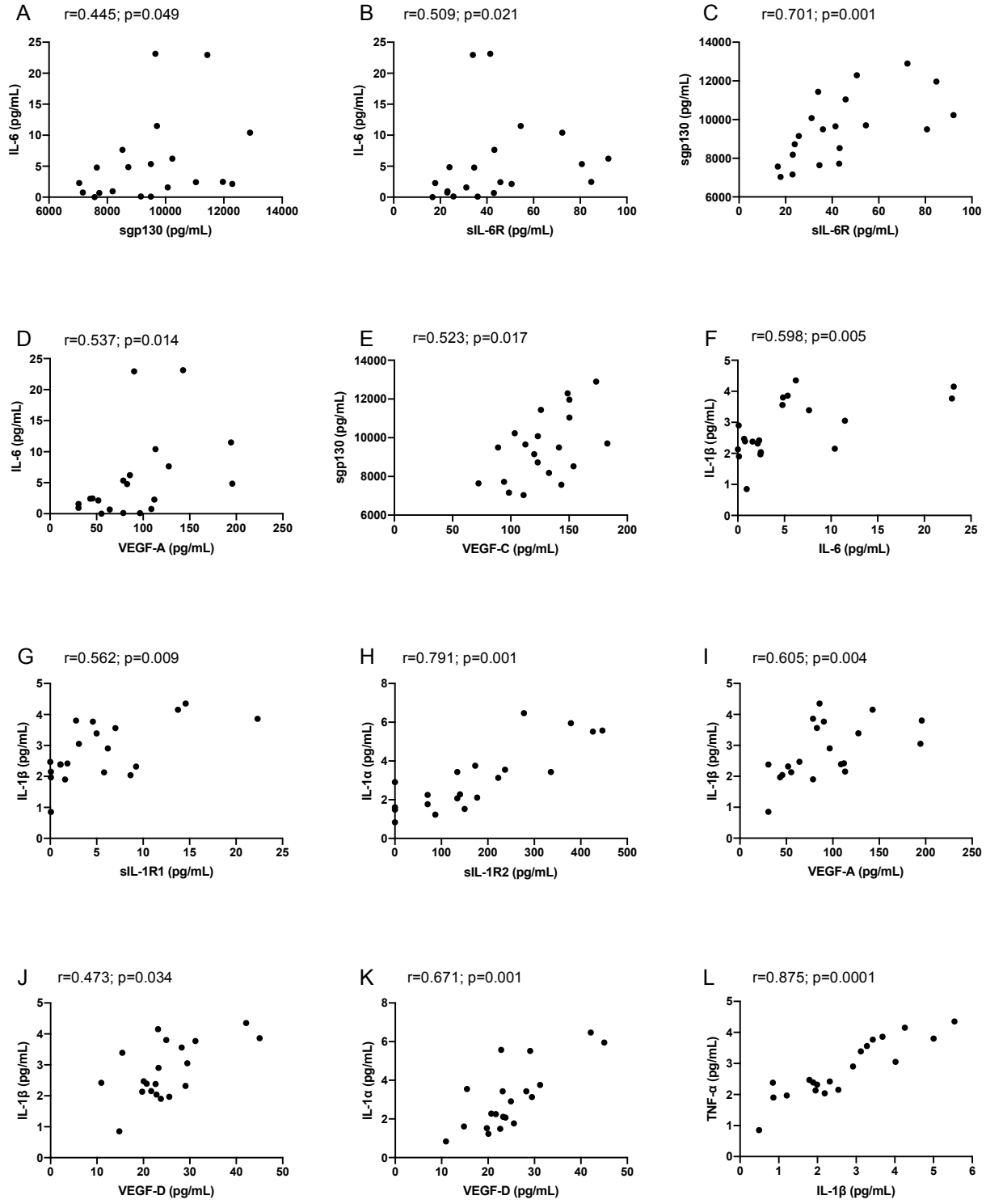


Table 7. Post-treatment CMT and VA correlations. Post-treatment values in CMT and VA were correlated with post-treatment levels of pro- and anti-inflammatory markers and values for visual parameters with correlation coefficient r>0.5 and p<0.05.

Post-Treatment CMT Values Correlated with		
Post-Treatment Cytokines Levels	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
IP-10	0.563 (0.089)	0.539 (0.107)
IL-18	0.552 (0.097)	0.442 (0.200)
VEGF-C	**0.614 (0.058)**	**0.709 (0.021)**
VA	-0.557 (0.094)	-0.563 (0.089)
Post-Treatment VA Values Correlated with		
Post-Treatment Cytokines Levels	**Pearson Correlation Coefficient (p-value)**	**Spearman Correlation Coefficient (p-value)**
sgp130	**-0.805 (0.004)**	**-0.769 (0.009)**
sIL-1R2	**-0.716 (0.019)**	**-0.615 (0.058)**
sIL-6R	**-0.768 (0.009)**	**-0.733 (0.015)**
IL-10	**-0.727 (0.017)**	**-0.589 (0.072)**
Leptin	**-0.852 (0.001)**	**-0.915 (0.001)**
IL-17A	**0.744 (0.013)**	**0.612 (0.060)**
IL-1α	**-0.642 (0.045)**	**-0.793 (0.006)**
IL-6	**-0.512 (0.129)**	**-0.733 (0.015)**
IP-10	**-0.702 (0.023)**	**-0.854 (0.001)**
IL-18	**-0.782 (0.007)**	**-0.684 (0.028)**
VEGF-C	-0.597 (0.067)	-0.600 (0.066)
CMT	-0.557 (0.094)	-0.563 (0.089)

Figure 28. IL-6 and IL-1 signaling pathway correlations. Paired correlations of combined pre- and post-treatment values of individual mediators known to regulate the IL-6 (A-F) and IL-1 (F-L) pathways are presented as scatter plots with r>0.5 and p>0.05 (Spearman correlation).



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Chapter 5. Summary, Translational Implications, and Future Directions

5.1 Summary of Data Regarding IL-6 Signaling on Human Müller Cells

IL-6 has become a target of interest for drug development in DR, yet very little is known about IL-6 signaling in the retina. Therefore, it has become absolutely crucial to identify retinal cell types that are capable of IL-6 signaling by investing the expression IL-6 signaling receptors. It is well established that Müller cells play a major role in the development of DR. However, prior to this project very little was known about Müller cell's IL-6 signaling capabilities and how they would be affected by hyperglycemia. The data of this project demonstrate:

- Human Müller cells express both mIL-6R and gp130 under normal glucose conditions and their expression is upregulated in response to hyperglycemia. These data demonstrate the ability of IL-6 to signal through both classical IL-6 and IL-6 trans-signaling.
- Activation of classical IL-6 signaling protects human Müller cells from hyperglycemia-induced cell death. Furthermore, the neuroprotective effects of IL-6 are mediated through IL-6-induced VEGF-A signaling.
- On the other hand, IL-6 trans-signaling on human Müller cells had slight protective effects that were VEGF-A independent. These data suggest an alternative mechanism which may be dependent on IL-10 release. However

further experiments are needed in order to determine the validity of this mechanism of action.

5.2 Summary of Data Regarding the Function of IL-6 Trans-signaling on Human Retinal Endothelial Cells.

Neovascularization and retinal edema are two vision threatening complications of DR. Previous studies have shown the pro-angiogenic abilities of IL-6 trans-signaling in tissues other than the retina, including the cornea and choroid. Therefore, our study focused on identifying pro-inflammatory and pro-angiogenic capabilities of classical IL-6 and IL-6 trans-signaling on human retinal endothelial cells (HRECs). Moreover, it aimed to provide novel insight into the action of IL-6 signaling on HRECs under hyperglycemic conditions. Our results indicate:

- HRECS express gp130 but do not express mIL-6R under normal or hyperglycemic conditions. These data establish that due to the lack of mIL-6R, HRECs are only able to signal through IL-6 trans-signaling.
- Furthermore, HRECs are able to synthesize and release low levels of sIL-6R and high levels sgp130. The data indicate that HRECs express the necessary receptors to initiate IL-6 trans-signaling (sIL-6R and gp130). However, due to high levels of sgp130 it is not sufficient to effectively activate IL-6 trans-signaling, signifying an alternative source of sIL-6R is needed.

- Initiation of IL-6 trans-signaling on HRECs with lower concentrations of IL-6/sIL-6R (10ng/mL) and not IL-6 alone increased release of pro-inflammatory cytokines and decreased HREC viability. These data provide insight into a potential mechanism of blood retinal barrier disruption and subsequent retinal edema.
- Finally, higher concentrations of IL-6/sIL-6R (50ng/mL) promoted VEGF-A release in a dose-dependent manner. Interestingly, trans-signaling promoted the formation of HREC tubular networks. These data demonstrate the pro-angiogenic capabilities of IL-6 trans-signaling and its potential as a targeted therapy to prevent neovascularization.

5.3 Summary of Data Regarding the Effects of Ranibizumab on Soluble Cytokine Receptors and Members of the VEGF family in Patients with Diabetic Macular Edema

Diabetic macular edema (DME) is one of the most prominent vision threatening complications of diabetic retinopathy for which there are limited treatment options available. Although ranibizumab is well-established for the treatment of DME, it is only effective in about one-third of the patient population. Surprisingly, very little is known about its effect on regulating retinal inflammation and various members of the VEGF family other than VEGF-A. Our study was unique in that it aimed to identify the effects of ranibizumab treatment on regulating known inflammatory signaling pathways, such as IL-6 and IL-1, and how they correlate to various VEGF family members. Our data show:

- Treatment with ranibizumab lowered levels of VEGF-A, but changes (post-minus pre-treatment) in VEGF-A levels were not correlated with decreased central macular thickness or increased visual acuity. This suggests that increased visual acuity in response to ranibizumab treatment may be dependent on alternative mechanisms, such as an IL-6/VEGF-A axis.
- Surprisingly, ranibizumab had a strong effect on soluble cytokine receptors, indicating that modulating inflammatory pathways by means of regulating soluble receptors may be more efficient than regulating the cytokines themselves.
- Changes in VEGF-A and VEGF-C correlated to changes in members of IL-6 signaling, such as sgp130 and sIL-6R. On the other hand, changes in VEGF-D correlated with changes in sIL-1R2.
- Finally, post-treatment levels of VEGF-C but not VEGF-A correlated to post-treatment levels of central macular thickness. This implies that VEGF-A alone is not sufficient to lower central macular thickness, however, the ratio of VEGF-A to VEGF-C may be of importance with regard to the severity of macular edema.

For the first time, this dissertation demonstrates that activation of IL-6 signaling pathways, classical IL-6 and IL-6 trans-signaling, leads to diverse outcomes on two retinal cell types that play an integral role in the development of diabetic retinopathy, human Müller and human retinal endothelial cells. The most important finding of this dissertation

is that the production of VEGF-A from both of these cell types is linked to IL-6, and more importantly their production of VEGF-A is due to triggering of opposing IL-6 signaling pathways. The activation of IL-6 classical signaling, but not IL-6 trans-signaling, leads to VEGF-A production from human Müller cells. In contrast, human retinal endothelial cells only release VEGF-A in response to IL-6 trans-signaling. Interestingly, our clinical study showed strong correlations between members involved in IL-6 signaling and VEGF-A, further highlighting the importance of an IL-6/VEGF-A signaling axis. These discoveries provide novel clinical insight into alternative mechanisms of VEGF-A production, which is known to play a prominent role in the development of neovascularization and diabetic macular edema. Taken together, these findings have significant translational implications for the field of diabetic retinopathy.

5.4 Translational Implications and Future Directions

For the last decade, advancements in treatment strategies for DR have been narrowly focused on anti-VEGF therapy including Avastin, Lucentis, and Eylea. Although anti-VEGF therapy is well established for the treatment of DR, it is only effective for about one-third of the patient population[1]. More importantly, the long-term effects of VEGF-A blockage on the retina's health and well-being are far less understood. Classically, VEGF-A is known as a neurotrophic factor and under physiological conditions is important for cell maintenance and survival. For example, previous studies have shown that VEGF-A signaling through VEGF receptor 2 is necessary for Müller cell survival *in vivo*, and Müller cell dropout is accompanied by thinning of the inner and outer nuclear layers[2,3].

Furthermore, our data demonstrate that the protective effects of IL-6 signaling on human Müller cells are mediated by IL-6 induced VEGF-A signaling. The overall goal of anti-VEGF therapy is to reduce VEGF-A levels below the pathological threshold and regain physiological function[3]. However, it is rarely taken into consideration that the physiological range of VEGF-A is most likely vastly different from patient to patient. For example, a patient who naturally has lower VEGF-A levels is going to respond drastically different to anti-VEGF therapy compared to a patient whose normal physiological range is much higher. The short-term use of anti-VEGF therapy is useful for reducing VEGF-A in attempt to regain physiological control. However, the use of anti-VEGF therapy long-term may not only reduce VEGF-A to normal physiological levels, but also reduce VEGF-A to a concentration where it can no longer promote its neuroprotective effects. As a consequence, the retina is left vulnerable and unprotected. Ultimately, anti-VEGF therapy may have short-term gains, but also long-term consequences, a fact that is slowly gaining some awareness.

Similar to anti-VEGF therapy, anti-IL-6 therapy is being considered for the treatment of DR. The idea for the development of a neutralizing IL-6 antibody was based off the assumption that elevated levels of IL-6 in the vitreous and aqueous humor of diabetic patients is pathological. This way of thinking was further driven by reports that IL-6 is detrimental for vascular function in other tissues. However, our data suggest that while IL-6 negatively affects the retinal vasculature via IL-6 trans-signaling, it is also absolutely crucial for the survival of human retinal Müller cells. The use of a neutralizing IL-6 antibody for the treatment of DR may prevent IL-6 trans-signaling and its detrimental effects on the

retinal vasculature, but it will also ultimately block the protective effects of classical IL-6 signaling within the neuroretina. Moreover, data from this dissertation have shown that production of VEGF-A is dependent on classical IL-6 signaling. Therefore, neutralization of IL-6 would lower IL-6 and IL-6-induced VEGF-A production subsequently reducing the protective capabilities of both neuroprotectants in the retina. Rather than neutralizing IL-6 itself, it may be more effective to block IL-6 trans-signaling directly through the use of soluble gp130, the natural antagonist to IL-6 trans-signaling. It seems that developing an "ideal" treatment (if there is one) against retinal damage has to be a careful undertaking thoroughly evaluating the good versus bad effects of a potential drug on the different retinal functions and cautiously weighing the needs of the neuroretina versus the needs of the retinal vasculature. Currently, it looks like that any treatment strategy that is good for the retinal vasculature is not beneficial for proper neuroretina function.

While much is known about the role of VEGF-A in the healthy and diabetic retina, very little is known about the other VEGF family members. Our studies indicate VEGF-C and VEGF-D may also play a role in diabetic macular edema. Previous studies have shown that in other tissues outside the retina, VEGF-C and VEGF-D promote lymphangiogenesis[4–6]. In addition, both VEGF-C and VEGF-D have been implicated in promoting lymphangiogenesis in the meninges of the brain[7]. Classically it has been thought that the retina does not have a lymphatic system. More recently, it has been hypothesized that the retina has a lymphatic-like system termed glymphatics. It is proposed that this glymphatics system is located in the paravascular space between retinal endothelial cells and the surrounding Müller cells. The presence of glymphatics

may be responsible for intermittent volume changes within the retinal layers[8,9]. Due to the fact that VEGF-C and VEGF-D are known to promote lymphangiogenesis in other tissues, it can be hypothesized that they control the formation of glymphatics as well. The overall goal of a lymphatic systems is to recycle interstitial fluid. Therefore, due to the fact that Müller cells are one of only two cell types found within the macula and wrap around the vasculature it is feasible that they potentially participate in the formation of glymphatics. It is possible that malfunctioning or leaky glymphatic systems may play a role in the development of diabetic macular edema. Whether or not VEGF-C or VEGF-D play a role in glymphatic physiology has yet to be determined. Our study gives rise to the idea that other VEGF family members most likely play just as an important of a role as VEGF-A. Therefore, it is crucial that future studies are aimed at determining the retinal function of all members of the VEGF family in order to have a more comprehensive understanding of the roles of VEGF signaling in the healthy and diseased retina. Increased knowledge pertaining to the roles of the various VEGF family members may give rise newly targeted treatment strategies other than VEGF-A.

Developing effective strategies for the treatment of DR also remains difficult due to the lack of proper animal models. The most common models for studying DR include: STZ-induced diabetes, galactosemia, and oxygen induced retinopathy (OIR). The STZ-induced diabetic mouse model is used for studying the effects type 1 diabetes, while the galactosemia mouse model allows for studying the direct effects of hexose sugars on DR associated pathology. Both of these models develop acellular capillaries, which are considered to be the hallmark of retinal pathology in DR, and are effective for studying

the earlier stages of DR. However, neither of these models develop proliferative retinopathy or macular edema. On the other hand, the OIR model uses oxygen supplementation and subsequent oxygen deprivation to promote neovascularization. Although the OIR model allows for the study of neovascularization it lacks the hyperglycemia component of DR (Figure 29).[10–14] To date, no proper model exists for the study of DR. As a consequence, very few mouse DR studies translate into reproducible human studies.

Figure 29. Animal models of diabetic retinopathy. The three most common animal models for studying DR include: streptozotocin (STZ) induced diabetes, galactosemia, and oxygen induced retinopathy (OIR). Each model has pros and cons, but no model is perfect for studying DR.

Streptozotocin

Induction:

- STZ (IP injection)
- 65-80 mg/kg body weight
- 5 consecutive days

Pros:

- T1D model
- Develops acellular capillaries
- Early stages of DR

Cons:

- No PDR or DME

Galactosemia

Induction:

- 30% D-galactose rich diet.

Pros:

- Role of hexose sugars
- Develops acellular capillaries
- Early stages of DR

Cons:

- No PDR or DME

Oxygen Induced Retinopathy

Induction:

- Alternating O_2
- 12hr cycles (80% to 40%)
- post-natal days 0-14.

Pros:

- Develops neovascularization
- Advanced stages of DR

Cons:

- No hyperglycemia

The lack of translatable data further establishes the importance of future studies utilizing human cell based systems. In addition to primary cultures, the use of dual or multi cell culture systems will allow for a better understanding of how certain pharmacological agents interact with certain cell types in a model that more closely represents the human retinal microenvironment. Our laboratory has started to develop a flow based system that mimics the retinal vasculature and can be used to study the effects of certain drugs and other various treatments on vascular permeability. The system contains hollow porous fibers that are connected to a pump in order to mimic retinal blood flow. Human retinal endothelial cells are cultured on the inside of the fibers while matching Human Müller cells are cultured on the outside of the fibers. The interaction between human retinal endothelial and Müller cells simulates an established blood retinal barrier. What makes this system even more unique is the ability to inject pharmacological agents into the endothelial cell compartment or the Müller cell compartment. Injections into the endothelial cell compartment would mimic systemic drug treatments, while injections into the Müller cell compartment would mimic tissue-specific drug treatments. In addition to measuring vascular permeability, media samples can be taken from either compartment and be measured for inflammatory cytokines, chemokines, and various growth factors. Another example of a multi-culture human based approach is the organ on a chip. The organ on a chip is multicellular human based microfluidic chip that acts as an artificial organ. This technology is currently being used to study the brain, lungs, heart, kidneys, and can be adapted for any organ system[15–17]. The use of human based multicellular systems, such as the retinal vasculature flow based system and the organ on a chip, will

allow for more efficient and reliable testing of therapeutic agents and potentially increase the probability of developing more efficient strategies for treating DR.

5.5 Conclusions

The development of effective therapeutic strategies for the treatment of DR remains difficult due a lack in translatable knowledge. It is important to understand that inflammatory cytokines and growth factors, like IL-6 and VEGF-A, have important physiological functions. Once concentrations exceed the normal physiological range their functions can become pathological. The majority of knowledge regarding inflammatory cytokines and growth factors pertains to their functions in the context of DR. Therefore, it is crucial to understand the function of a given cytokine not only in the disease state, but also in the context of normal physiological function.

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