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Inflammation In The Pathogenesis Of Diabetic Retinopathy

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INFLAMMATION IN THE PATHOGENESIS OF DIABETIC RETINOPATHY

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DEDICATION

My Beloved Parents, Chunyu Yuan and Binhai Shi,

My Dear Grandparents, Bainian Shi and Fengqin Liu

My Best Friend, Teng Wang

My "Family" Here, Douglas and Sally Cochrane,

Without Whom None of My Success Would Be Possible

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CHAPTER 1 “ROLE OF INFLAMMATION IN THE PATHOGENESIS OF DIABETIC RETINOPATHY”

1.1 Diabetic Retinopathy

Diabetic retinopathy (DR) is a visually debilitating eye disease that affects patients diagnosed with diabetes mellitus (DM). In brief, diabetes is a chronic, metabolic disease resulting in high blood glucose levels in the body. As of 2014, it was estimated that 387 million people are diabetic worldwide (Update 2014). This is expected to increase by 55% in the next 20 years, reaching epidemic levels by 2035 (Fig. 1). There are three major types of diabetes: type 1, type 2 and gestational diabetes.

Type 1 diabetic mellitus (T1DM) is an autoimmune disease, during which the host immune system mistakenly attacks pancreatic beta cells, thus rendering them incapable of producing insulin. T1DM is referred to as “insulin-dependent” associated, in large part, with genetic risk factors. On the other hand, multifactorial causes drive type 2 diabetic mellitus (T2DM) which include obesity, unhealthy diet, age, and family history resulting in reduced sensitivity to insulin among patients. T2DM diabetes is considered “non-insulin-dependent” and affects roughly 90% of patients diagnosed with DM [1].

Diabetic retinopathy is known to be the leading cause of irreversible blindness among people of working age in the United States with more than 10,000 new cases annually [2]. This disease progresses through four stages, which feature a number of pathological events associated with the retina. Early stage DR is called background or non-proliferative diabetic retinopathy (NPDR);

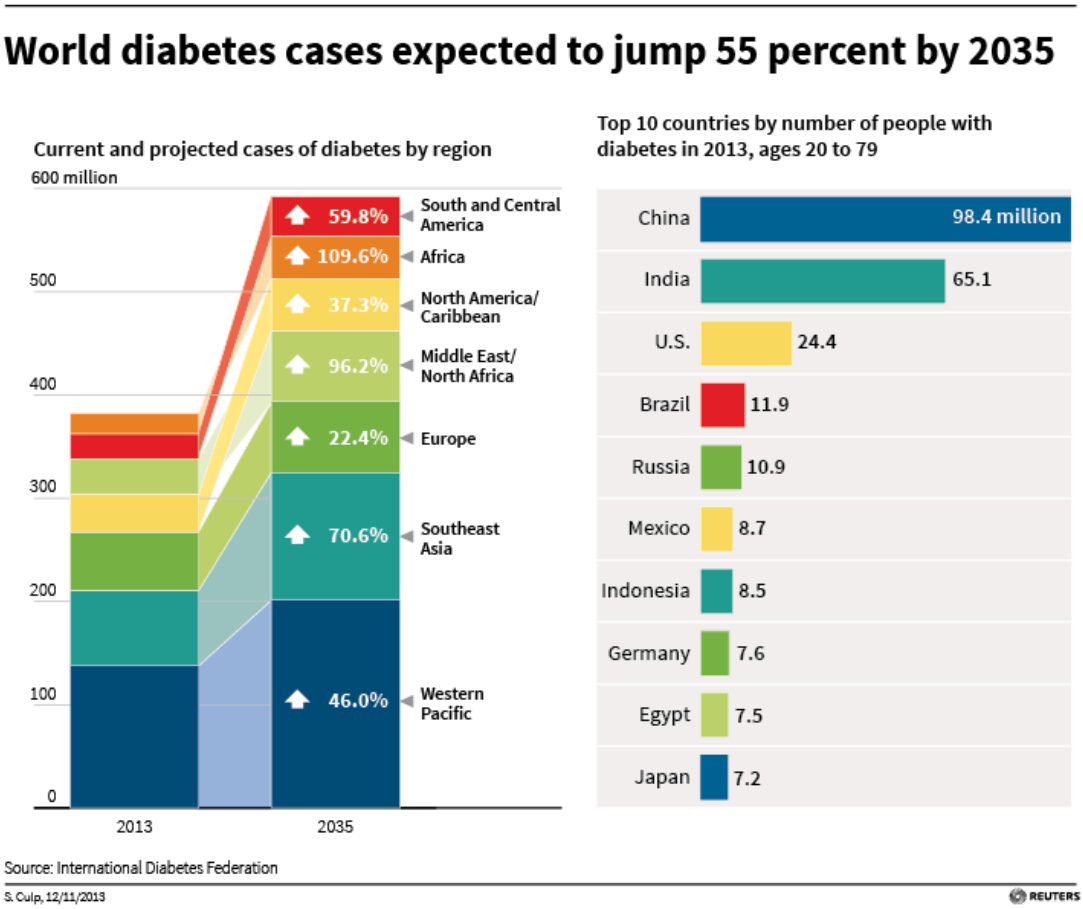

Figure 1. Current and projected cases of diabetes worldwide from 2014 – 2035. Source: International Diabetes Federation.

patients are typically asymptomatic, but retinal blood vessels become damaged and begin to leak fluid. NPDR features microaneurysms, retinal hemorrhages (flame, dot), hard exudates (cholesterol/fat deposits from vasculature), macular ischemia, and macular edema (the most common cause of vision loss in diabetes) (www.geteyesmart.org/eyesmart/diseases/diabetic-retinopathy) (Fig. 2). These latter changes are considered moderate to severe NPDR. Most of the time, vision is still not affected with mild NPDR, except in cases where macular edema occurs because swelling of the macula interferes with clear vision processing.

Proliferative diabetic retinopathy (PDR) mainly develops as a result of retinal ischemia. Once blood vessels begin to close and reduce blood flow, the retina tries to compensate through neovascularization. During the advanced stages of PDR, blood vessels expand and grow (Fig. 2). These new vessels are abnormal however; they are more fragile and can be associated with scar tissue. As a result, the new vessels are prone to bleeding into the vitreous and the scar tissue can cause the retina to wrinkle or detach. Both of these situations result in severe vision loss affecting both central and peripheral vision. In addition, neovascularization can result in secondary glaucoma (neovascular glaucoma) [3].

Despite the high incidence of DR and its expected increase to epidemic levels in the next couple of decades, no existing treatments can significantly reverse either early or late stage DR. T1DM typically develop early diagnostic

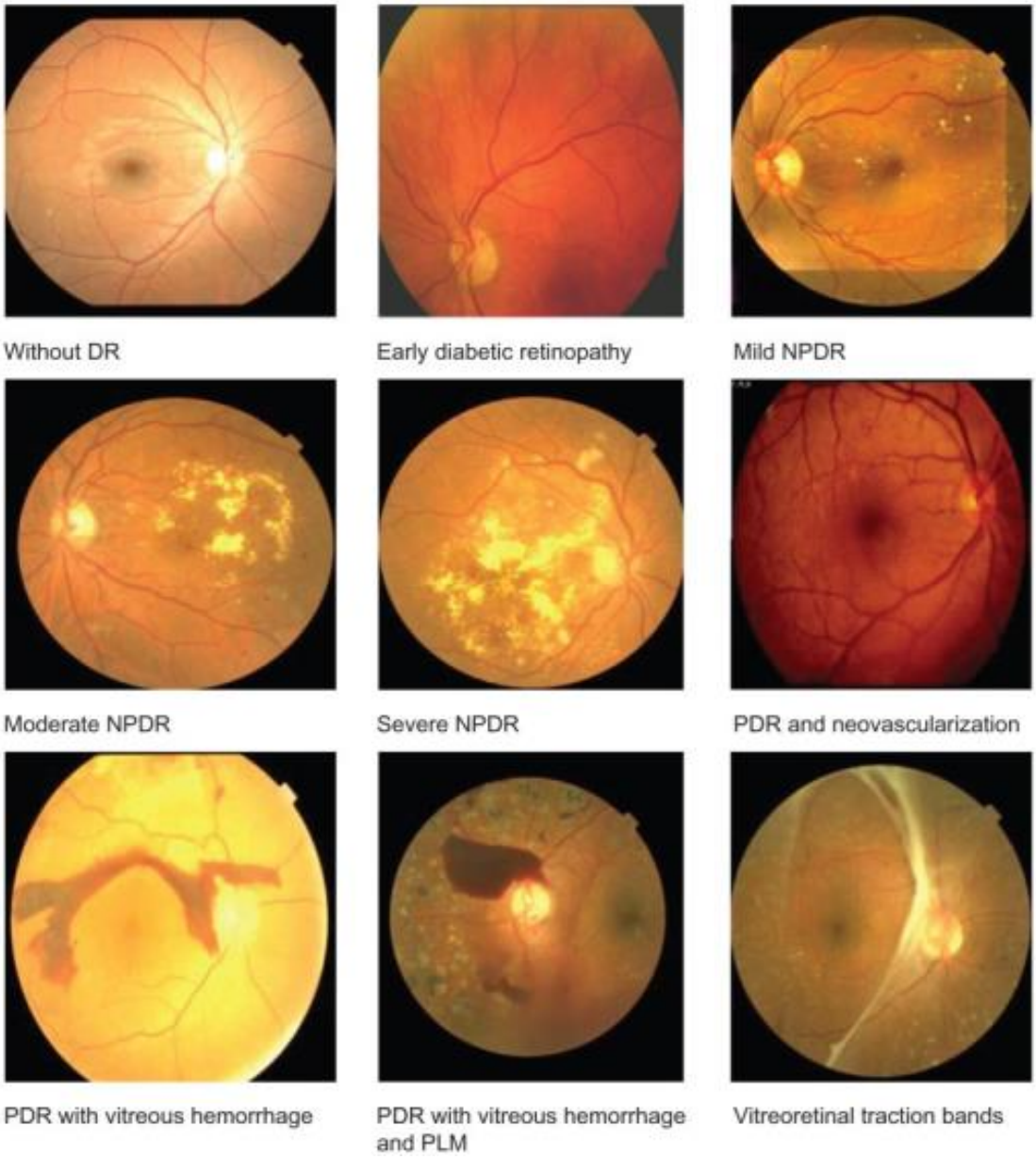

Figure 2. Fundus images demonstrating the different grades of diabetic retinopathy. Abbreviations: PDR, proliferative diabetic retinopathy; NPDR, non-proliferative diabetic retinopathy; PLM, previous laser marks. Source: El-Bab MF et al., *Clin Ophthalmol* (2012).

DR symptoms after roughly five years of onset, advancing into various degrees of retinopathy during the subsequent 20 years. In contrast, T2DM patients commonly present signs of early stage retinopathy concurrent with the initial diagnosis of diabetes, of which 60% will develop progressing degrees of retinopathy [4]. Strict control of blood sugar levels still remains the most effective approach to slow the onset of DR. As a result, mild or moderate NPDR is only monitored, while treatment is reserved for PDR. Current treatment options for the later stages include laser surgery and pan-retinal photocoagulation (PRP). Laser surgery is typically used to treat macular edema, PDR and neovascular glaucoma. PRP is aimed at shrinking new vessels and inhibiting further formation. This procedure also helps to reduce the chances for vitreous bleeding or retinal detachment. Vitrectomy is performed on patients who have had a vitreous hemorrhage; it removes both the blood and scar tissue in an effort to restore normal retinal function. The aforementioned procedures are invasive and have adverse side effects including reduced color vision, fovea damage, and Bruch's membrane rupture. In light of the role of angiogenesis in the DR pathology, ophthalmologists are applying anti-angiogenic medications as well [5]. Particularly, anti-VEGF has revolutionized the treatment of diabetic macular edema. Ranibizumab currently has FDA approval, while bevacizumab is commonly used off-label and FDA approval is pending for afilbercept [6]. However, even anti-VEGF treatment has limited therapeutic outcomes as not all patients respond and it can cause many devastating complications, as well.

These intravitreal injections can result in increased incidence of endophthalmitis, intraocular inflammation, elevated intraocular pressure, ocular hemorrhage, tractional retinal detachment and age-related macular degeneration [7]. Therefore, the development of more effective treatment modalities for both early and late stages of DR is of major concern.

1.2 Pathogenesis of DR

As stated, patients rarely develop clinical symptoms during early stage DR [4]; while the more advanced and complicated progressive stages feature hallmarks such as stimulated release of VEGF, retinal edema, retinal microaneurysms, capillary nonperfusion and degeneration with neovascularization, and dysregulated neural function and structure [8-12]. In order to develop effective treatments, it is essential to go beyond the clinical manifestations of disease and understand the events associated with the development and progression of DR. In fact, it has been through better understanding of the biochemical events that take place that led to the development of anti-angiogenic treatments. To this end, the current project seeks to focus on the role of inflammation in driving the cellular and molecular events associated with DR to identify therapeutic points of intervention leading to improved disease outcome.

Extensive research using animal models has allowed for the association of clinical symptoms with different pathological events known to occur during the development and progression of DR (Fig. 3). Firstly, oxygen metabolism is an

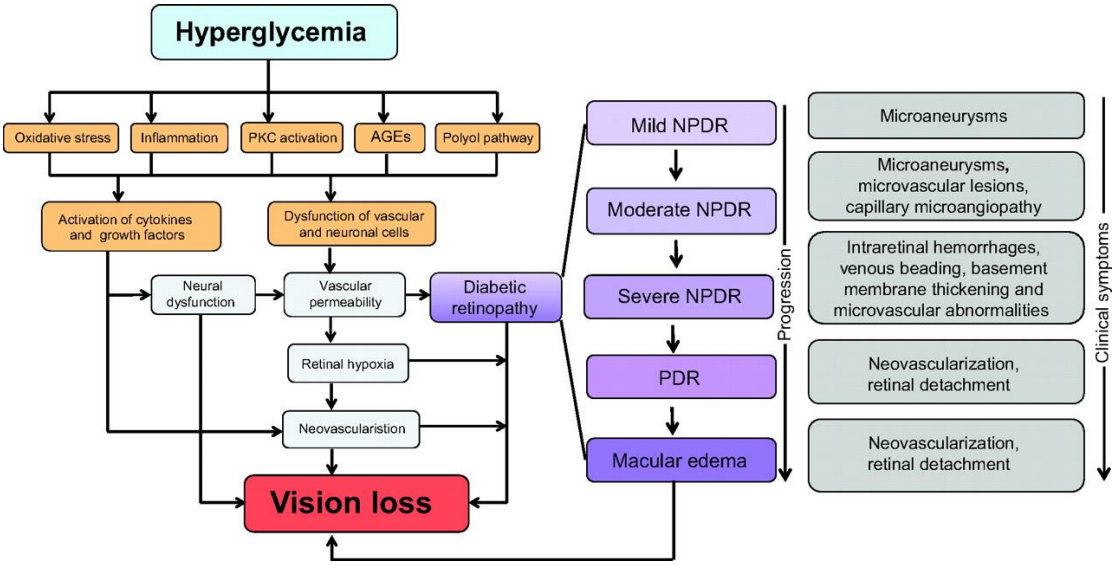

Figure 3. Diagrammatic representation of key factors known to be involved in DR pathogenesis. Source: Robinson R et al., *Disease Models & Mechanisms* (2012).

important component of cellular homeostasis. Under normal physiological conditions, low levels of reactive oxygen species (ROS) are produced by the mitochondrial electron transport chain – up to 5% of oxygen that enters into this reaction [13]. Cytochrome P450, NADPH oxidase and nitric oxide synthases also contribute to this normal cellular function. During diabetes however, the ROS scavenging system is impaired, resulting in unbalanced, excessive ROS levels termed “oxidative stress”. Kowluru and Chan have described possible causes such as auto-oxidation of glucose, shifted redox balance, reduced glutathione (GSH), and impaired superoxide dismutase (SOD), leading to further enhanced ROS production, vascular inflammation, protein/lipoprotein oxidation and overall impaired cellular homeostasis [13].

Protein kinase C (PKC) comprises a family of serine/threonine kinases containing a Ca2+ and/or diacylglycerol (DAG) binding domain and takes part in various intracellular pathways when phosphorylated and translocated. Under a high glucose environment, DAG is up-regulated and then stimulates activation of the PKC pathway, which is associated with multiple cardiovascular abnormalities in diabetic mellitus. It has been reviewed by Das Evcimen and King that the DAG-PKC pathway affects the vascular system in multiple aspects, including endothelial permeability, cell growth, angiogenesis, cytokine metabolism and leukostasis [14].

Advanced glycation end products (AGEs) is another pathogenic factor; these modified proteins/lipids are up-regulated and accumulated during DR.

AGEs contribute to the vasculopathy associated with DR. Regarding inflammation, the most important pathway is the receptor for advanced glycation end products (RAGE) pathway, in which AGE-modified proteins, such as N_{ε}-carboxy-methyl-lysine (CML) interact with the vascular endothelium via the RAGE receptor, thus up-regulating NF-κB and its target genes, pro-inflammatory cytokines such as VEGF and TNF-α [15]. This series of pathophysiological alterations will proceed to cross-linking and result in activation of cytokines, cellular dysfunction and apoptosis of vascular and neuronal cells, which will further destroy healthy retinal microvascular endothelial cells and compromise the blood-retinal barrier, ultimately leading to vascular permeability. It is important to note that all of the above-mentioned pathogenic pathways at least partially contribute to the development of local inflammation in the retina, which is the major interest in this series of studies.

1.3 Inflammation

Inflammation is, for the most part, a protective host response that includes chemotaxis of immune cells and activation of various molecular mediators, such as cytokines and chemokines. During this process, both infiltrated immune cells (macrophages and PMN) and local residential cells secretes inflammatory mediators: chemokines, interferons (IFN), interleukins (IL) and tumor necrosis factors (TNF) and further drives progress of inflammation. The activation of the endogenous pro-inflammatory cytokine network is counterbalanced by the subsequent generation of anti-inflammatory and pro-resolving agents. Despite

the beneficial role of pro-inflammatory factors in host defense, their sustained production can potentiate chronic inflammation leading to deleterious pathological conditions [16, 17]. As a result, inhibition of pro-inflammatory mediators by endogenous molecules such as anti-inflammatory molecules and specialized pro-resolving mediators (SPM) typically occurs during the normal progression of a healthy inflammatory response. If acute inflammation persists, sustains and remains unresolved, chronic inflammation will develop. In any case, a state of chronic inflammation results in disease pathogenesis.

Inflammation has been found to be associated with DR as early as the 1960's when it was discovered that diabetic patients, who were administered salicylates for rheumatoid arthritis, demonstrated a lower incidence of retinopathy [18]. However, the immune response and pro-/anti-inflammatory mediators have only recently received considerably more attention. Leukostasis, which is the excessive influx of leukocytes into the retina leading to obstruction of blood vessels and increased blood viscosity, has been found to be classically associated with DR. Other evidence implicating the participation of an activated inflammatory response includes the upregulation of adhesion molecule ICAM-1 and its ligand, CD18, located on retinal cells and neutrophils, respectively [19-22]. In addition, studies have described the involvement of potent pro-inflammatory markers in the pathology of DR, including NF-κB, iNOS, cyclooxygenases, VEGF, IL-1β, TNF-α, Fas, FasL, and complement factors [23-39] - all of which contribute to an environment that is characteristic of

chronic inflammation. However, roles of anti-inflammation and SPMs in DR pathogenesis are still largely unknown. The studies described herein will expand the traditional view of DR pathogenesis into the influence of type 1/type 2 dominant inflammatory responses and SPMs that will be examined using in vivo and in vitro approaches, including C57BL/6J (B6) and BALB/cJ (BALB/c) mice, residential retinal cell types (retinal endothelial cells and Müller cells) and leukocytes (neutrophils/PMN) that are associated with DR pathogenesis.

The type 1 and type 2 immune response paradigm describes the predominance of differentially programmed innate immune activities. Type 1/type 2 refers to the cytokine and chemokine expression from different kind of cell types in local retinal inflammation instead of limiting to traditional polarized CD4+ T helper cells. BALB/c (type 2 dominant) and B6 (type 1 dominant) strains of mice have been demonstrated to be suitable models in experiments of investigating type 1/type 2 immunity [40]. In type 1 responders, inflammatory activity tends to be sustained and exacerbated, developing into chronic inflammation, if left untreated. While in type 2 responders, the local inflammation is less severe and resolves overtime [41]. Using a *Pseudomonas aeruginosa*-induced bacterial keratisis model, previous work in our lab has demonstrated benefits of type 2 dominant immune response in BALB/c mice in terms of local homeostasis and reconsitution after infection [42, 43]. It has been shown using a type 1 diabetic model that, type 1 cytokines are correlated with desturction of pancreatic islets, while type 2 cytokines are thought to provide

protection [44]. Hence, the current experiments will expand upon what is known regarding inflammation in diabetes, the potential influence of a type 1 versus a type 2 immune respsone and its featured cytokines in DR. It is expected that by studying the anti-inflammatory response and role of SPMs in the diabetic mouse model will reveal key intervention points for the treatment of early stage diabetic retinopathy.

1.4 Retinal Endothelial Cells

REC line retinal capillaries and are responsible for maintaining homeostasis of the blood-retinal barrier, with pericytes to structurally support them (Fig. 4). To maintain the active retina, the retinal endothelium ensures the nutrient requirements and oxygen supply. It also helps to isolate the retina from toxins, microorganisms and pro-inflammatory leukocytes [45]. Therefore, the retinal endothelium is one of the major components involved in retinal ischemic pathology and retinal inflammation associated with DR, as they are the first line of residential cells that directly interact with docked and infiltrating immune cells. Studies have revealed that retinal capillary cells undergo accelerated apoptosis before clinical signs of DR are apparent [46, 47]. During DR, the tight junctions among REC are interrupted by high glucose-induced pressure resulting in leaking retinal microvasculature [13]. In addition, REC play an essential role during neovascularization upon ischemic stimulation, with elevated levels of the angiogenic factor, VEGF, identified as a hallmark event [48]. In an experimental autoimmune uveitis model, scanning laser ophthalmoscopy has demonstrated

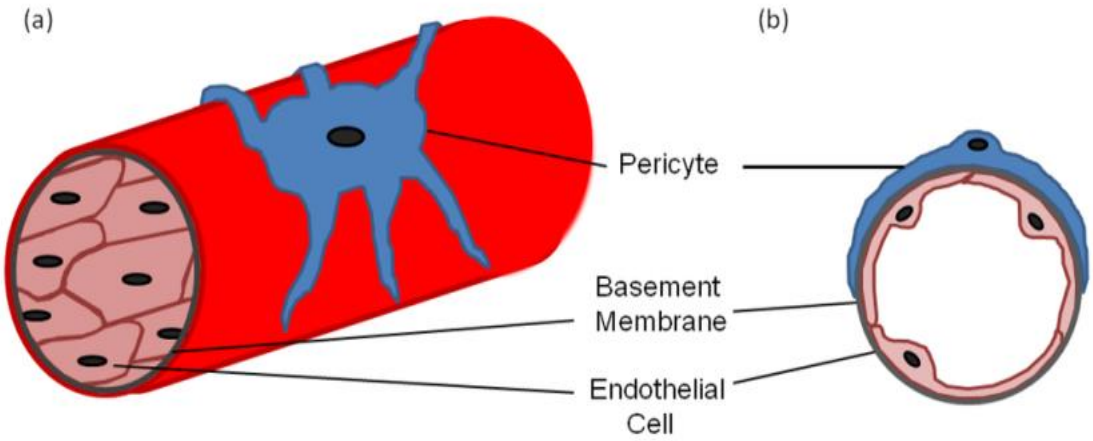

Figure 4. Diagram of longitudinal section (A) and cross-section (B) of a retinal blood vessel. Source: Mills et al., *Cells*, 2013.

that leukocytes migrate through the retinal microvasculature [49]. During inflammation, retinal endothelial cells respond to the extracellular molecules secreted by these immune cells. But more importantly, REC contribute to the inflammatory response by secreting cytokines themselves. The intraocular levels of TNF-α and IL-1β have been shown to be upregulated in patients with posterior ocular inflammation [50, 51]. Human REC produce TNF-α and IL-1β, as well as IL-6, in response to the presence of extracellular cytokines [52, 53]. It also has been demonstrated that human REC are capable of immunomodulation through IFNs and toll-like receptors (TLRs) [54]. In addition, REC possess enzyme synthesis activity, such as matrix metalloproteinases (MMPs) [52], of which MMP-2 and MMP-9 play important roles both in vivo and in vitro [55, 56]. In the current research plan, due to the aforementioned significance regarding pro-inflammatory, angiogenic and chemotactic roles of REC, they will be used as one of the major cell types to study type 1/type 2 responses during the development of DR.

1.5 Müller Cells

Müller cells or Müller glia are one of three resident glial cell types in the retina. Müller cells serve as the supporting cells for the neuronal retina. Müller cells span the full retinal thickness with the somata located in the inner nuclear layer (INL) (Fig. 5). From the somata arise two major trunks; the inner trunk projects to other Müller cells and photoreceptors, while the outer trunk extends into the vitreous [57]. This close anatomical relationship allows for an intimate

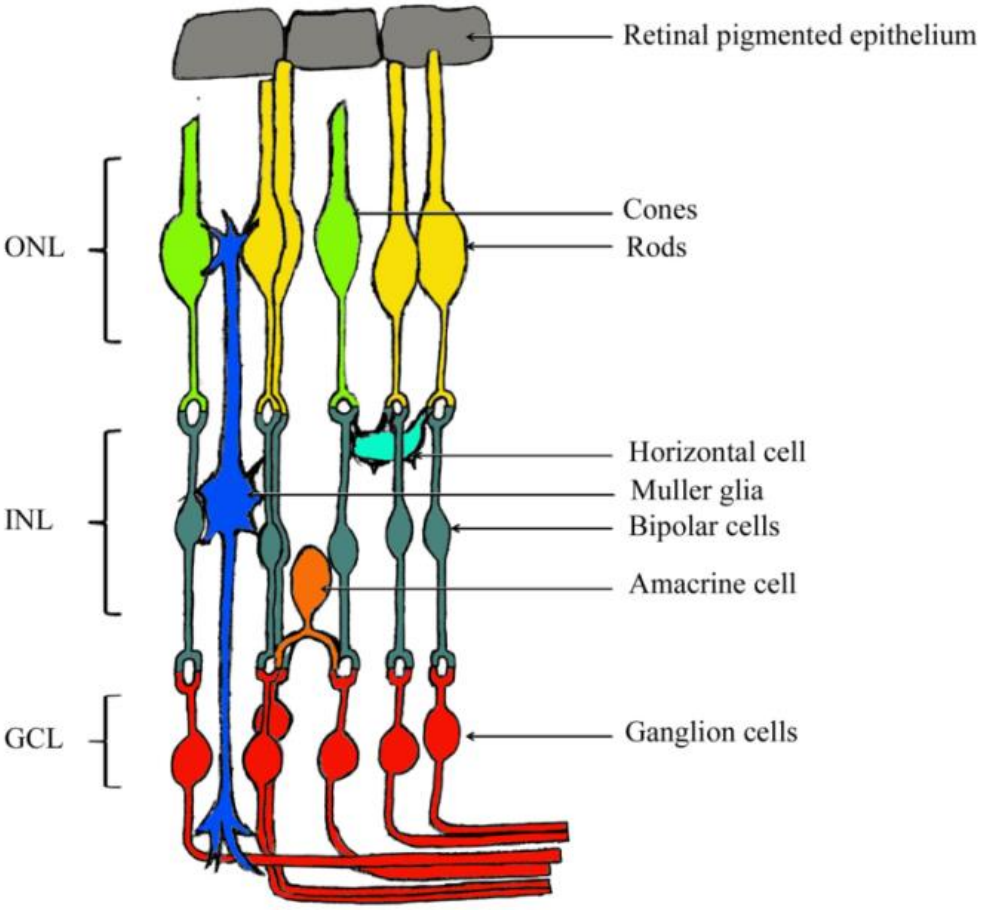

Figure 5. Illustration representing the organization of the mature retina. Muller glial cell is shown in blue. Source: Belecky-Adams et al., *Neural Stem Cells – New Perspectives*, 2013.

physiological regulation among Müller cells, neurons and blood vessels in the retina [58, 59]. As a type of neural supporting cell in the retina, Müller cells produce signaling proteins for neural survival, growth and differentiation, such as brain derived neurotrophic factor (BDNF) [60, 61]. Clinical studies concerning fibrovascular membranes have demonstrated high expression levels of various pro-inflammatory cytokines by this cell type, including TNF-α, VEGF and IL-6 [62, 63]. Moreover, stimulation of β-adrenergic receptors on Müller cells leads to decreased levels of TNF-α, IL-1β, iNOS and pro-inflammatory lipid mediator PGE2 [64]. As TNF-α, IL-1β, NO and PGE2 are all important inflammatory mediators upregulated under hyperglycemic conditions, these results suggest the involvement of Müller cells during the pathogenesis of DR. Müller cells are also associated with the mediation of extracellular matrix deposition by producing MMP-2 and MMP-9, regulated with TNF-α [65]. However, it remains unclear how Müller cells are influenced under type 1 and type 2 immune responses. In light of the aforementioned inflammatory and angiogenic effects and its supporting network with REC, this resident retinal cell type is another key player in the pathogenesis of DR.

1.6 Leukostasis and PMN

Neutrophils are the first leukocytes recruited to inflammatory sites and then neutralize invading pathogens by phagocytosis, degranulation and the release of neutrophil extracellular traps. [66] In terms of inflammation, PMN react to different cytokines, growth factors and bacterial stimulation [67, 68], further

expand their lifespan and drive resolution of inflammation by activating adaptive immune responses in normal physiological condition. Diabetes results in recruitment of inflammatory mediators into the retina. Studies have indicated that neutrophils are significantly increased in retinal (and choroidal) vessels from diabetic patients and other animal models. [69, 70]

The notion that inflammation is involved in the pathogenesis of DR is largely supported by retinal leukostasis. Furthermore, the static infiltration of leukocytes into the retinal vasculature is thought to play a significant role during early DR and contributes to vascular leakage, increased permeability of the blood-retina barrier and then ischemia followed by angiogenesis. As mentioned, ICAM-1 and CD18 expressed by resident retinal cell types and infiltrating inflammatory cells, such as neutrophils, were found to be significantly upregulated in DR models, further supporting the leukostasis process during DR [19-22].

1.7 Pro-Resolving Lipid Mediators

Over the last decade, research regarding SPMs has introduced a new mechanism of inflammation that suppresses inflammation and promotes resolution. Resolution of inflammation is an active process where the class switch of pro-inflammatory prostaglandins to SPMs plays an important role in resolution of inflammation [71]. Resolvins are a family of protective, pro-resolving compounds produced by docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) biosynthetic pathways [72]. Resolvin D1 (RvD1)

is converted by 15-lipoxygenase (LOX) and 5-LOX from D-series ω-3 polyunsaturated fatty acids and exert their function by binding to G protein-coupled receptors ALX/formyl peptide receptor 2 (FPR2) and G protein-coupled receptor 32 (GPR32) [73]. Resolvin E1 (RvE1) has another G-protein coupled receptor ChemR23, and is generated by acetylated COX-2 and 5-LOX. DHA can also be converted into neuroprotectin/protectin D1 (NPD1/PD1) via lipoxygenase activity. These PUFA-derived lipid mediators exert their anti-inflammatory function by driving resolution of inflammation, organ protection and anti-fibrotic activity, which contributes to their classification as “pro-resolving” lipid mediators or SPMs. As described, SPMs can be biosynthesized by LOX enzymes, which comprise the main pathways for SPM biosynthesis. In particular, 15-LOX is largely protective and is a key enzyme for the generation of SPMs [74]. Detailed diagram of illustrated lipid mediator pathways are shown in Figure 6.

It has been widely accepted that SPMs exert their anti-inflammatory and resolving effect by limiting infiltration of inflammatory cells; RvD1 reduces PMN infiltration and increases nonphlogistic phagocytosis of apoptotic PMN [75]. LXA4, first discovered to be pre-resolving, provides stop signals for PMN infiltration thus limiting the intensity of inflammation [76]. PGD2, along with the PGE2, can switch their pathway during acute inflammation and then generate enzymes essential for the production of lipoxins in PMN [77]. RvE1 is another important regulator of PMN chemotaxis and activation under inflammation in

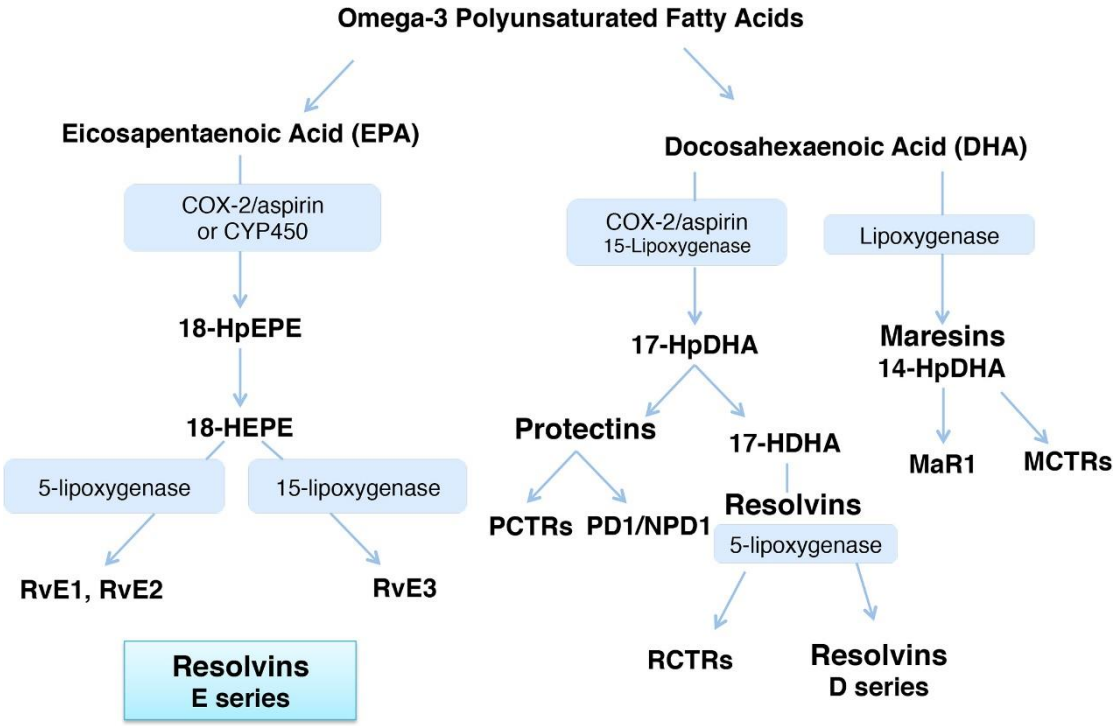

Figure 6. Synthetic pathways of pro-resolving lipid mediators. Source: Serhan, C.N. et al., Discovery of specialized pro-resolving mediators marks the dawn of resolution physiology and pharmacology. *Mol Aspects Med*, 2017. 58: p. 1-11

vivo [78]. Endogenous PD1/NPD1 has been demonstrated to mediate PMN infiltration, while other D-series resolvins can downregulate TNF-α and IL-1β, limiting PMN transmigration and activation into inflammatory tissues [72, 79, 80]. Regarding DR, DHA-rich diet in fish oil was shown to decrease complications in diabetic retinopathy by inhibiting retinal microvascular damage, downregulating expression of pro-inflammatory molecules IL-1β, IL-6 and ICAM-1 [81]. A protective effect of RvD1 against diabetic neovascularization has been demonstrated, in part through the suppression of the pro-inflammatory cytokine TNF-α [82]. In addition, an intermediate metabolite of DHA synthesis pathway, 4-HDHA reduced retinal neovascularization [83]. In the current studies, we looked at the whether these pathways were involved in the pathogenesis of DR.

1.8 NF-κB

NF-κB is composed of a family of transcription factors that regulates transcription of broad range of genes to control physiological and pathophysiological activities, such as inflammation and immune activity, cell growth, apoptosis and cancer. It contains 5 subunits - p65 (RelA), RelB, p50, p52 and c-Rel, which dimerize before nuclear translocation and trigger transcription of target genes. Traditionally, the NF-κB pathway is activated by IL-1 receptor (IL-1R), TNF receptor (TNFR) and TLRs, known to be the canonical NF-κB pathway. Other relatively newly found activators include lymphotoxins, CD40 ligand and B-cell activating factor, which activate the non-

canonical NF-κB pathway [84]. In the canonical NF-κB pathway, IKKα, IKKβ and IKKγ are activated, promoting phosphorylation, ubiquitination and degradation of IκBα, which binds to the p65-p50 dimer, isolates it and inhibits its activity in the cytosol. Upon release from IκBα, the p65-p50 dimer is phosphorylated and translocated into the nucleus, then after recruiting transcription factors such as p300/CBP and HDAC, binds to target genes and stimulates their transcription. The p65-p50 dimer is well studied and described as the major contributor of NF-κB activity [85]. On the other hand, IKKα itself was demonstrated to play important role in the non-canonical NF-κB pathway, followed by the activation and translocation of RelB-p52 dimer. For the interest in present studies, differential phosphorylation of NF-κB p65 in high glucose, pro- versus anti-inflammatory cytokines and its regulation on pathogenic inflammatory genes are explored here.

In diabetes, NF-κB starts to play an important role from very early stages of pathogenesis. In T1DM, NF-κB is activated by IL-1β and induces pancreatic beta cells dysfunction and death [86]; in T2DM, NF-κB is constitutively activated as a result of the low-grade, chronic inflammatory state [87]. NF-κB plays a pivotal role in DR pathogenesis by responding to hyperglycemia/hypoxia activated pathways and further activating more pathogenic cascades. First of all, ROS is produced by residential cells and inflammatory cells during DR, damage DNA and activates NF-κB [88]. AGEs, by activating RAGE receptors, interacting with PKC pathway, both activate NF-κB [89, 90]. Next, NF-κB

stimulates further transcription of inflammatory mediators that exacerbate DR pathogenesis in multiple aspects (Fig. 7): 1) pro-inflammatory cytokines and chemokines including IL-1β, TNF-α, interleukin-6 (IL-6), IL-8 and monocyte chemotactic protein-1 (MCP-1) [91-95]; 2) inducible nitric oxide synthase (iNOS) [96]; 3) cyclooxygenase-2 (COX-2) for the production of pro-inflammatory prostaglandins [97]; 4) adhesion molecules ICAM-1 and VCAM-1 [98]; 5) apoptotic molecules - Fas and Fas ligand (FasL) [99]. Additionally, NF-κB pathway has long been known to play central role in not only directing inflammation, but also in terms of reacting to metabolic pathogens [87]. For example, IKK kinase was used for treatment target in T2DM [100], which features low grade, chronic systemic inflammation. IKKβ activation is also implicated in diabetes by regulating insulin signaling and insulin sensitivity [101].

1.9 Ischemia/Reperfusion

Ischemia/reperfusion (I/R) injury, also known as reperfusion injury, is a tissue damaging process that is caused by reperfusion of the blood supply into previously ischemic tissue. During this process, instead of recovering to normal physiological conditions, restoration of the blood supply induces oxidative stress, thus resulting in inflammation [102]. Cardiovascular system dysfunction, stroke and thrombotic damage can take place at the beginning stage of ischemia [103, 104], creating an aerobic local environment. The maximum time length each tissue could survive from ischemic damage varies and can result in necrosis [105], yet restoration of blood supply usually removes ischemic

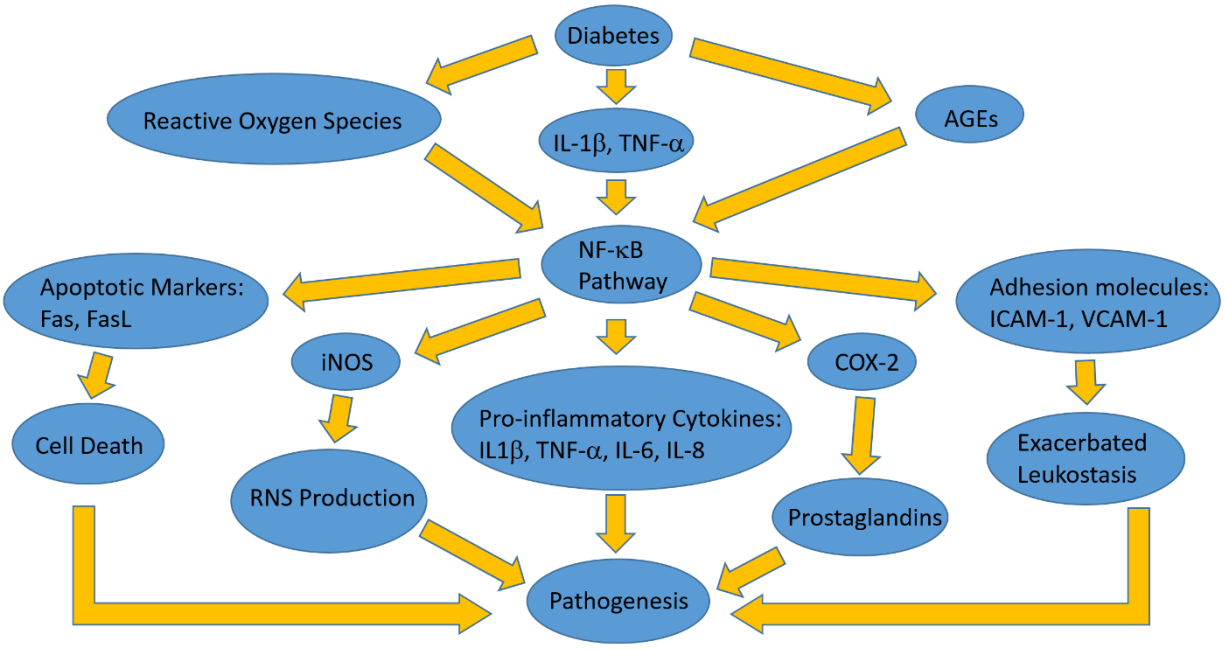

Figure 7. Pivotal regulatory role of NF-κB in pathogenesis of DR.

damage and instead, promotes free radical-induced reperfusion damage [106]. It is hypothesized that absence of oxygen during the ischemic stage leads to production of various intermediates, which are converted to tissue-damaging free radicals during oxygen resupply [107, 108]. The oxygen radicals activate different pathogenic cascades that evoke the inflammatory response. Production of IL-8 and adhesion molecules promote docking and invasion of inflammatory cells, most importantly neutrophils and macrophages. As a result, local inflammation in I/R tissues is exacerbated, causing further pathophysiological responses and tissue damage.

During DR, apoptosis of pericytes and REC, disruption of tight junctions, and breakdown of the blood-retinal barrier results in microvascular leakage, creating an ischemic condition [109]. This induces production of pathogenic molecules such as oxygen radicals [110], hypoxia inducible factor-1 (HIF-1) [111] and cytokines/chemokines. Earlier studies have demonstrated that retinal ischemia/reperfusion leads to microvascular damage that is similar to what is observed in DR and glaucoma [112]. Moreover, free radicals were also found to promote neurodegeneration in retinal I/R [113]. Thus, I/R surgery is suitable to mimic neurodegeneration, microvascular leakage and inflammation in DR, which have been demonstrated in different studies [112, 114]. To this end, I/R is used to mimic DR-like changes in a short time frame in order to investigate our type 1/type 2 immune response paradigm.

CHAPTER 2 “VIP PROTECTS PRIMARY HUMAN RETINAL MICROVASCULAR ENDOTHELIAL CELLS AGAINST HYPERGLYCEMIA-INDUCED INCREASES IN TNF-α AND ENHANCES PRO-RESOLVING RVD1”

2.1 Abstract

The purpose of our study was to evaluate the therapeutic effect of VIP on human retinal endothelial cells (HREC) under high glucose conditions. Diabetes affects almost 250 million people worldwide. Over 40% of diabetics are expected to develop diabetic retinopathy, which remains the leading cause of visual impairment/blindness. Currently, treatment is limited to late stages of retinopathy with no options available for early stages. To this end, the purpose of the current study is to evaluate the therapeutic effect of vasoactive intestinal peptide (VIP) on HREC under high glucose conditions. Primary HREC were cultured in normal (5mM) or high (25mM) glucose medium +/- VIP treatment. Protein levels of TNF-α, resolvin D1 (RvD1), formyl peptide receptor 2 (FPR2), G protein-coupled receptor 32 (GPR32), VEGF, and VIP receptors, VPAC1 and VPAC2 were measured. High glucose-induced changes in TNF-α and RvD1 were restored to control levels with VIP treatment. RvD1 receptors, ALX/FPR2 and GPR32, were partially rescued with VIP treatment. VPAC2 expression appeared to be the major receptor involved in VIP signaling in HREC, as VPAC1 receptor was not detected. In addition, VIP did not induce HREC secretion of VEGF under high glucose conditions. Our results demonstrate that VIP's therapeutic effect on HREC, occurs in part, through the balance between the pro-inflammatory cytokine, TNF-α, and the pro-resolving mediator, RvD1.

Although VPAC1 is considered the major VIP receptor, VPAC2 is predominantly expressed on HREC under both normal and high glucose conditions.

2.2 Introduction

Diabetic retinopathy (DR) continues as the leading cause of irreversible blindness in the United States resulting in over 10,000 new cases annually [2]. With both type 1 and type 2 diabetics at risk, over 40% of all adult diabetic patients are expected to develop this visually debilitating disease. Despite the fact that diabetes is projected to reach epidemic levels by 2030, there remains no available treatment for early stage DR, save for maintaining glycemic control. Hallmark features of DR are of both vascular and neural natures, including leukocyte adhesion to retinal vasculature, vascular occlusions, endothelial cell damage and pericyte and photoreceptor loss with underlying degenerative and inflammatory changes [115]. Inflammation has been linked to DR as early as the 1960's when it was found that diabetic patients, who were administered salicylates for rheumatoid arthritis, demonstrated a lower incidence of retinopathy [116]. However, only more recently has the inflammatory response come to the forefront as a major contributing factor to the development and progression of DR.

TNF-α is a well-characterized cytokine known to play a role in a wide spectrum of biological activities, predominately pro-inflammatory in nature. It has been reported that TNF-α levels are increased in retinas of both type 1 and

type 2 diabetic rodents, as well as during the development of DR [37]. It has been indicated as a major cytokine involved in driving leukocyte adhesion. Furthermore, it has been shown that this molecule induces endothelial and pericyte cell injury and apoptotic cell death [115]; key events in the progression of DR.

In contrast, resolvins are a family of protective, pro-resolving compounds produced by docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) biosynthetic pathways [72]. RvD1 is derived from D-series ω-3 polyunsaturated fatty acids and binds to G protein-coupled receptors ALX/FPR2 and GPR32 [73], leading to reduced polymorphonuclear leukocytes (PMN) infiltration and increased nonphlogistic phagocytosis of apoptotic PMN [75]. A protective effect of RvD1 against diabetic neovascularization has been demonstrated, in part through the suppression of the pro-inflammatory cytokine TNF-α [82].

VIP is an endogenous immunoregulatory neuropeptide synthesized by neurons throughout the central and peripheral nervous systems, in addition to immune cells [117]. Focusing on the retina, VIP immunoreactivity has been detected in amacrine cells and other interneurons of the inner nuclear layer (INL) and inner plexiform layer (IPL) [118, 119]. The immunoregulatory activities of VIP are mediated predominately by two G protein-coupled receptors, VPAC1/VIPR1 and VPAC2/VIPR2. VPAC1 is constitutively expressed in lymphocytes, macrophages, dendritic cells, microglia, monocytes and mast cells, whereas VPAC2 is thought to require activation [120]. VPAC1 serves as

the major immunoregulatory receptor for VIP in various immune cells, while VPAC2 is thought to play a role in immune homeostasis and tissue restoration [121, 122].

Recent studies have reported decreased expression of VIP and both VPAC1/VPAC2 receptors in the retina during early stage DR [121, 123]. Moreover, in diabetic macular edema, activation of the protective VIP/PACAP pathway has been shown to prevent the breakdown of the outer blood retinal barrier by mediating tight junction integrity [124]. However, the modulatory mechanism and potential therapeutic effect of VIP during the development of DR is largely unknown. The current study seeks to demonstrate a potential pro-resolving role for VIP during DR by preliminarily investigating its interaction with TNF-α and RvD1 in HREC under high glucose conditions.

2.3 Materials and Methods

Retinal Endothelial Cell Culture

Primary HREC were acquired from Cell System Corporation (CSC, Kirkland, WA). Cells were grown in M131 medium containing microvascular growth supplements (MVGS; Invitrogen, Carlsbad, CA), 10 mg/mL gentamycin, and 0.25 mg/mL amphotericin B. All primary cells were used within six passages. Prior to experimentation, cells were transferred for three days to high (25 mM) or normal (5 mM) glucose medium (M131 medium supplemented with glucose) with MVGS and antibiotics, then quiesced by removing MVGS for 24h. Cells were exposed to VIP (10^{-9} M) for 4h [125-127], followed by rinsing with cold

PBS and collection into lysis buffer containing protease and phosphatase inhibitors. Cellular extracts were prepared by sonication, and total protein concentration was determined for analyses as described below.

To evaluate whether VIP acts directly via VPAC2 and/or ALX/FPR2 regarding TNF-α levels, cells were treated with the VIP receptor antagonist, [D-p-Cl-Phe^{6},Leu^{17}]-VIP (Leu) (VPAC antagonist; R&D Systems, Minneapolis, MN) or a selective antagonist of ALX/FPR2 signaling, WRW4 (Tocris, Pittsburg, PA). Cells cultured under normal and high glucose conditions were exposed to VIP (10^{-9} M) in the presence of each antagonist (VPAC antagonist at 2M or WRW4 at 1M) for 4h, then processed for protein analyses as described above.

Previously, high osmolar conditions have been included as an additional control to determine whether the observed in vitro effects were a result of high glucose treatment or increased osmolarity of the treatment media [128]. Since it has been established that no differences were observed between high osmolarity and normal glucose, this control was omitted from the current study.

ELISA

Protein levels for TNF-α and RvD1 were determined using ELISA kits (Thermo Fisher Scientific, Waltham, MA; Cayman Chemical, Ann Arbor, MI). Cells were collected and processed as described above. All samples were centrifuged at 5,000 × *g* for 5 min and an aliquot of each supernatant was assayed in duplicate or triplicate per the manufacturer's instruction. Equal protein was loaded into all wells. The reported sensitivities of these assays

are as follows: <2.0 pg/mL for TNF-α and 3.3 pg/mL for RvD1.

Western Blotting

Proteins were separated on 4–12% tris-glycine gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. After blocking membranes in TBST (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) BSA at r.t. for 60 min, membranes were incubated overnight at 4℃ with antigen-specific primary antibodies. The primary antibodies were used as follows: GPR32, ALX/FPR2 and VEGF (Abcam, San Francisco, CA); VPAC1 and VPAC2 (Santa Cruz, Santa Cruz, CA). Blots were then incubated with species-specific HRP-conjugated secondary antibodies for 2 h at r.t. Proteins were visualized by incubation with a chemiluminescence substrate kit (Thermo Fisher Scientific, Waltham, MA). Western blot images were collected (Azure Biosystem C500, Dublin, CA) and target protein expression was quantified (Image Studio Lite software) after normalizing to β-actin. One representative blot is shown. Treatment groups were normalized to β-actin levels and then compared to normal glucose, which was normalized to 1.0.

Statistical analysis

All assays were carried out at least twice from two independent experiments and the data are presented as mean ± SEM. Data were analyzed by the Kruskal-Wallis test, followed by Dunn's testing. $P < 0.05$ was considered to be statistically significant.

2.4 Results

VIP reduced levels of high glucose-induced TNF-α

Changes in TNF-α protein levels were assessed under high glucose conditions and after VIP treatment, as shown in Figure 8. As expected, TNF-α protein levels were significantly increased by HREC under high glucose compared to normal glucose conditions (control). This effect was abrogated with VIP treatment, whereby TNF-α levels were similar to controls. No significant effect was observed with VIP treatment in cells cultured in normal glucose.

Effect of VIP on pro-resolving mediators

Our previous research has demonstrated VIP's therapeutic effect to be both anti-inflammatory and pro-resolving [42, 127, 129, 130]. As such, we next addressed a potential mechanism by which VIP might mediate these pro-resolving effects by looking at RvD1 and corresponding receptors, ALX/FPR2 and GPR32. As illustrated in Figure 9A, RvD1 protein levels were significantly reduced in high glucose versus normal glucose conditions. However, levels were increased with VIP treatment in high glucose. No notable changes were observed in HREC after VIP treatment under normal conditions.

In addition, RvD1 receptors ALX/FPR2 and GPR32 were significantly down-regulated (approximately 50% and 40%, respectively) after high glucose exposure compared to normal glucose controls (Fig. 9B and 9C, respectively), which is consistent with the changes observed in their ligand, RvD1. Further, VIP treatment enhanced protein levels of both receptors, albeit only GPR32

was significantly increased (28%) over high glucose only. ALX/FPR2 and GPR32 levels after VIP treatment of normoglycemic cells remained comparable to controls.

VIP receptor expression by HREC

As the two predominant receptors of VIP, levels of VPAC1 and VPAC2 were evaluated in HREC. Although described as the major VIP receptor on most cell types [120], VPAC1 was not detected in HREC in either normal or high glucose. In contrast, VPAC2 was constitutively expressed under both high and normal glucose conditions (Fig. 10). However, VPAC2 protein levels were significantly reduced in high glucose versus normal glucose conditions, despite VIP treatment.

VIP-induced effects are carried out by VPAC2 receptors

Despite that VPAC2 was predominantly expressed on HREC, we next confirmed whether the VIP-induced changes in TNF-α levels were directly mediated by this receptor. To do so, HREC were cultured under high glucose conditions, then exposed to a receptor antagonist for either ALX/FPR2 (WRW4) or VPAC2 (Leu) prior to VIP treatment. As shown in Figure 11, results indicate that VIP-induced down-regulation of TNF-α was abrogated in the presence of the VPAC antagonist, but not the ALX/FPR2 antagonist. In fact, TNF-α levels from HREC cultured in HG+VIP+Leu were comparable to HG only and significantly higher than NG, NG+VIP and HG+VIP. While the ALX/FPR2 antgonist, WRW4, did result in a slight increase in TNF-α levels compared to

HG+VIP, the difference was not significant.

VEGF levels are not affected by VIP treatment

Considerable clinical effort has been put forth to inhibit VEGF, as it is well known to cause retinal permeability and neovascularization in diabetes. As VIP was previously reported to enhance growth factor production [130], we investigated VEGF protein levels after VIP treatment under both normal and high glucose conditions to evaluate this potential side effect. As depicted in Figure 12, VIP treatment did not have any effect on VEGF levels under normal glucose conditions. Remarkably, high glucose-induced VEGF levels, however, were significantly reduced after VIP treatment.

2.5 Discussion

Studies have indicated TNF-α is an important mediator of the retinal pathology observed under hyperglycemic conditions, including leukocyte adherence in retinal blood vessels [131], retinal endothelial cell apoptosis [132], pericyte loss and capillary degeneration [133, 134] and vascular permeability and leukostasis [135]. It has been previously shown by Jiang et al. that TNF-α levels are increased in HREC cultured under hyperglycemic conditions [132]. Therefore, as an initial step towards characterizing the retino-protective effects of VIP during DR, we examined whether VIP can regulate levels of this potent pro-inflammatory cytokine. As expected, high glucose significantly induced TNF-α expression in HREC, suggesting its involvement in the diabetic inflammatory response. VIP abrogated this effect, thus returning TNF-α to

control levels. This anti-inflammatory effect of VIP has been reported in other diabetic systems; as VIP was shown to inhibit TNF-α induced apoptosis in acinar cells isolated from submandibular glands of non-obese diabetic mice with salivary dysfunction through functional VPAC1 receptors coupled to the protein kinase A signaling pathway [136]. The current study suggests that VIP, as an alternate treatment against DR, may effectively ameliorate the initial pro-inflammatory cytokine release observed in vivo; however this effect appears to be VPAC1-independent and associated with the pro-resolving molecule, RvD1.

The balance between TNF-α and RvD1 appears to be an important factor in disease pathogenesis. Previous research also indicates that TNF-α can suppress RvD1 expression [82]. Using a uveitis model, topical ocular application of RvD1 was shown to reduce levels of TNF-α resulting in improved disease outcome [137]. Likewise, in a diabetic mouse model, RvD1 expression was enhanced with the reduction of TNF-α following entanercept treatment, leading to a reduction of pathological retinal angiogenesis [82]. This resolvin has been demonstrated to reduce angiogenesis and protect against retinopathy [82]. Therefore, we next sought RvD1 as a potential mechanism by which VIP might mediate its pro-resolving effects. To this end, the current study showed that high glucose conditions decreased RvD1 levels in HREC. More importantly, VIP treatment effectively up-regulated RvD1 production after high glucose exposure similar to observed normal glucose levels. In addition, high glucose-induced reduction of ALX/FPR2 and GPR32 was partially rescued after

VIP treatment. Both of these RvD1 receptors are important in carrying out the resolution of acute inflammation by mediating PMN recruitment [138], and promoting D1-miRNA circuits [138]. These data support the idea that VIP's pro-resolving effects are carried out, at least in part, via lipid mediator circuits. Further, the inverse relationship between TNF-α and RvD1 enhance the efficacy of VIP as a potential therapeutic.

Although VPAC1 is broadly expressed on different cell types, most notably immune cells [120], and has received considerable attention for its anti-inflammatory effects, it was not detected in HREC. VPAC1 has been detected in rat brain microvascular endothelial cells [139], as well as transformed murine endothelial cells derived from heart (H5V) [140], indicating that a lack of VPAC1 detection could be unique to retinal endothelial cells. In contrast, VPAC2 appeared to be constitutively expressed under normal glucose conditions, yet decreased after exposure to high glucose. We have previously shown in a bacterial keratitis model that VPAC2 is more strongly correlated with tissue homeostasis and disease resolution [129]. Regarding diabetes, Ma et al. have indicated that VPAC2 activation leads to improved glucose and lipid metabolism, while increasing insulin sensitivity in *db/db* mice [141]. In the current study, we demonstrate that VPAC2, not VPAC1, is expressed by HREC, thus suggesting a potential mechanism by which VIP treatment could ameliorate disease progression of DR. Although VIP treatment was not able to rescue high glucose-induced down-regulation of VPAC2, it is possible that this receptor could be

associated with RvD1 expression/activation. In this regard, VIP-induced changes in TNF-α levels were abrogated by a VPAC antagonist. These findings support the notion that the observed VIP-mediated effects regarding this pro-inflammatory mediator are carried out primarily both of this receptor pathway, which will be further explored in future in vivo studies.

In light of our previous research highlighting VIP's ability to enhance growth factor expression during corneal wound healing and reconstitution of the extracellular matrix [42], we next determined whether this neuropeptide increases VEGF levels. VEGF has been implicated as a major causative factor in diabetic macular edema, retinal neovascularization and related complications [142]. Remarkably, HREC expression of VEGF was significantly downregulated in high glucose with VIP treatment compared to high glucose only. Similar to the cornea, which must remain clear for accurate visual processing, VIP treatment does not appear to induce angiogenesis via VEGF expression. These findings are essential in moving forward with investigating VIP as an alternative therapy for DR.

Overall, the current study reports the expression of VPAC2, but not VPAC1, on retinal endothelial cells. Additionally, it indicates a novel regulatory role for VIP over RvD1 levels. Taken together, these findings provide rationale to further explore the therapeutic potential of VIP in the development and progression of DR. This neuropeptide not only reduced anti-inflammatory mediators, but is tied to important lipid mediator circuits, as well. In addition, these data suggest that

VPAC2 (not VPAC1) and ALX/FPR2 are the major receptors involved in VIP signaling in HREC.

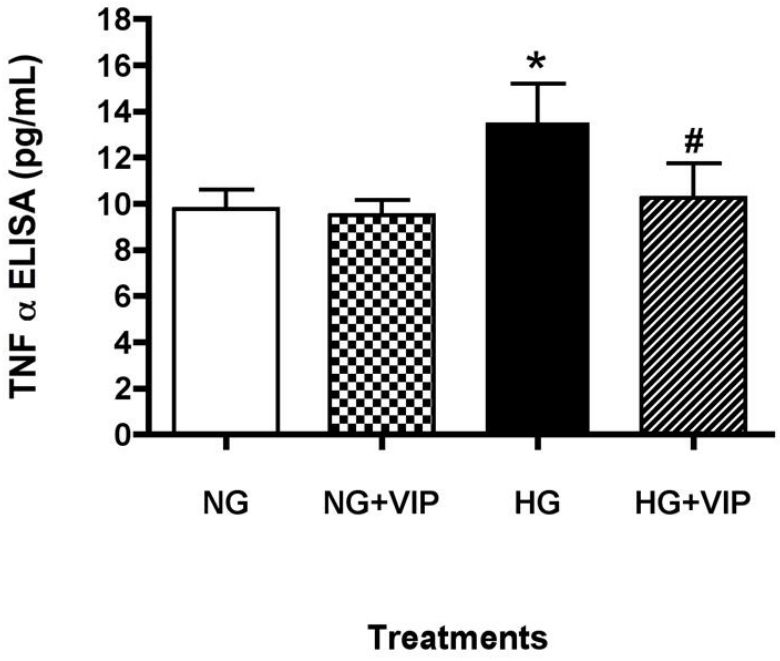

Figure 8. TNF-α protein levels as detected by ELISA. HREC were cultured under normal glucose (NG, 5 mM) and high glucose (HG, 25 mM) conditions +/- VIP treatment (10^{-9} M) for 4 hours. Data shown are representative of two independent experiments in duplicate and are expressed as mean ± SEM. *$P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

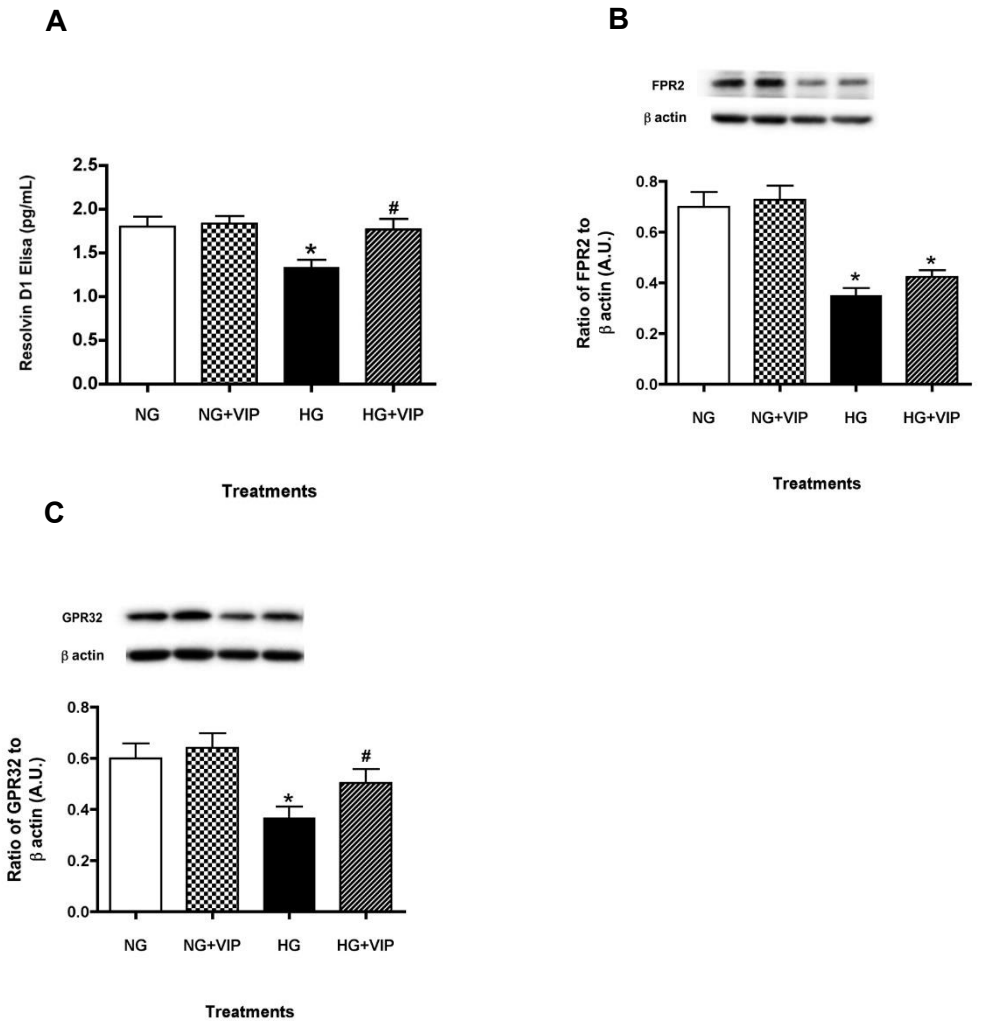

Figure 9. Protein levels of RvD1 and its receptors in HREC treated with VIP. HREC were cultured under normal glucose (NG, 5 mM) and high glucose (HG, 25 mM) conditions +/- VIP treatment (10^{-9} M) for 4 hours. Protein levels of RvD1 (A) were measured by ELISA and its receptors ALX/FPR2 (B) and GPR32 (C) were detected by Western blot. Data shown are representative of two independent experiments in duplicate and are expressed as mean + SEM. *P < 0.05 vs NG, #P < 0.05 vs HG.

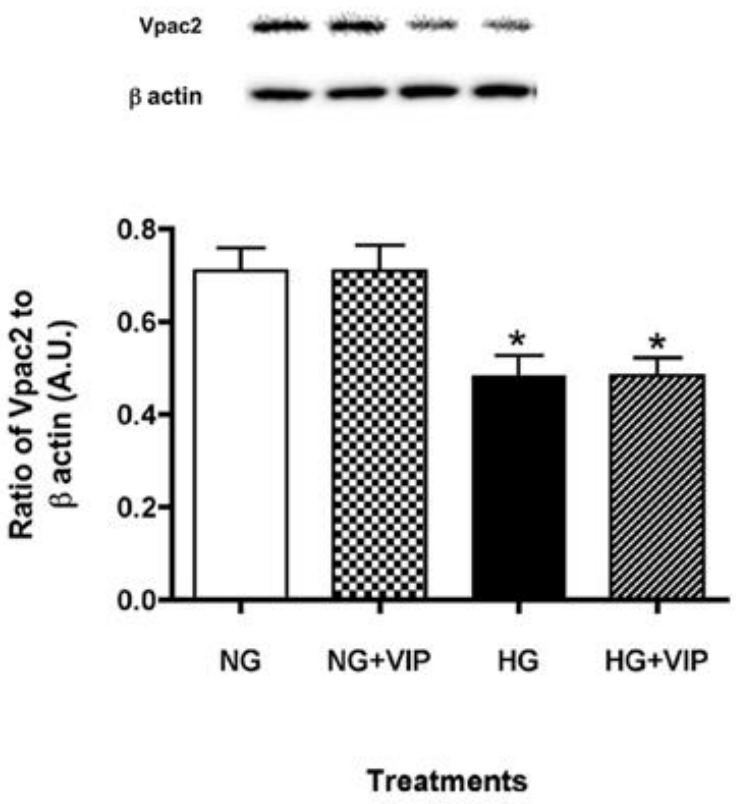

Figure 10. VPAC2 receptor levels. HREC were cultured under normal glucose (NG, 5 mM) and high glucose (HG, 25 mM) conditions +/- VIP treatment (10^{-9} M) for 4 hours. Protein levels of VPAC2 were examined by Western blot. Data shown are representative of two independent experiments in duplicate and are expressed as mean + SEM. **P* < 0.05 vs NG, $^{\#}P$ < 0.05 vs HG.

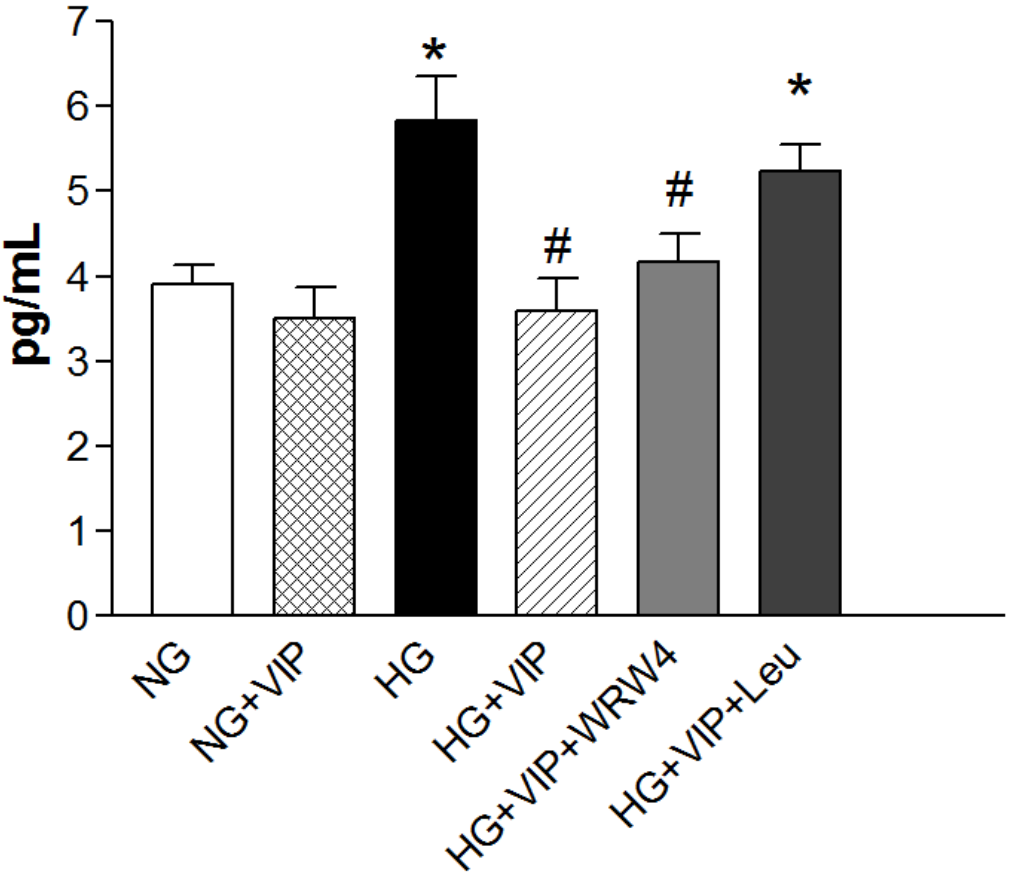

Figure 11. TNF-α protein levels as detected by ELISA. HREC were cultured under normal glucose (NG, 5 mM) and high glucose (HG, 25 mM) conditions +/- ALX/FPR2 antagonist (WRW4) or VPAC antagonist (Leu) +/- VIP treatment (10^{-9} M). Data shown are representative of two independent experiments in duplicate and are expressed as mean + SEM. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

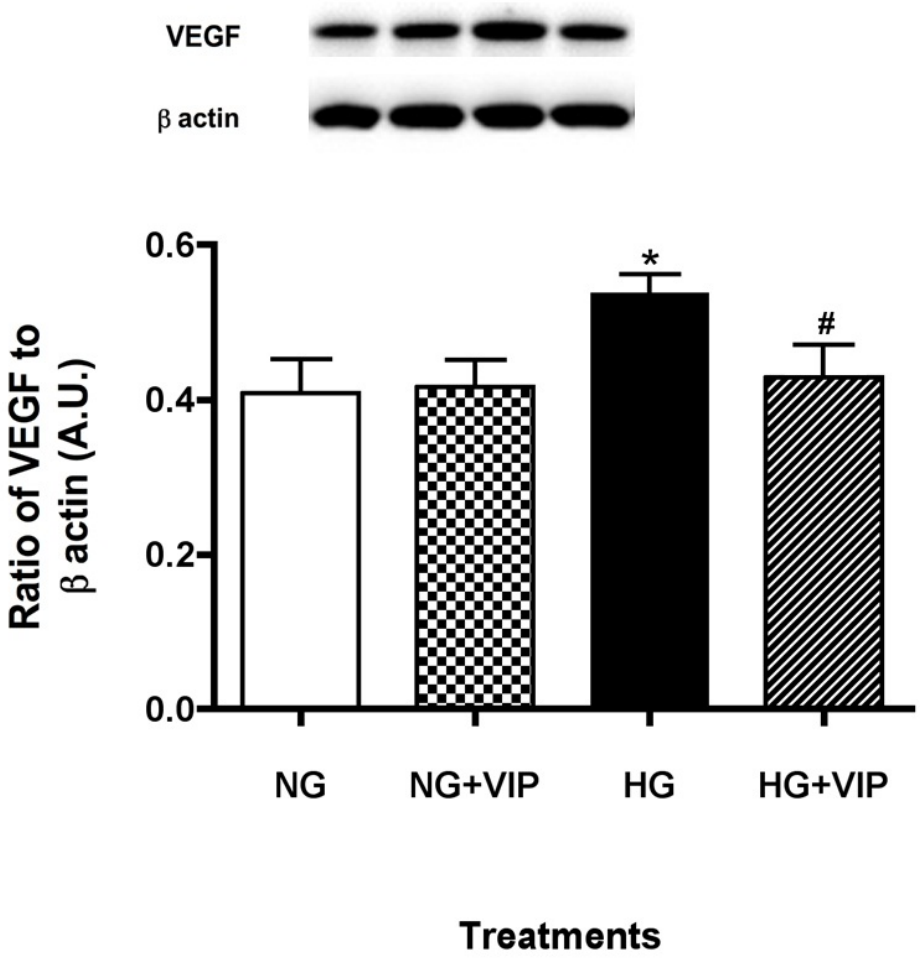

Figure 12. Protein levels of VEGF as detected by Western blot. HREC were cultured under normal glucose (NG, 5 mM) and high glucose (HG, 25 mM) conditions +/- VIP treatment (10^{-9} M) for 4 hours. Data shown are representative of two independent experiments in duplicate and are expressed as mean + SEM. *P < 0.05 vs NG, $^{\#}P$ < 0.05 vs HG.

CHAPTER 3 “A REGULATORY ROLE FOR β-ADRENERGIC RECEPTORS REGARDING THE RESOLVIN D1 (RvD1) PATHWAY IN THE DIABETIC RETINA”

3.1 Abstract

Diabetic retinopathy is a visually debilitating disease with limited treatment options available. Compound 49b, a β-adrenergic receptor agonist, has been demonstrated to effectively reduce disease pathogenesis associated with diabetic retinopathy. While the exact mechanisms are not fully understood, previous studies have determined that it reduces the pro-inflammatory cytokine, TNF-α, and inhibits apoptosis of the retinal microvasculature. As inflammation becomes more recognized in driving disease pathogenesis, so does the regulation by pro-resolving pathways as therapeutic points of intervention. The current study sought to explore whether Compound 49b had any influence on pro-resolving pathways, thus contributing to improved disease outcome. Using in vivo (animal model of type 1 diabetes) and in vitro (retinal endothelial cells, Müller cells, neutrophils/PMN) techniques, it was determined that high glucose lowers pro-resolving lipid mediator, resolvin D1 (RvD1) levels and differentially alters required enzymes, 5-lipoxygenase (5-LOX), 15-LOX-1 and 15-LOX-2. RvD1 receptors formyl peptide receptor 2 (ALX/FPR2) and G-protein coupled receptor 32 (GPR32) were also downregulated in response to hyperglycemic conditions. Moreover, it was observed that β-adrenergic receptor activation restored high glucose-induced decreases in both enzyme activity and RvD1 levels observed in vivo and in vitro. The current study is the first to describe a

regulatory role for β-adrenergic receptors on pro-resolving pathways.

3.2 Introduction

Roughly 10% of Americans were diagnosed with diabetes in 2012 (National Diabetes Statistics Report, 2014). Given that nearly 60% of diabetic patients will develop some complications related to diabetic retinopathy, generation of novel therapies is of paramount importance. While it is clear that the retinal response to high glucose is multifactorial, including oxidative stress, vascular endothelial cell growth factor (VEGF), protein kinase C, inflammatory mediators, endoplasmic reticulum stress, and epigenetic changes, therapies to prevent or delay progression of diabetic retinopathy continue to elude scientists. It has been previously reported that docosahexaenoic acid (DHA) is abundant in the retina [143]. This omega-3 fatty acid has recently been linked to the activation of a number of enzymes and proteins reported to be anti-inflammatory, as well as protective to tissues [144, 145]. Among them includes resolvin D1 (RvD1), a D-series resolvin [72, 146], which has been shown to display potent anti-inflammatory activity and control the inflammation-resolution balance in host defense. Hypoxia was one of the early signals found to stimulate the production of pro-resolving mediators, such as RvD1 and RvE1, as demonstrated in hypoxic endothelial cells [72]. Work in the oxygen-induced retinopathy (OIR) model of proliferative retinopathy has suggested that RvD1 is protective within the retina, which was correlated with reduced TNF-α levels [82]. Furthermore, 4-hydroxy-docosahexaenoic acid (4-HDHA), an intermediate metabolite of DHA

through 5-lipoxygenase (5-LOX) activity, also reduced retinal neovascularization [83]. This response was subsequently blocked when 5-LOX was eliminated [83].

In order to generate RvD1, DHA is metabolized by both 5- and 15-LOX [72] [146], which selectively interacts with receptors ALX/FPR2 and GPR32 [147]. Using the streptozotocin model of type 1 diabetes, it was found that loss of 5-LOX (a key lipid mediator enzyme) resulted in reduced vascular damage, oxidative stress, and leukostasis [148]. Work in *db/db* mice has revealed that local application of RvD1 accelerated wound closure and decreased apoptotic cell accumulation [149]. Further studies using *db/db* mice revealed that RvD1 treatment was protective against development of type 2 diabetes [150]. Additionally, studies in HepG2 cells demonstrated that RvD1 attenuated endoplasmic reticulum stress-induced apoptosis in a model of non-alcoholic fatty liver disease [151]. Taken together, data from multiple tissues suggest that the pathological markers associated with diabetes lead to a significant reduction in resolvins, which may further contribute to exacerbated retinal damage.

We have previously reported that Compound 49b, a β-adrenergic receptor agonist, demonstrates both anti-apoptotic and anti-inflammatory properties in the diabetic retina and in retinal cells under hyperglycemic conditions [152]. For that reason, we questioned whether β-adrenergic receptor signaling may regulate lipoxygenase enzyme expression and resultant RvD1 production, particularly in two resident retinal cell types, REC and Müller cells.

Polymorphonuclear leukocytes (PMN) were examined as well, in light of increased leukostasis and the mounting pathogenic role of inflammatory cells during the development of diabetic retinopathy. There is little on the role of β-adrenergic receptor regulation of lipid mediators in the eye. *In vitro* studies have demonstrated that protein kinase A (PKA) can phosphorylate 5-LOX in PMN [153]. In contrast, work in human airway endothelial cells suggests that 15-LOX-1 can decrease β2-adrenergic receptor phosphorylation, leading to decreased cAMP levels [154]. Additionally, mouse models of Alzheimer's disease have shown that norepinephrine induces expression of formyl peptide receptor 2 (ALX/FPR2) [155], which is one of two known receptors for RvD1 [73, 156]. Thus, the potential regulatory role for β-adrenergic receptor signaling on RvD1 or lipoxygenase enzymes in a diabetic retinopathy model remains unknown.

We have previously reported that Compound 49b can reduce TNF-α, as well as SOCS3, under hyperglycemic conditions [152, 157]. Therefore, we hypothesized that diabetes or high glucose culturing conditions would decrease enzymatic levels of 15-LOX and downstream production of RvD1, which could be ameliorated by Compound 49b. Indeed, we found that high glucose and diabetic conditions significantly decreased 15-LOX, as well as RvD1 levels. In addition, lipoxygenase enzymes and RvD1 were increased following Compound 49b treatment.

3.3 Experimental procedures

Animals

All mouse experiments were approved by the Institutional Animal Care and Use Committee at Wayne State University (Protocol# 11-08-14). C57BL/6J wildtype mice were purchased from Charles River Laboratories. Mice were made diabetic by injections of 60 mg/kg of streptozotocin dissolved in citrate buffer for 5 consecutive days. Control mice received citrate buffer only. Glucose measurements were done weekly, with glucose levels >250 mg/dL considered diabetic. At 2 months of diabetes, 10 control and 10 diabetic mice received Compound 49b (4μL containing 1 mM) (formulated by Dr. Duane Miller, University of Tennessee Health Science Center, Memphis TN, in collaboration with Dr. Jena Steinle) topically onto each eye for 14 days. After 14 days of Compound 49b treatment, all mice were sacrificed and analyzed as described below.

Retinal endothelial cell culture

Primary human retinal microvascular endothelial cells (REC) were acquired from Cell System Corporation (CSC, Kirkland, Washington). Cells were grown in M131 medium containing microvascular growth supplements (Invitrogen), 10 g/mL gentamycin, and 0.25 g/mL amphotericin B. Prior to the experiment, cells were transferred to high (25 mM) or normal (5 mM) glucose medium (M131 medium with added glucose), supplemented with MVGS and antibiotics for 3 days. Only primary cells within passage 6 were used. Cells were quiesced by incubating in high or normal glucose medium without MVGS for 24 hr. Compound 49b treatment was then added at 50 nM for 24 hours, as done

previously [152].

Müller cell (rMC-1) culture

Müller cells (rMC-1; kindly provided by Dr. Vijay Sarthy at Northwestern University) were thawed and cultured in DMEM medium under normal glucose (5mM) conditions. Medium was supplemented with 10% FBS and antibiotics. Once cells reached ~ 80% confluency, they were passed into dishes containing either high (25 mM) or normal glucose medium. Once ready for experimentation, cells were moved to the appropriate medium without FBS to induce serum starvation for 18-24 hours. Compound 49b was then applied at 50 nM for 24 hours prior to cell collection.

PMN isolation

Peritoneal PMN from C57BL/6 mice were harvested as previously described [158]. In brief, mice received an intraperitoneal (IP) injection (1.0 mL) of a 9% casein solution (Difco, Detroit, MI) administered 27 hours prior to cell harvest, followed by a second injection 24 hours later. Cells were collected by peritoneal lavage 3 hours after the second injection, washed 3× (200 ×*g*, 10 min), and then isolated using a Percoll gradient (100,000 ×*g*, 20 min). Cell viability (>95%) and purity (>90%) were determined. Cells were resuspended in media (RPMI 1640 supplemented with 3% FCS and antibiotics) containing either normal glucose (5 mM) or high glucose (25 mM) for 24 hours (37□C, 5% CO_2). Cells were then exposed to Compound 49b treatment (50 nM) for an additional 24 hours, prior to harvest for protein analyses.

Cell treatment

REC were selectively treated with either propranolol, a β-adrenergic receptor antagonist, or PKA siRNA (Dharmacon, Lafayette, CO). For propranolol work, cells were cultured under normal and high glucose conditions, and treated with propranolol (50 nM) 30 minutes prior to 49b treatment. To block the PKA pathway, cells were similarly cultured and transfected with PKA siRNA at a final concentration of 20 nM as previously described [64] using GenMute siRNA transfection kit (SignaGen, Rockville, MD) followed by 49b treatment.

Western blotting

After appropriate treatments and rinsing with cold phosphate-buffered saline, REC, rMC-1 and PMN were collected in lysis buffer containing protease and phosphatase inhibitors and scraped into tubes. Retinal extracts were prepared by sonication. Equal amounts of protein from the cell or tissue extracts were separated on pre-cast tris-glycine gels (Invitrogen, Carlsbad, CA), and then blotted onto nitrocellulose membranes. After blocking in TBST (10mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) BSA, membranes were treated with the following primary antibodies: 5-LOX, 15-LOX-1, 15-LOX-2, ALX/FPR2, GPR32 (Abcam, San Francisco, CA) and -actin (Santa Cruz, Santa Cruz, CA), followed by incubation with appropriated secondary antibodies (Fisher Scientific, Pittsburgh, PA) labeled with horseradish peroxidase. Antigen-antibody complexes were detected using a

chemilluminescent reagent kit (Thermo Scientific, Pittsburgh, PA). Western blot images were collected on an Azure Biosystem C500 machine (Azure Biosystems, Dublin, CA) and densitometric analysis was performed.

ELISA

RvD1 ELISA kits (Cayman Chemical, Ann Arbor, MI) were used to measure RvD1 expression in REC, rMC-1, PMN and retinal lysates. Equal protein concentrations were added to all wells. Assay protocol was performed according to the manufacturer's instructions. Cross reactivity of this assay for RvD2 and RvE1 is 0.05% and <0.01%, respectively.

Statistics

All experiments were repeated in triplicate and data are presented as the mean ± SEM. Non-parametric Kruskal-Wallis with Dunn's post-hoc tests were used for the cell culture data. One-way ANOVA with a Student Newman Keul's post-hoc test was done for animal studies using Prism 7.0 software. $P<0.05$ was considered significant. Representative blots are shown for all Western blot analyses.

3.4 Results

Compound 49b significantly increases RvD1 levels and receptors ALX/FPR2 and GPR32 in the diabetic retina.

Since DHA is highly abundant in the retina [143], we determined whether RvD1 levels and associated receptors, ALX/FPR2 and GPR32 [73, 156] were altered under diabetic conditions (Fig 13A-C). In 2-month diabetic mice, levels

of RvD1 (A), ALX/FPR2 (B) and GPR32 (C) were significantly decreased. In contrast, Compound 49b treatment significantly enhanced expression of all three proteins when compared to untreated diabetic mice. These results suggest that Compound 49b may be protective to the diabetic retina through key pro-resolving pathways.

Compound 49b prevents high glucose-induced decrease in RvD1 and associated receptors in REC.

To determine which cell types may contribute to the observed RvD1 profile in the diabetic retina, REC in normal and high glucose were treated with Compound 49b. Parallel to the diabetic retina, RvD1 levels were significantly decreased in REC exposed to high glucose conditions (Fig 14A). RvD1 receptors ALX/FPR2 and GPR32 were similarly downregulated with high glucose (Fig 14B and 14C, respectively). After treatment with Compound 49b, RvD1, ALX/FPR2, and GPR32 levels were significantly upregulated in REC, despite hyperglycemic conditions, to levels similarly observed with normal glucose.

Compound 49b results in differential expression of lipoxygenase enzymes in REC.

5-LOX, 15-LOX-1 and 15-LOX-2 enzymes were significantly increased in REC cultured in high glucose (Fig 15A-C). When REC grown in high glucose were treated with Compound 49b, 5-LOX enzyme expression returned to levels observed in normal glucose (Fig 3A), with further enhanced expression of 15-

LOX-1 and 15-LOX-2 (Fig 15B and 15C), suggesting that β-adrenergic receptor signaling may differentially regulate key lipid mediator enzymes in REC.

Compound 49b did not affect RvD1, ALX/FPR2 or GPR32 in Müller cells.

Since Müller cells are the primary residential inflammatory cell type in the retina, we examined whether they respond likewise to high glucose via changes in the D-series resolvin pathway. As illustrated in Fig 16, neither high glucose nor Compound 49b altered RvD1 levels in Müller cells (A). Similar responses were displayed regarding ALX/FPR2 and GPR32 when compared to REC and whole retinal lysates – levels were decreased in Müller cells in response to high glucose (Fig 16B and 16C). However, Compound 49b had no apparent effect.

Compound 49b exhibited limited effects on high glucose-induced changes in lipoxygenase enzymes in Müller cells.

As shown in Fig 17, 5-LOX (A) was significantly upregulated, while 15-LOX-1 enzyme (B) was downregulated in Müller cells grown in high glucose; no differences were observed with 15-LOX-2 (C). Compound 49b effectively reduced 5-LOX levels to those similar to control. However, unlike REC and the diabetic retina, Compound 49b treatment had no effect on either 15-LOX expression in Müller cells cultured in high glucose.

High glucose conditions decreased RvD1, ALX/FPR2 and GPR32 levels in PMN.

To investigate a systemic immune cell type that may respond to retinal damage, isolated murine PMN were exposed to normal and high glucose +/-

Compound 49b treatment. Protein levels of RvD1, ALX/FPR2 and GPR32 were detected in PMN (Fig 18A-C). Exposure to high glucose resulted in significant downregulation of all three molecules. Compound 49b did not appear to affect RvD1 or GPR32 – levels remained unchanged under both normal glucose and high glucose conditions. However, hyperglycemia-induced decreases in ALX/FPR2 were restored after Compound 49b treatment.

Compound 49b had limited effect on lipoxygenase levels in PMN in high glucose.

High glucose conditions significantly reduced 15-LOX-1 levels in PMN, with no effect observed with 5-LOX or 15-LOX-2 (Fig 19A-C). Following Compound 49b treatment, levels for all three enzymes high glucose remained unchanged. These results suggest that systemic immune cells may respond differently to β-adrenergic receptor agents compared to residential retinal cells.

Compound 49b effects on RvD1 in REC are dependent on β-adrenergic receptor signaling.

Compound 49b activity is carried out through β-adrenergic receptor mediated increases in PKA activity [152]. To determine the specificity of Compound 49b's observed influence on the pro-resolving RvD1 pathway, REC were exposed to propranolol, a non-specific β-adrenergic receptor antagonist, or PKA siRNA in the presence of high glucose and/or Compound 49b. As shown in Fig 20A, propranolol abrogated the effects of Compound 49b and resulted in RvD1 levels similar to those observed with high glucose alone. Similar effects

were seen with PKA siRNA (Fig 20B), whereby RvD1 levels were significantly reduced comparable to high glucose alone despite Compound 49b treatment, thus further suggesting a β-adrenergic receptor signaling pathway-dependent effect.

3.5 Discussion

Work in the OIR model has suggested that lipid mediators may reduce retinal neovascularization, with lipoxygenase enzymes expressed primarily by circulating cells [83]. Additionally, it has been demonstrated that RvD1 and RvE1, as well as neuroprotectin, are protective in the OIR model, potentially through reduced TNF-α levels [82]. It is well established that early diabetic retinopathy following streptozotocin (STZ) injections leads to increased TNF-α levels [8, 115, 152]. Work in diabetic-induced 5-LOX knockout mice demonstrated that loss of this key enzyme resulted in reduced degenerate capillaries, leukostasis, and NF-κB levels, while reduced leukostasis only was observed in diabetic 12/15-LOX knockout mice [148]. Furthermore, work in humans has suggested that patients with diabetic retinopathy or diabetic macular edema have decreased levels of resolvins and protectins [159]. Thus, it appears that diabetes (both proliferative and non-proliferative) may alter lipoxygenase levels, as well as resolvin activity. However, the regulation and cellular source of these enzymes and resolving pathways is less clear. Regarding the former, there are limited and conflicting data regarding the potential regulatory role for β-adrenergic receptor signaling in relation to RvD1

or lipoxygenase enzymes [153-155]. Based upon these studies, it is likely that different cell types and/or systems could potentially activate different signal transduction pathways for resolvin production specific to each system. Hence, the current study sought to define potential regulatory pathways associated with RvD1 expression in the diabetic retina and begin to identify the cellular source(s) related to these pathways.

Compound 49b is a β-adrenergic receptor agonist based structurally on isoproterenol with chemical modifications to increase its ocular potency for use as a topical treatment [152]. It has been previously shown to significantly reduce cleaved caspase 3 in the diabetic retina and retinal endothelial cells [152, 160]. Moreover, we have demonstrated that hyperglycemia-induced increases in TNF-α in REC and Müller cells [157, 161] can be effectively reduced by Compound 49b [157, 161]. It has also been found that the protective effect of RvD1 against diabetic retinopathy occurs in part through the suppression of TNF-α [82]. Therefore, we determined whether Compound 49b could regulate lipoxygenase enzymes and resultant RvD1 expression, ultimately contributing to the reduction in TNF-α both *in vitro* (REC and Müller cell culture) and *in vivo* (STZ-induced diabetic retina). We also examined PMN to extend our findings to circulating inflammatory cells, given the role of leukostasis during development and progression of diabetic retinopathy. Overall, our data indicate that high glucose significantly reduces RvD1 and corresponding receptor levels in the diabetic retina. Our findings in the STZ-induced diabetic mouse retina are

in contrast to findings in the OIR model, where RvD1 levels were not altered [83]. The observed differences in RvD1 could be due to glucose-induced damage versus oxygen-induced damage. However, ALX/FPR2 and GPR32 (RvD1 receptors) were also decreased in the diabetic retina. In addition, similar results were consistently observed for these same molecules tested in REC after exposure to high glucose conditions. Correspondingly, treatment with Compound 49b significantly increased RvD1 expression similar to normal glucose both in the diabetic retina and *in vitro* using REC accompanied by decreased 5-lipoxygenase and increased 15-lipoxygenase enzyme levels.

Upon further examination of retinal Müller cells, a slightly different response was observed. While high glucose resulted in increased 5-LOX and decreased 15-LOX, ALX/FPR2 and GPR32 levels, RvD1 remained unchanged. This suggests that Müller cells have a different profile in response to high glucose than the whole retina or REC, with no change in RvD1 levels, but downregulated receptor expression. Similar to REC and diabetic retina; however, Compound 49b significantly reduced 5-LOX enzyme levels in Müller cells cultured in high glucose, as well as increased ALX/FPR2 levels, suggesting a potential regulatory pathway in Müller cells, albeit much less responsive. Compound 49b did not affect GPR32 levels, indicating that RvD1 would most likely signal through ALX/FPR2 in Müller cells and warrants further investigation. In the diabetic retina, REC and Müller cells, β-adrenergic receptor signaling appears to be pro-resolving, at least in part, through actions carried

out by RvD1 and ALX/FPR2. However, this needs to be further confirmed using ALX/FPR2 antagonists and/or silencing of the PI3K/Akt signaling pathway to determine the extent of RvD1/ALX/FPR2 in mediating the protective effects of Compound 49b. Further, given the increased 5-LOX enzyme levels, it does not rule out the potential role Müller cells may have in producing 5-lipoxygenase-driven pro-inflammatory lipid mediators, such as leukotrienes.

When we expanded our work to circulating inflammatory cells (namely PMN) that are known to enter the retina during leukostasis, yet a different paradigm appeared. Effects of high glucose culturing conditions resulted in decreased RvD1, ALX/FPR2 and GPR32 levels; though were limited to decreased 15-LOX-1 regarding lipoxygenase enzymes. Despite no response of 15-LOX-2 to high glucose exposure, these results are not surprising given that 15-LOX-1 is highly expressed by circulating leukocytes compared to 15-LOX-2, which is thought to be more restricted to tissue expression. And though 5-LOX (and 15-LOX-2) remain unchanged under high glucose conditions, these results suggest that 15-LOX-1 may be the limiting enzyme in the production of RvD1 within the PMN. Furthermore, while RvD1, 15-LOX-1, ALX/FPR2 and GPR32 were significantly decreased in high glucose, only ALX/FPR2 was restored toward normal glucose levels after Compound 49b treatment. Albeit a limited receptor effect, it may have significant implications as RvD1 has been shown to potently regulate both human and mouse neutrophils [4] [146].

These results suggest that although retinal cells appear to lose pro-

resolving and lipoxygenase enzyme responses in high glucose, restoration through stimulation of β-adrenergic receptor signaling pathways may occur. As such, we next confirmed that the observed effects of Compound 49b in RECs were carried out through activation of β-adrenergic receptor signaling, as indicated using propranolol – a non-specific β-adrenergic receptor antagonist. In addition, this specificity was further confirmed using PKA siRNA to silence the downstream β-adrenergic receptor signaling pathway, as both approaches resulted in blunted RvD1 expression under high glucose conditions despite Compound 49b treatment. In contrast, while circulating PMN are partially responsive to high glucose, they did not appear to be influenced by the β-adrenergic receptor agonist. This raises two points as to why PMN alone are only partially responsive to high glucose compared to the response of retinal cells. One potential explanation for the limited response to high glucose may be related to the stimuli. PMN are designed to circulate throughout the body and respond to specific damage. The 25mM glucose used in this study may not be an appropriate damage stimulus for PMN. Likewise, it is possible that other pro-inflammatory signals released from retinal cells or cell-cell contact with REC may be required to elicit a more robust response from neutrophils. To this end, the microenvironment of the retinal vasculature may allow for interplay between REC and PMN that can be influenced by both high glucose conditions and Compound 49b treatment.

Another key question arising from this study is why β-adrenergic receptors

may regulate RvD1? In the retina, it is clear that maintenance of β-adrenergic receptor signaling reduces inflammatory pathways [152, 162-164]. Although it has been reported in activated microglia that isoproterenol (from which Compound 49b is derived) increases (as opposed to decreases) levels of inflammatory cytokines [165] [166], studies were carried out in chronic stress and surgical trauma models – not models of high glucose or hyperglycemia. To this end, we have shown a different effect of 49b on REC and Müller cells. Compound 49b works through β1-adrenergic receptors in REC and β2-adrenergic receptors. In both cases of these cell types, Compound 49b reduces TNF-α [152, 167]. Once we established the actions on TNF-α, we then moved to show its actions on TLR4. From these findings, we hypothesized it may work on pro-resolving pathways, as well. To date, little to no information exists on β-adrenergic receptors and RvD1. Our findings that Compound 49b can increase RvD1 in diabetic whole retina and REC grown in high glucose strongly indicate that this may occur through β-adrenergic actions on cAMP. Work in rat brain astrocytes demonstrated that DHA release can be regulated by calcium-independent phospholipase A2 (iPLA2) [168]. DHA release was amplified by PKA agonist application [168]. Since we have shown that Compound 49b significantly increases PKA [152], it is possible that our findings on lipoxygenase enzymes and RvD1 may relate to actions on PKA and iPLA2. These will be the focus of further study.

Collectively, these data indicate for the first time a regulatory role for β-

adrenergic receptors regarding pro-resolving pathways in the diabetic retina. Activation of β-adrenergic receptor signaling pathway via Compound 49b rescued hyperglycemic-induced decreases in RvD1, its key enzymes 5- and 15-LOX, as well as receptors ALX/FPR2 and GPR32 in the diabetic retina and REC. Future insight into how β-adrenergic receptor signaling pathways influence pro-resolving mediators in the diabetic retina may contribute to the development of therapeutic modalities targeted at the resolution of inflammation, not simply anti-inflammatory in nature.

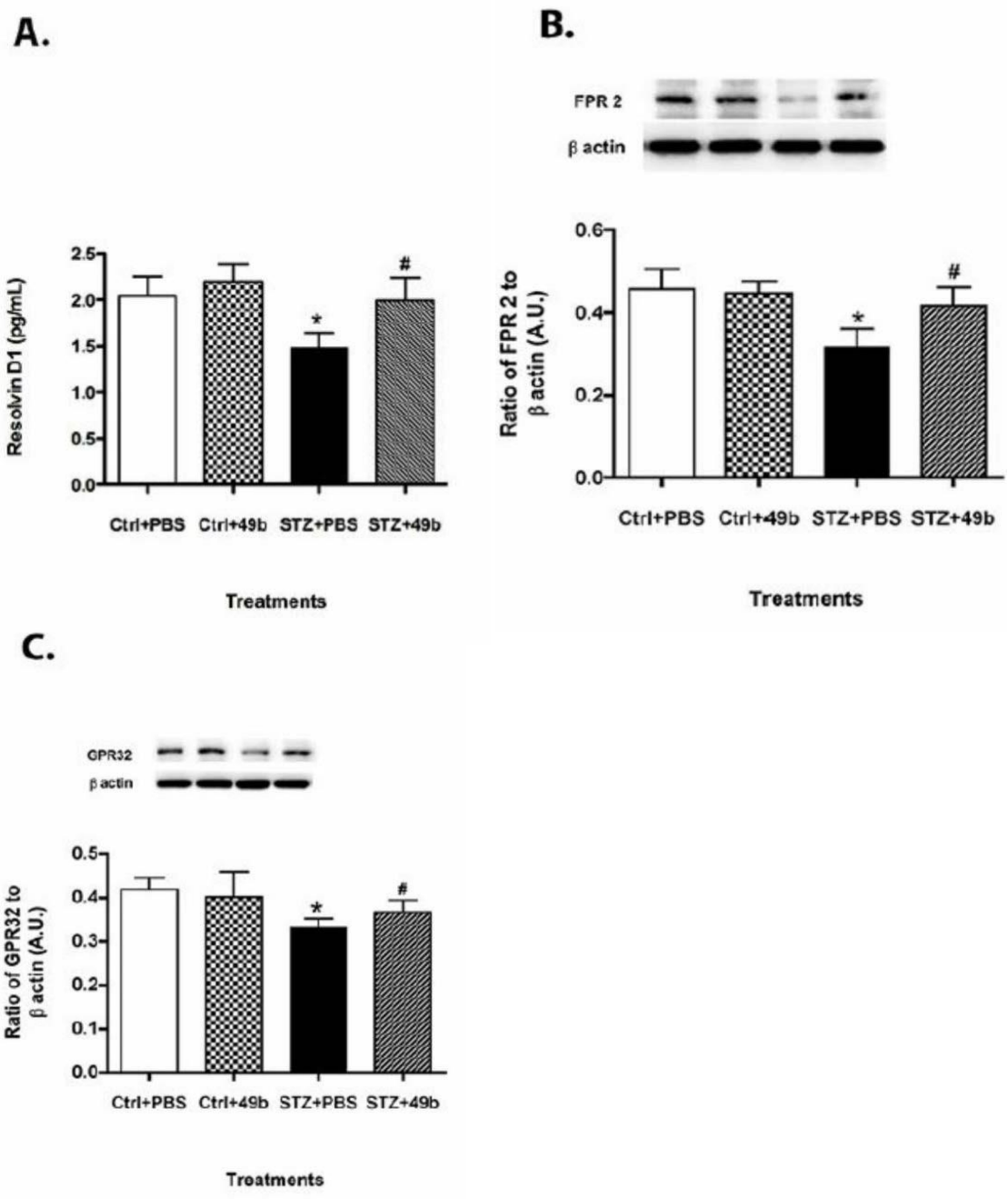

Figure 13. Compound 49b significantly increased RvD1 (A), ALX/FPR2 (B) and GPR32 (C) in the diabetic retina. Data from whole retinal lysates from C57BL/6 mice untreated (Ctrl+PBS), control mice treated with Compound 49b (Ctrl+49b), streptozotocin-induced diabetic mice (STZ+PBS), or diabetic mice treated with Compound 49b (STZ+49b). *P<0.05 vs. ctrl+PBS, $^{\#}$P<0.05 vs. STZ+PBS. N=5 mice in each group.

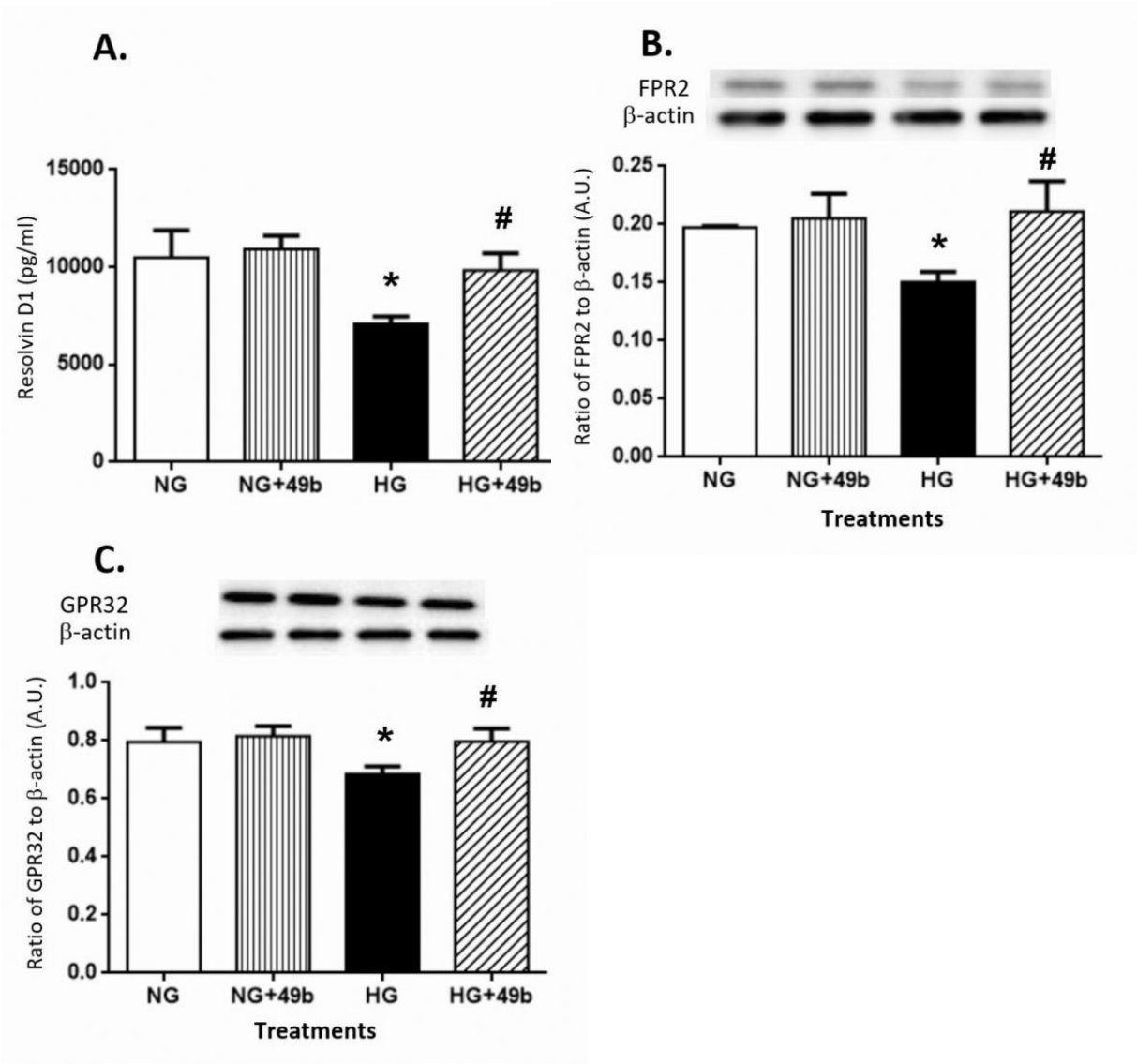

Figure 14. Compound 49b increased levels of RvD1 (A), ALX/FPR2 (B) and GPR32 (C) in REC exposed to high glucose. REC cells were grown in normal glucose (NG), normal glucose+Compound 49b (NG+49b), high glucose (HG) or high glucose treated with Compound 49b (HG+49b). *P<0.05 vs. NG, #P<0.05 vs. HG. N=4 for each treatment.

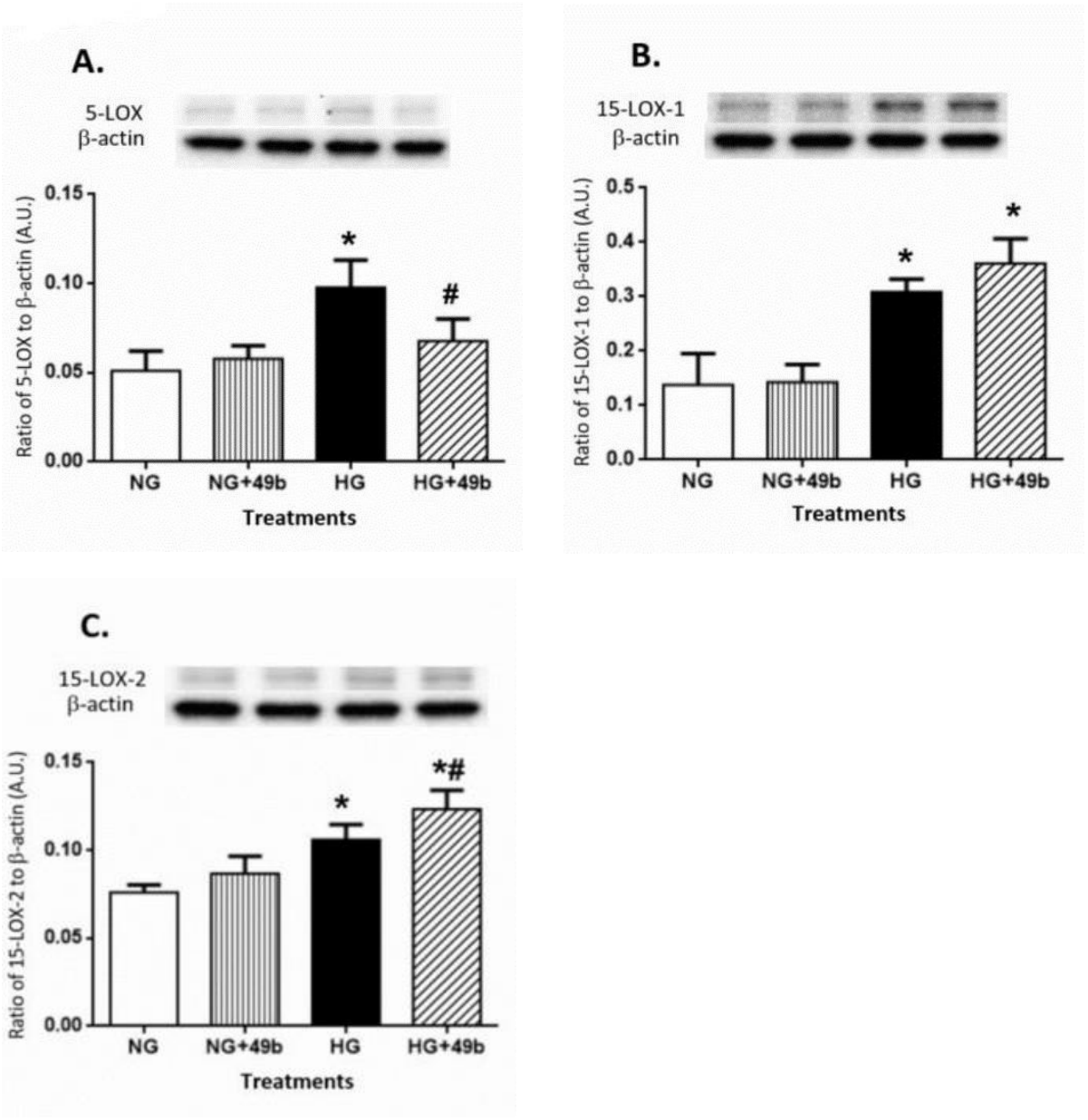

Figure 15. Lipoxygenase enzymes were differentially expressed in REC cells grown in high glucose and after Compound 49b treatment. 5-LOX (A), 15-LOX-1 (B) and 15-LOX-2 (C) in REC cells grown in normal glucose (NG), normal glucose+Compound 49b (NG+49b), high glucose (HG) or high glucose treated with Compound 49b (HG+49b). *P<0.05 vs. NG, #P<0.05 vs. HG. N=4 for each treatment.

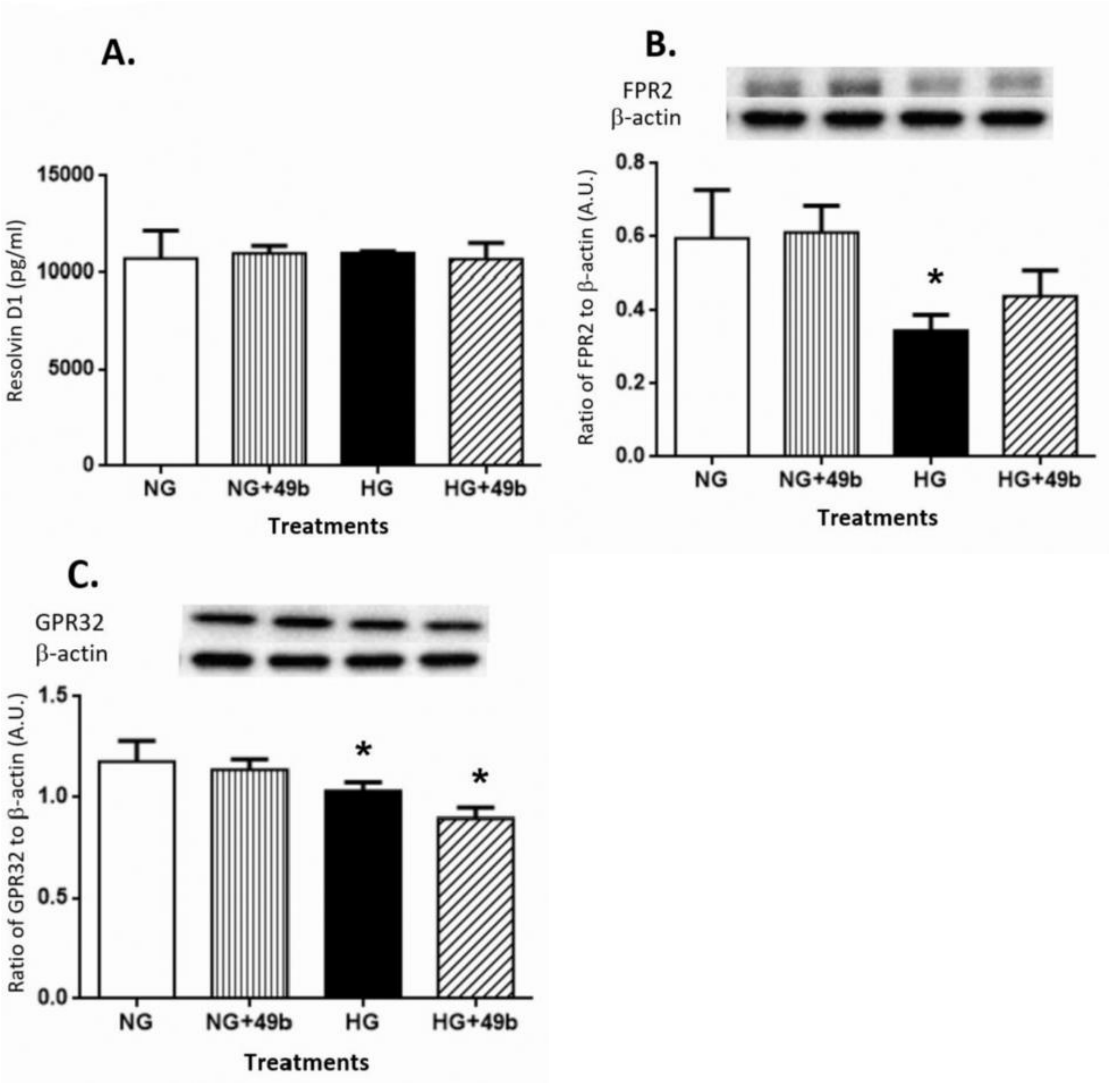

Figure 16. Müller cells grown in high glucose did not increase RvD1 levels after Compound 49b treatment. Müller cells were grown in in normal glucose (NG), normal glucose+Compound 49b (NG+49b), high glucose (HG) or high glucose treated with Compound 49b (HG+49b). Panel A shows RvD1 protein expression, panels B and C show Western blot results for ALX/FPR2, and GPR32 levels in Müller cells. *P<0.05 vs. NG, $^{\#}$P<0.05 vs. HG. N=4 for each treatment.

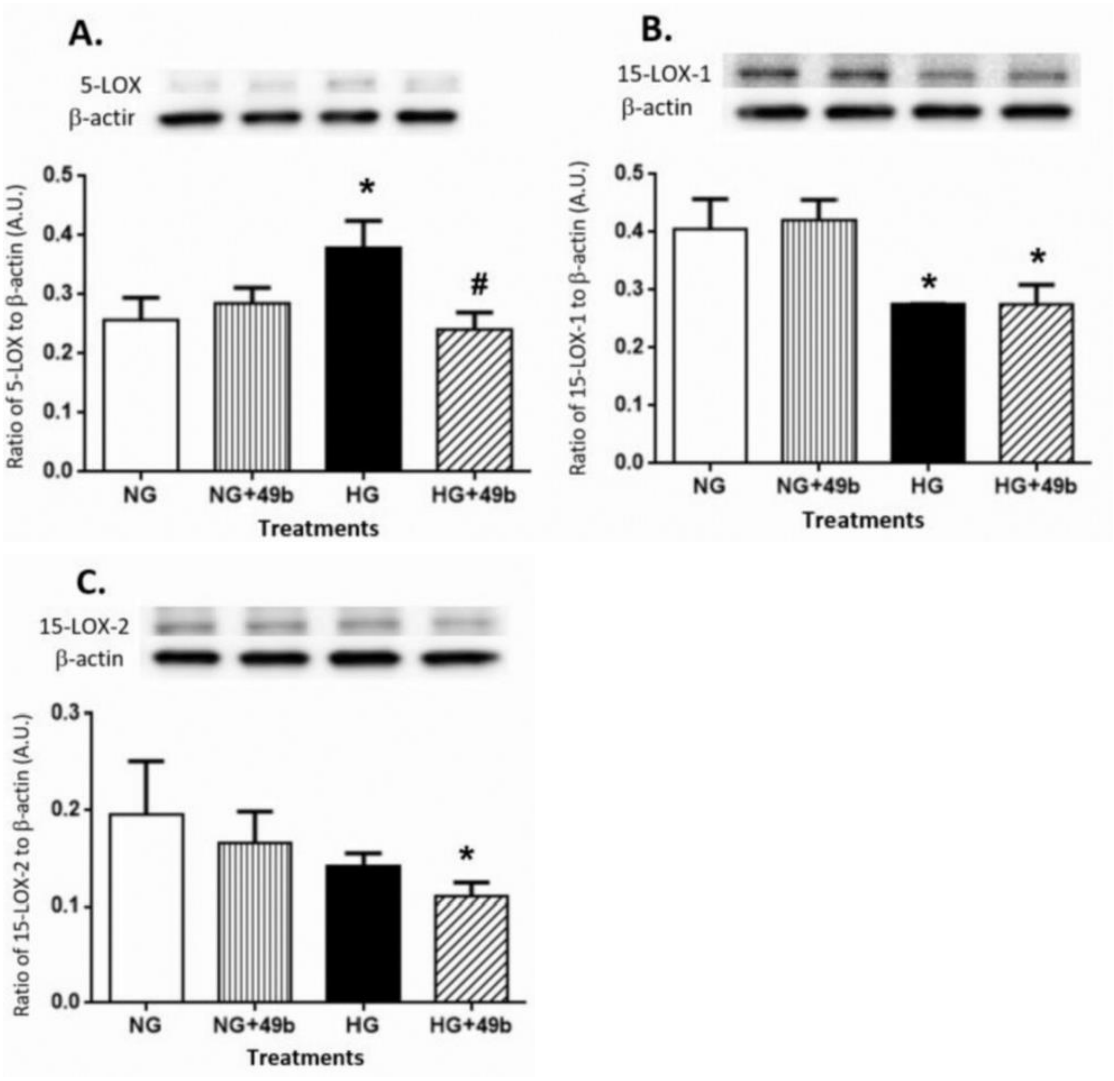

Figure 17. Compound 49b displayed limited effects on 5-LOX in Müller cells in high glucose. Müller cells were grown in in normal glucose (NG), normal glucose+Compound 49b (NG+49b), high glucose (HG) or high glucose treated with Compound 49b (HG+49b). Panels A – C illustrate Western blots for 5-LOX, 15-LOX-1 and 15-LOX-2, respectively. *P<0.05 vs. NG, $^{\#}$P<0.05 vs. HG. N=4 for each treatment.

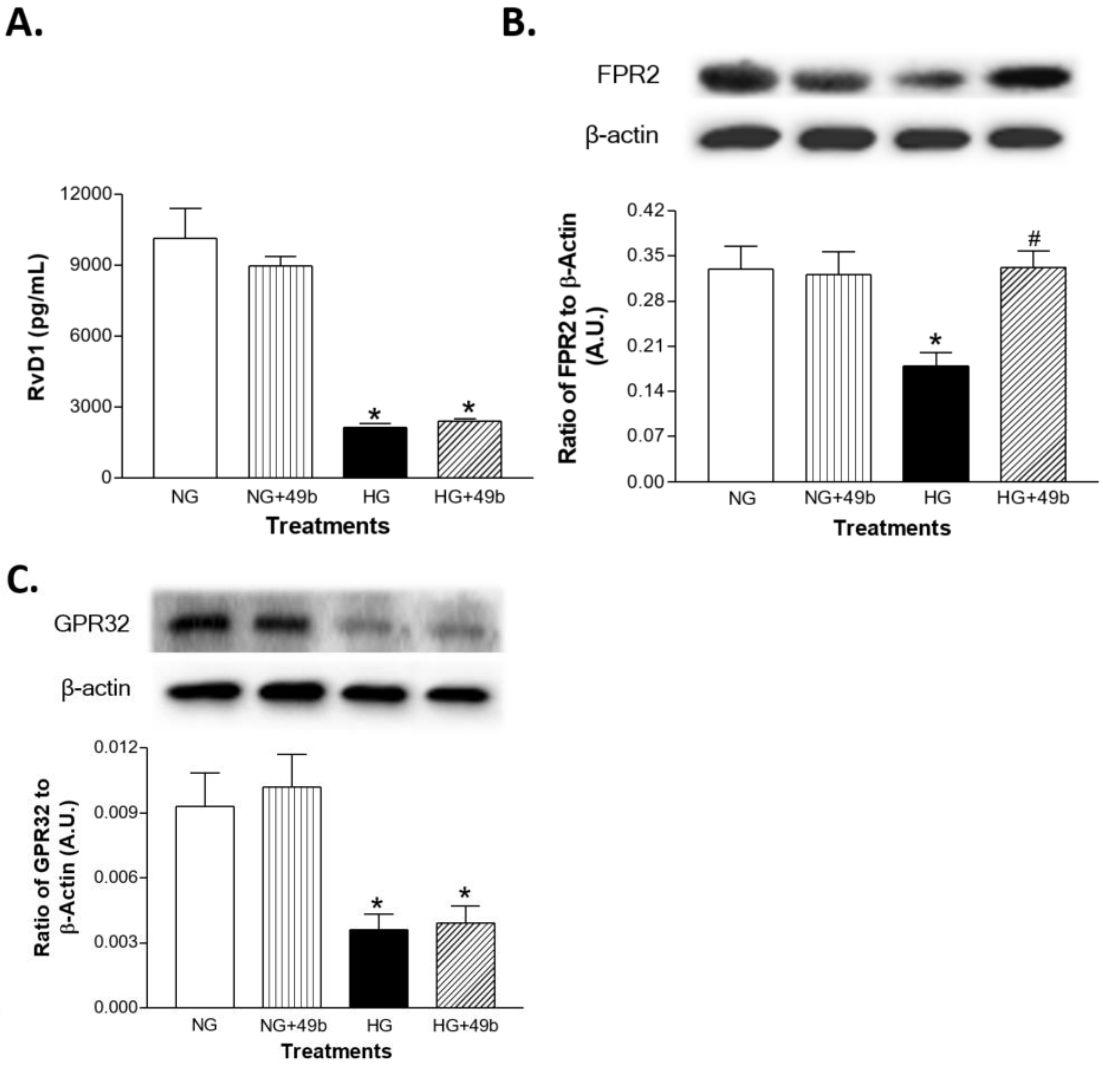

Figure 18. Compound 49b significantly increased ALX/FPR2 levels, with no influence on RvD1 or GPR32 levels. RvD1 (A), ALX/FPR2 (B) and GPR32 (C) expression in PMN exposed to normal glucose (NG), normal glucose+Compound 49b (NG+49b), high glucose (HG) or high glucose treated with Compound 49b (HG+49b). *P<0.05 vs. NG, #P<0.05 vs. HG. N=4 for each treatment.

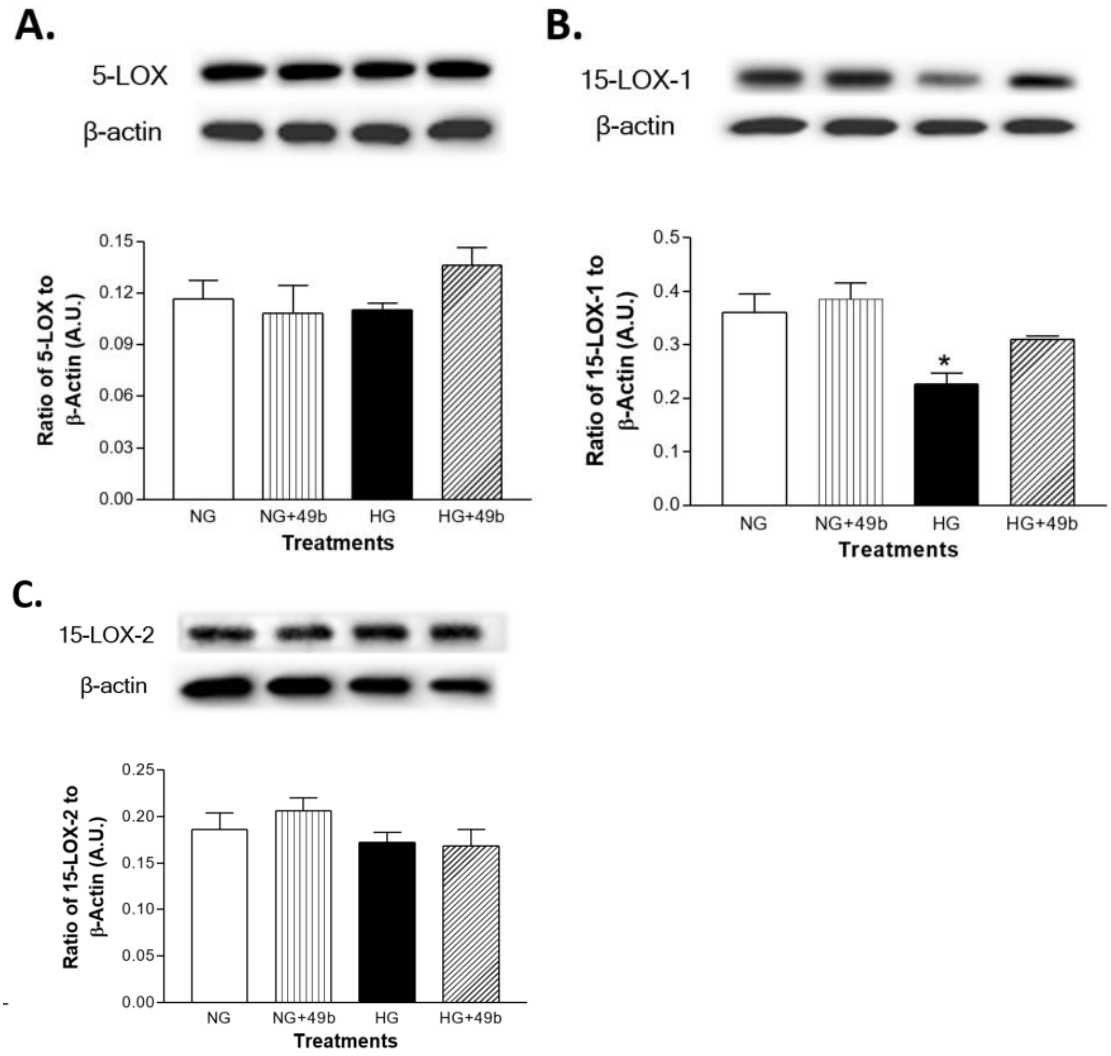

Figure 19. Compound 49b had no effect on lipoxygenase levels in PMN exposed to high glucose. Mouse PMN cells were exposed to normal glucose (NG), normal glucose+Compound 49b (NG+49b), high glucose (HG) or high glucose treated with Compound 49b (HG+49b). Western blots results are shown for 5-LOX (A), 15-LOX-1 (B) and 15-LOX-2 (C) expression. *P<0.05 vs. NG, #P<0.05 vs. HG. N=4 for each treatment.

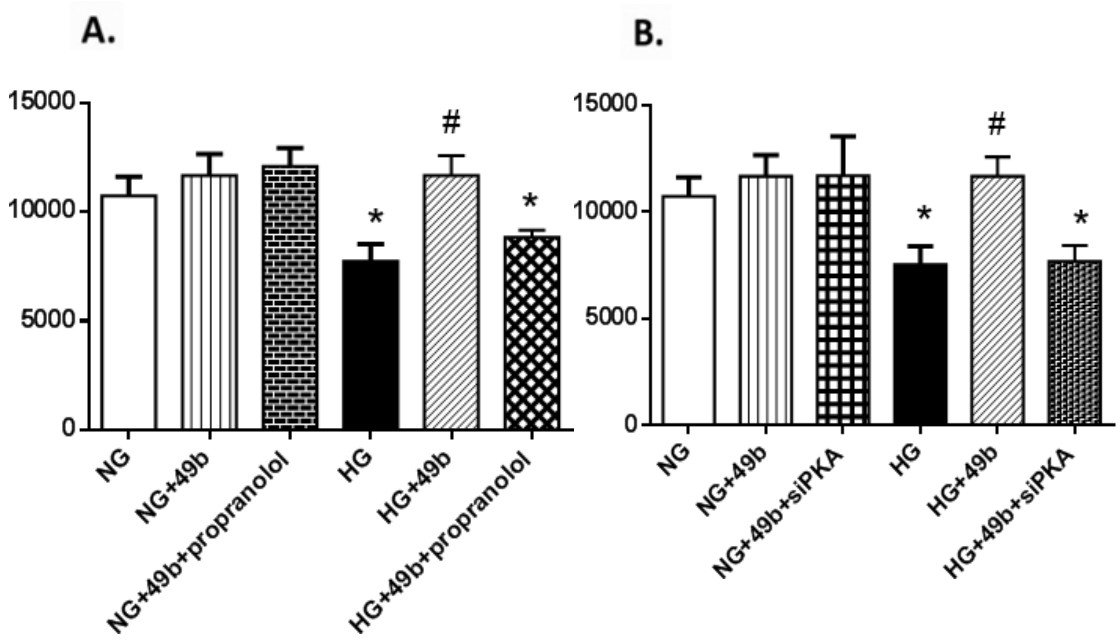

Figure 20. Increased RvD1 levels after Compound 49b treatment in REC were β-adrenergic receptor pathway specific. REC were exposed to normal glucose (NG), normal glucose+Compound 49b (NG+49b), normal glucose+Compound 49b+propranolol or PKA siRNA(NG+49b+propranolol/siPKA), high glucose (HG), high glucose treated with Compound 49b (HG+49b) and high glucose+Compound 49b+propranolol/PKA siRNA (HG+49b+propranolol/siPKA). ELISA results are shown for RvD1 levels after treatment with propranolol (A) and PKA siRNA (B). *P<0.05 vs. NG, #P<0.05 vs. HG. N=4 for each treatment.

CHAPTER 4 “CHARACTERIZATION OF SITE SPECIFIC PHOSPHORYLATION OF NF-κB P65 IN RETINAL CELLS IN RESPONSE TO HIGH GLUCOSE AND CYTOKINE POLARIZATION”

4.1 Abstract

Background

Inflammation is an important contributor to the pathogenesis of DR, a severe blinding eye disease affecting nearly 60% of diabetic patients. NF-κB is a master transcriptional regulator for a wide spectrum of inflammatory genes. Although NF-κB is comprised of multiple subunits, p65 has received the most attention. However, the p65 subunit can be phosphorylated at numerous sites, for which the effects of DR-related conditions are not well characterized. Since dysregulation of NF-κB has been linked to chronic inflammation, the current study examines site specific p65 phosphorylation in retinal cells exposed to high glucose and investigates the effects of cytokine polarization in the regulation of the high glucose-induced inflammatory response.

Methods

Phosphorylation of NF-κB p65 was examined in human primary retinal endothelial cells (HREC) and MIO-M1 Müller cells after exposure to high glucose and pro- or anti-inflammatory cytokines. Cells were incubated in high (25 mM) or normal (5 mM) glucose, then treated with IL-1β, TNF-α or IL-4. Cells were then harvested at different time points to assess phosphorylation levels at multiple p65 sites, including Thr-254, Ser-276, Ser-281, Ser-311, Ser-468, Ser-529, Ser-536 and Thr-435. Related downstream gene activation was selectively

measured by real-time RT-PCR, ELISA and/or Western blot.

Results

High glucose exposure resulted in differential phosphorylation of p65 subunit sites between HREC and Müller cells. Pro-inflammatory cytokines further exacerbated the phosphorylation of these sites while influencing additional sites that were not altered in high glucose. In contrast, IL-4 exhibited a generally suppressive effect on the phosphorylation of p65 sites in both HREC and Müller cells, and promoted expression of IκBα. Downstream inflammatory mediators were more activated in response to pro-inflammatory cytokine treatment than high glucose exposure. Anti-inflammatory IL-4 inhibited expression of downstream NF-κB regulated inflammatory genes, while IL-10 levels were significantly enhanced even in the presence of high glucose.

Conclusion

The current study is the first to characterize high glucose-induced NF-κB p65 phosphorylation after cytokine polarization. By understanding NF-κB phosphorylation and cytokine influence during hyperglycemic conditions, intervention points can be identified for early stage treatment of DR.

Keywords: diabetic retinopathy, NF-κB, p65, phosphorylation, interleukin-4, inflammation

4.2 Background

Diabetes mellitus (DM) is a chronic, metabolic disease resulting in high blood glucose levels in the body. Diabetic retinopathy (DR) is a visually

debilitating eye complication of diabetes that is the leading cause of blindness among working age patients in the United States [2]. This disease exhibits several pathological events associated with the retina. The early stage of DR, with its featured complications of microaneurysms, hard exudates and progressing hemorrhages, continues to lack effective treatments [169]. Although intensive efforts have been put into understanding pathogenic mechanisms of DR, remarkable intervention points for treatment against non-proliferative stages of DR have yet to be found. Current anti-vascular endothelial growth factor (VEGF) treatments risk deleterious side effects such as endophthalmitis, intraocular inflammation and elevated intraocular pressure [7], with compromised efficacy around 50% and requires repetitive administration [170].

Inflammation was found to be associated with diabetes back in 1960s [18]; since then, leukostasis and associated local inflammatory activities have been demonstrated to be key contributors for retinal non-perfusion, retinal ischemia and resultant retinal vascular leakage during DR [19-22]. The retinal endothelium is part of the blood-retinal barrier which isolates the retina from toxins, microorganisms and pro-inflammatory leukocytes [45]. Retinal endothelial cells (REC) line the microvasculature with surrounding pericytes [171]. They are directly exposed to hyperglycemic conditions and interact with infiltrating leukocytes. During inflammation, RECs respond to extracellular molecules secreted by both residential retinal cells as well as immune cells.

While DR was traditionally characterized as a microvascular disease, it is now being viewed as a neural degenerative disease [116], as well. Müller cells are supporting glia in the retina and one of the earliest residential cellular responders during the pathogenesis of DR [172]. They actively react to inflammatory cytokines and secret various molecules to modulate the microenvironment during the development of DR, including RECs [173]. Additionally, it has been demonstrated that reactive oxygen species (ROS) toxicity in RECs was derived from retinal Müller cells and pigment epithelial cells through paracrine effects rather than a direct effect of high glucose [174], suggesting a role for this cell type during the development of this disease.

NF-κB is a major transcription factor evoked by a number of stimuli, including pro-inflammatory cytokines. Activation of NF-κB further enhances the inflammatory response by inducing the transcription of wide spectrum of inflammatory mediators related to leukocyte recruitment and cytokine production [85]. Five NF-κB subunits have been well characterized and associated with its activity, including RelA (p65), RelB, p50, p52 and c-rel [175]. Under normal conditions, NF-κB is sequestered within the cytoplasm through the direct interaction with inhibitor proteins such as IκBα. Upon activation, the IKK complex phosphorylates IκBα. Further ubiquitination and degradation of IκBα releases the transcription activating subunits of NF-κB [175]. The classic pathway of NF-κB activation is triggered by IL-1 receptor (IL-1R), TNF receptor (TNFR) and pattern recognition receptors (PRRs), through downstream

activation of IKKβ, IκBα and release of p65-p50 [175]. In contrast, the non-canonical NF-κB pathway is activated by CD40 ligand, lymphotoxin-β and depends on activation of IKK and release of p52-RelB [175]. When released, p65-p50 and RelB-p52 heterodimers translocate into the nucleus, recruiting transcription co-activators such as cAMP-response element binding protein (CREB) binding protein (CBP)/p300 and histone acetyltransferase (HATs), and activate transcription of downstream molecules by binding to target DNA elements [175].

Site-specific phosphorylation of NF-κB p65 subunit leads to the selective transcription of downstream pro-inflammatory genes [176]. As the best characterized subunit of NF-κB, p65 and its phosphorylation is a pivotal point for canonical NF-κB activation. However, multiple phosphorylation sites have been mapped in both the N-terimal Rel homology domain and C-terminal transactivation domain of p65; Ser-205, Thr-254, Ser-276, Ser-281 and Ser-311 are located in N-terminal Rel homology domain, while Thr-435, Ser-468, Thr-505, Ser-529, Ser-535, Ser-536 and Ser-547 are found in C-terminal transactivation domain [176]. It has been shown that during inflammation, phosphorylation of Ser-276, Ser-281, Ser-311, Ser-468, Ser-529, Ser-536 and Thr-435 stimulate transcriptional activity, while Thr-254 is involved in stabilization and nuclear translocation [84]. Since the high glucose-induced influence on different NF-κB p65 phosphorylation sites is unknown, we first sought to characterize these sites in RECs and Müller cells under such

conditions.

Inflammation is a protective immunomodulatory response, during which pro-inflammatory mediators are counterbalanced by anti-inflammatory agents. During DR, pro-inflammatory cytokines, mainly interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) are produced by residential retinal cells, neutrophils and macrophages and shape the inflammatory response that contributes to disease pathogenesis [23, 33]. Both of these inflammatory mediators are canonical NF-κB pathway activators. In type 1 diabetes, IL-1β activates NF-κB and induces pancreatic beta cell dysfunction and death [86], while in type 2 diabetes NF-κB is constitutively activated by a low-grade, chronic state of inflammation [87]. Regarding DR, NF-κB has been shown to be activated early and remain activated for up to 14 months in experimental animal models and cultured retinal cells [23]. In contrast, interleukin-4 (IL-4) is associated with anti-inflammatory immune responses and influences further differentiation of T cells into Th2 cells. It has potent anti-inflammatory effects on other leukocytes such as polymorphonuclear leukocytes (PMN) and monocytes [177]. It has been reported that IL-4 inhibits insulitis and diabetic mellitus by stimulating a Th2 response [178]. In terms of DR, clinical studies have indicated significantly elevated levels of IL-4 in both vitreous and aqueous humor in patients, together with other pro-inflammatory cytokines [179, 180]. However, little is known on the anti-inflammatory effect of IL-4 in DR. In addition, whether IL-4 has any regulatory effect on NF-κB phosphorylation has yet to be found.

As such, we sought to investigate the effects of cytokine polarization in human primary retinal endothelial cells (HREC) and Müller cells exposed to high glucose in regulating NF-κB p65 site specific differential phosphorylation and downstream inflammatory mediators.

4.3 Methods

Cell Culture & Cytokine Time Course Treatment

Primary HRECs (Cell System Corporation; Kirkland, WA) were grown in HREC medium containing microvascular growth supplements (MVGS; Invitrogen, Carlsbad, CA), 10 mg/mL gentamycin, and 0.25 mg/mL amphotericin B. All cells were used within six passages. The MIO-M1 Müller cell line was obtained from the UCL Institute of Ophthalmology, London UK. MIO-M1 Müller cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 10 mg/mL gentamycin, and 0.25 mg/mL amphotericin B. Prior to experimentation, cells were transferred for four days to high (25 mM) or normal (5 mM) glucose medium (HREC medium or DMEM medium supplemented with glucose) with MVGS or FBS and antibiotics. Cells were then quiesced by removing MVGS or FBS for 24h. Cells were treated with pro-inflammatory cytokines IL-1β (10 ng/mL, R&D Systems, Minneapolis, MN) or TNF-α (10 ng/mL, R&D Systems, Minneapolis, MN) versus anti-inflammatory cytokine IL-4 (20 ng/mL, R&D Systems, Minneapolis, MN) for 10 min (MIO-M1 only), 30 min, 2 h, 24h, followed by rinsing with cold PBS. Since Müller cells are early responders in DR and

have similar characteristics with macrophages [181], an earlier time point of 10 minutes was added for the analysis of these cells. Cell collection was carried out as detailed below.

Western Blotting

Cells were collected in lysis buffer containing protease and phosphatase inhibitors for protein isolation. Cellular extracts were then prepared by sonication, and total protein concentration was determined for Western blot analyses. Proteins were separated on 4–20% tris-glycine gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. After blocking membranes in TBST (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) BSA at r.t. for 60 min, membranes were incubated overnight at 4°C with antigen-specific primary antibodies. The primary antibodies were used as follows: anti-NF-κB p65 (phospho Thr-254), anti-NF-κB p65 (phospho Ser-276), anti-NF-κB p65 (phospho Ser-281), anti-NF-κB p65 (phosphor Ser-311), anti-NF-κB p65 (phospho Ser-468), anti-NF-κB p65 (phospho Ser-529), anti-NF-κB p65 (phospho Thr-435), anti-IκBα (Abcam, San Francisco, CA); Anti-NF-κB p65 (phospho Ser-536), anti-vascular cell adhesion protein 1 (VCAM-1) (Cell Signaling Technology, Danvers, MA); anti-COX-2, anti-β-actin (Santa Cruz, Santa Cruz, CA). Blots were then incubated with species-specific HRP-conjugated secondary antibodies for 2h at r.t. Proteins were visualized by incubation with a chemiluminescence substrate kit (Thermo Fisher Scientific, Waltham, MA). Western blot images were collected (Azure

Biosystem C500, Dublin, CA) and target protein expression was quantified (Image Studio Lite software) after normalizing to β-actin. One representative blot is shown.

Real-time RT-PCR

RNA was extracted by RNA STAT-60 (Tel-Test, Friendswood, TX, USA) per the manufacturer's protocol and subjected to real-time RT-PCR analyses. Total RNA extracted for HREC and MIO-M1 was quantitated by spectrophotometric determination (260 nm). Total RNA (100 ng) was reverse transcribed and used to produce a cDNA template as previously described [182]. cDNA products were diluted 1:20 with DEPC-treated water, and 2 µL cDNA (10-µL total reaction volume) was used for semi-quantitative real-time RT-RT-PCR analysis (CFX Connect Real-Time RT-RT-PCR Detection System; BioRad, Hercules, CA, USA). All human primer pair sequences designed in the laboratory (PrimerQuest, Integrated DNA Technologies, Coralville, IA, USA) are listed in Table 1. RT-PCR amplification conditions were determined using routine methods [183]. Relative transcript levels were calculated using the relative standard curve method comparing the amount of target normalized to an endogenous reference, β-actin. Data are shown as the mean ± SD for relative transcript levels and represent at least two individual experiments.

ELISA

Intercellular adhesion molecule-1 (ICAM-1), interleukin-8 (IL-8), interleukin-10 (IL-10) (R&D Systems, Minneapolis, MN), IL-1β and TNF-α(Thermo Fisher

Table 1. Primer sequences.

Genes	Definition	Forward (5' to 3')	Reverse (5' to 3')
ICAM-1	Intercellular adhesion molecule 1	CTTCGTGTCCTGTATGGCCC	CACATTGGAGTCTGCTGGGA
VCAM-1	Vascular cell adhesion protein 1	GTCAATGTTGCCCCCAGAGATA	ACAGGATTTTCGGAGCAGGA
IL-1β	Interleukin 1 beta	AGAAGTACCTGAGCTCGCCA	CTGGAAGGAGCACTTCATCTGT
IL-6	Interleukin 6	TACAGGGAGAGGGAGCGATA	CTCAGACATCTCCAGTCCTCT
IL-8	Interleukin 8	AGAGCCAGGAAGAAACCACC	GGCAAAACTGCACCTTCACAC
IL-10	Interleukin 10	AAGACCCAGACATCAAGGCG	AATCGATGACAGCGCCGTAG
TNF-α	Tumor necrosis factor alpha	AGGCGCTCCCCAAGAAGACA	TCCTTGGCAAAACTGCACCT
TNFR	Tumor necrosis factor receptor	CCAGTGCGTTGGACAGAAGG	GAAGAATCTGAGCTCCCGGTG
CXCL-11	C-X-C motif chemokine 11	TTGTTCAAGGCTTCCCCATGT	CCACTTTCACTGCTTTTACCCC
CCL-23	C-C motif chemokine ligand 23	CTGGACATGCTCTGGAGGAGA	GGAGTGAACACGGGATGCTT
COX-2	Cyclooxygenase 2	GCTGTTCCCACCCATGTCAA	AAATTCCGGTGTTGAGCAGT
BAX	Bcl-2-associated X protein	CATGGGCTGGACATTGGACT	GGCAGCCCCCAACCAC
IFNγ	Interferon gamma	TGGAAAGAGGAGAGTGACAGA	ACACTCTTTTGGATGCTCTGGT
IL-17a	Interleukin 17A	CCTTGGAATCTCCACCGCAA	GTGGTAGTCCACGTTCCCAT
iNOS	Inducible nitric oxide synthase	GGACCCTGCAGACAGGC	TTCTTCACTGTGGGGCAAGG
cFLIP	Cellular FLICE-inhibitory protein	CAGCAGGTCTGAGCTTGTCC	AGTGGGGGAGTTGCCCG

Scientific, Waltham, MA) ELISAs were used to measure protein expression in HREC and Müller cells. Cells were collected and processed as described above. All samples were assayed in duplicate or triplicate per the manufacturer's instruction. Equal protein was loaded into all wells. The reported sensitivities of these assays are 0.254 ng/mL for ICAM-1, 7.5 pg/mL for IL-8, 3.9 pg/mL for IL-10, 1 pg/mL for IL-1β and 1.7 pg/mL for TNF-α.

Statistical analysis

All assays were carried out at least twice from three independent experiments and the data are presented as mean ± SD. Data were analyzed by the Analysis of variance (ANOVA) test following by Fisher's LSD test. $P < 0.05$ was considered to be statistically significant.

4.4 Results

IκBα levels are reduced in response to IL-1β/TNF-α, yet restored with IL-4 after HG exposure

Levels of IκBα, a regulatory protein that inhibits NF-κB, were assessed in HREC and Müller cells after high glucose exposure and cytokine treatment over time (Fig. 1). Regarding HREC, there was no difference in IκBα levels between normal and high glucose. However, in the presence of pro-inflammatory cytokines, IL-1β and TNF-α, IκBα was significantly downregulated early (30 minutes). These levels increased at 2h, but remained significantly reduced over NG and HG treatment groups at 24h. In contrast, IL-4 treatment maintained IκBα levels similar to controls.

In contrast to HREC, HG reduced IκBα levels in Müller cells. However, similar trends were observed after cytokine treatments, where pro-inflammatory IL-1β and TNF-α significantly reduced IκBα at early time points, which then appeared to peak at 2h and decrease at 24h (significant for TNF-α only). IL-4 treatment restored HG-induced downregulation of IκBα, which was significantly higher than HG at 2h and both NG and HG at 24h.

Differential phosphorylation of NF-κB p65 subunits when exposed to high glucose versus inflammatory cytokines in HREC and Müller cells

Next, we characterized the phosphorylation of NF-κB p65 subunits in response to high glucose, pro-inflammatory NF-κB activators (TNF-α and IL-1β) and anti-inflammatory IL-4 in HREC. As shown in Figure 22, high glucose upregulated phosphorylation at five out of the eight tested p65 sites, including Thr-254 (*A*), Ser-276 (*B*), Ser-468 (*E*), Ser-529 (*F*) and Thr-435 (*H*). No differences were observed for sites Ser-281 (*C*), Ser-311 (*D*) or Ser-536 (*G*) after exposure to high glucose only. When HREC were exposed to high glucose in the presence of either IL-1β or TNF-α, all sites tested showed significantly increased phosphorylation compared to normal glucose controls. Moreover, both of these cytokines further enhanced NF-κB activation over high glucose-induced effects at most of the time points tested. Additionally, IL-1β appears to be a more potent stimulator of Ser-311 compared to TNF-α at 30 min after treatment. On the contrary, anti-inflammatory cytokine IL-4 suppressed high glucose-induced phosphorylation of p65 subunits sites Thr-254, Ser-281, and

Thr-435. Although high glucose did not induce changes in Ser-281, Ser-311 or Ser-536, IL-4 treatment reduced activation of NF-κB at 30 minutes for Ser-281 and at 2 and 24h for Ser-311 below basal levels observed with normal glucose. No effect was observed with Ser-536. There was no indication that IL-4 treatment increased phosphorylation beyond the observed high glucose-induced effects for sites Ser-276, Ser-468 and Ser-529.

The human Müller cell line, MIO-M1, indicated similar trends as observed in HREC where not all sites resulted in increased phosphorylation with high glucose exposure. As shown in Figure 23, p65 subunit sites Thr-254 (*A*), Ser-281 (*C*), Ser-311 (*D*), Ser-468 (*E*) and Thr-435 (*H*) were activated due to high glucose alone; while no differences in phosphorylation were observed for Ser-276 (*B*), Ser-529 (*F*) and Ser-536 (*G*) compared to normal glucose controls. Although, both Ser-529 and Ser-536 sites did reveal significantly increased phosphorylation after treatment with IL-1β and TNF-α. Unlike HREC though, IL-1β and TNF-α treatment did not appear to have as strong of an effect on Müller cells; enhanced phosphorylation beyond high glucose-induced effects was limited to Ser-468, Thr-254 (IL-1β only at 24h) and Ser-311 (TNF-α only at 10 and 30 min). IL-4 treatment, however, significantly downregulated phosphorylation of NF-κB p65 at Thr-254, Ser-276, Ser-281, Ser-468 and Thr-435 compared to high glucose. In addition, although phosphorylation levels of Ser-276 did not change in response to high glucose or pro-inflammatory cytokine treatment, IL-4 downregulated phosphorylation beyond normal

glucose controls. Results of phosphorylation on different sites with high glucose versus cytokines treatment are summarized in Table 2.

Effects of high glucose and cytokine treatment on transcription of downstream genes associated with NF-κB activation in HREC and Müller cells

Transcription of selected genes known to be regulated by NF-κB as well as pathogenic in DR was assessed after high glucose exposure and cytokine treatment in both HREC and Müller cells. Analysis of gene expression in HREC (Fig. 24) indicated that high glucose upregulated transcript levels of interleukin-6 (IL-6) (*D*), IL-8 (*E*), IL-10 (*F*), TNF-α (*G*) and C-C motif chemokine ligand 23 (CCL23) (*I*). High glucose had no effect on the expression of ICAM-1 (*A*), VCAM-1 (*B*), IL-1β (*C*), C-X-C motif chemokine 11 (CXCL11) (*H*), cyclooxygenase-2 (COX-2) (*J*), or bcl-2 associated X protein (BAX) (*K*). When high glucose exposure was combined with IL-1β treatment, transcription levels of ICAM-1, VCAM-1, IL-1β, IL-6 (vs normal glucose only), IL-8, TNF-α, CXCL11, CCL23 and COX-2 were significantly upregulated over both normal glucose and high glucose alone. mRNA levels of IL-10 were lower than high glucose alone at 30 min post-treatment with IL-1β, while BAX did not change from basal expression levels. Treatment with TNF-α revealed similar trends compared to IL-1β and resulted in upregulation of ICAM-1, VCAM-1, IL-1β, IL-6, IL-8, TNF-α, CXCL11 and COX-2. Whereas, IL-10 expression was decreased compared to high glucose only at 30 min and no differences were detected regarding CCL23 or BAX compared to basal expression. Despite high glucose exposure,

Table 2. Summarization of NF-κB p65 phosphorylation sites.

NF-κB p65 phosphorylation sites	Main functions	HREC			Müller cells		
		HG	HG + IL-1β or TNF-α	HG + IL-4	HG	HG + IL-1β or TNF-α	HG + IL-4
Thr-254	p65 transactivation [184]	↑	↑↑↑	↓	↑↑↑	↑↑↑	↓↓
Ser-276	CBP recruitment, system inflammation [185, 186]; Translocation of p65 [187]	↑↑↑	↑↑↑	None	None	None	↓↓↓
Ser-281	Translocation of p65 [187]	None	↑↑	↓	↑↑↑	↑↑↑	↓↓↓
Ser-311	IL-6 transcription and CBP recruitment [188]	None	↑↑↑	↓	↑	↑↑↑	None
Ser-468	ICAM-1, VCAM-1 transcription [189]	↑↑↑	↑↑↑	None	↑	↑↑↑	↓
Ser-529	Translocation of p65 [190]	↑	↑↑↑	None	None	↑↑↑	None
Ser-536	ICAM-1, IL-8 transcription [191]; CBP recruitment [192]; Translocation of p65 [193]	None	↑↑↑	None	None	↑↑↑	↑
Thr-435	p65 transactivation [194]	↑↑↑	↑↑↑	↓	↑↑	↑↑↑	↓↓↓

↑/↓,↑↑/↓↓, ↑↑↑/↓↓↓ indicates P < 0.05, 0.01 and 0.0001, respectively, vs normal glucose/high glucose controls. None = indicates no changes were observed. Results were derived from the peak change observed among the different time points examined.

IL-4 treatment decreased mRNA levels for IL-6, IL-8 and TNF-α. No differences were observed in ICAM-1, VCAM-1, IL-1β or BAX when compared to normal glucose controls. In addition, IL-4 treatment upregulated IL-10 and CXCL11 at 24h, and COX-2 at 2h time points compared to high glucose only. High glucose-induced upregulation of CCL23 mRNA remained unchanged after IL-4 treatment.

In Müller cells, high glucose exposure resulted in upregulation of mRNA transcripts for a limited number of mediators (Fig. 25): TNF-α (*F*), interferon-gamma (IFNγ) and inducible nitric oxide synthase (iNOS) (*J*). Addition of pro-inflammatory IL-1β or TNF-α under high glucose conditions increased mRNA levels for IL-1β (*A*), IL-6 (*B*), IL-8 (*C*), interleukin-17A (IL-17A) (*E*), TNF-α (*F*), TNFR (TNF-α treatment only) (*G*), iNOS (*J*), COX-2 (*K*) and cellular FLICE inhibitory protein (cFLIP) (*L*). Similar to HREC, CXCL11 (*I*) was exclusively upregulated by TNF-α, not IL-1β, in Müller cells. IL-4 upregulated mRNA expression of IL-10 (*D*) and COX-2, yet decreased expression of TNF-α, IFNγ, CXCL11 and iNOS. No effect was observed regarding IL-6 or cFLIP expression after IL-4 treatment.

Protein analysis of NF-κB regulated genes in HREC and Müller cells after high glucose exposure and cytokine treatment

Based on mRNA expression, several genes were selected to further analyze protein levels after 26 h of high glucose exposure and pro-/anti-inflammatory cytokine treatments (Fig. 25). Consistent with mRNA results,

protein levels of ICAM-1 (*A*), VCAM-1 (*B*), IL-1β (*C*), IL-8 (*D*), TNF-α (*F*) and COX-2 (*G*) were similar in high and normal glucose. IL-10 (*E*), which was downregulated, was the only molecule that did not show corresponding mRNA expression and protein levels after high glucose exposure. When HREC were exposed to high glucose and pro-inflammatory cytokine IL-1β or TNF-α, protein levels were significantly elevated over both normal and high glucose for all molecules (except IL-10) – including ICAM-1, VCAM-1, IL-1β, IL-8, TNF-α and COX-2. As mentioned, IL-10 was significantly reduced in the presence of either pro-inflammatory cytokine compared to normal glucose controls. On the other hand, IL-4 treatment abrogated high glucose-induced changes in IL-8, TNF-α and upregulated IL-10. Further, IL-4 treatment had no effect on ICAM-1, VCAM-1 or IL-1β, which remained similar to basal levels observed in normal glucose. Similar to mRNA expression, COX-2 protein levels were significantly elevated with IL-4 treatment, but not as elevated after IL-1β or TNF-α treatments.

Protein levels were also examined at 24h in Müller cells exposed to high glucose and cytokine treatments, as shown in Figure 27 for IL-1β (*A*), IL-8 (*B*), IL-10 (*C*), TNF-α (*D*) and COX-2 (*E*). Exposure to high glucose had no effect on IL-1β and COX-2 when compared to basal levels observed in normal glucose. However, IL-8, TNF-α, and surprisingly IL-10 were significantly increased with high glucose. As expected, treatment with IL-1β and TNF-α resulted in significant increases of IL-1β, IL-8, TNF-α and COX-2 over both normal and high glucose. IL-10, on the other hand, was significantly decreased compared

to high glucose. These effects were consistently reversed following IL-4 treatment – reducing IL-8 and TNF-α to basal levels observed with normal glucose exposure. IL-10, however, was further enhanced after IL-4 treatment over high glucose.

4.5 Discussion

Phosphorylation is one of the prerequisite steps in NF-κB p65 activation. It has been found to be highly important for recruitment of transcription factors and subsequent binding of the p65 subunit with its target genes, as demonstrated by various NF-κB p65 site specific studies [185-193, 195-198]. Although it has been shown over a decade ago that high glucose activates NF-κB in both pericytes [199] and vascular smooth muscle cells [200], more recent reports have indicated that NF-κB activation in RECs is rather due to paracrine influences from other retinal cells such as Müller cells and pigment epithelial cells [174]. As a result, we hypothesized that high glucose induces differential phosphorylation of NF-κB in retinal cells. Specifically, we expected NF-κB p65 and downstream pathways associated with the pathogenesis of DR to be differentially induced in RECs and Müller cells after high glucose exposure. Beyond high glucose-induced activation of NF-κB, we also characterized the effects of two potent NF-κB activators, IL-1β and TNF-α, both of which are known to be produced early in the development of DR [115, 201, 202]. To this end, we found similar trends between HREC and Müller cells in the phosphorylation of 3 out of 8 sites of the p65 subunit – Thr-254, Thr-435 and

Ser-468. High glucose significantly increased the phosphorylation of these three sites compared to normal glucose. In addition, it appeared that high-glucose induced phosphorylation of Ser-276 and Ser-529 was specific to HREC, while phosphorylation of Ser-281 and Ser-311 was restricted to Müller cells. Ser-536, on the other hand, was not affected by high glucose exposure in either cell type. Moreover, pro-inflammatory cytokines were shown to be potent activators of all p65 subunit sites, indicating an important role for Ser-536 in particular regarding the canonical NF-κB pathway. Thus, we demonstrated that selected NF-κB p65 sites were differentially influenced by high glucose in HREC and Müller cells, and that the presence of pro-inflammatory cytokines IL-1β and TNF-α exhibited a more widespread, robust effect on p65 phosphorylation sites.

The influence of high glucose versus pro-inflammatory cytokines on NF-κB activity was further demonstrated by our studies of downstream target genes. The complex nature of DR pathogenesis includes a wide spectrum of mediators, such as advanced glycation end products (AGEs), ROS, protein kinase C (PKC) pathway, polyol pathway and inflammatory pathways [203]. NF-κB is an essential transcriptional regulator of numerous cytokines and is widely activated by the aforementioned pathways. ROS is produced by retinal and inflammatory cells during DR, which damages DNA and activates NF-κB [88]. AGEs, through RAGE receptor activation, interact with the PKC pathway and influences NF-κB activity [89, 90]. Upon activation by any of these pathogenic molecules related to DR, NF-κB is able to stimulate further transcription of various inflammatory

mediators that exacerbate the disease state in many aspects. This effect includes upregulation of pro-inflammatory cytokines/chemokines IL-1β, TNF-α, IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1) [91-95], iNOS [96]; COX-2 for the production of pro-inflammatory prostaglandins [97]; adhesion molecules ICAM-1 and VCAM-1 [98]; and apoptotic molecules, Fas and Fas ligand [99]. As such, based on an online NF-κB targeted gene list [204], NF-κB regulated genes known to contribute to the pathogenesis of DR were selected for further study. High glucose exposure led to the upregulation of pro-inflammatory molecules IL-8, IL-6, IFNγ, iNOS, and TNF-α, indicating that hyperglycemia is capable of stimulating an inflammatory response in these cell types. The fact that TNF-α was upregulated by high glucose alone in both cell types underlines the detrimental effects of diabetes. As a major contributor to the pathogenesis of DR, TNF-α mediates inflammation [205], regulates the breakdown of the blood-retinal barrier [135], and directs apoptosis of retinal residential cells [115]. As evidenced from pro-inflammatory cytokine treatments, once TNF-α (or IL-1β) is present in the microenvironment, the cascade of inflammatory events are exacerbated, which potentiates a state of chronic inflammation within the retina. In fact, TNF-α and IL-1β exhibited a much more robust effect on downstream inflammatory mediators compared to high glucose alone. These findings agree with the trends observed from our NF-κB p65 phosphorylation studies, further indicating the importance of pro-inflammatory cytokines in the early pathogenesis of DR, as high glucose appears to have a

markedly reduced effect, in comparison, on these same key inflammatory mediators.

Phosphorylation of Ser-311 by ζPKC has been associated with IL-6 transcription [188]. Both Ser-276 and Ser-311 phosphorylation has been found to promote interaction with CREB, enhancing the NF-κB response [188, 195]. Ser-536 phosphorylation defines separate pathways of NF-κB activation of canonical and non-canonical patterns, activating transcription of IL-8 and ICAM-1, both of which are important for leukostasis activation [191]. Mutation of Ser-536 prevents CBP recruitment [192]. Phosphorylation of Ser-529 and Ser-536 has been shown to be associated with translocation of NF-κB subunits [190, 193]. In the current study, we showed that Ser-311 and Ser-536 were not responsive to high glucose exposure in HREC. Similarly, Ser-529 and Ser-536 remained unchanged in Müller cells. Yet these three sites were significantly activated by inflammatory cytokines. It is possible that phosphorylation of these sites are necessary for transcription of wide spectrum of NF-κB target genes. Therefore, inhibiting phosphorylation on Ser-536, Ser-311 and Ser-529 could potentially abrogate, in part, the translocation of NF-κB p65 into the nucleus and influence the transactivation of p65, ultimately leading to a reduction in transcriptional activity of target genes, as well.

Regulation of NF-κB is limited to the level of phosphorylation. IκBα inhibits NF-κB by sequestering p65 in the cytoplasm. When phosphorylated, ubiquitinated and then degradated, IκBα releases NF-κB p65 so that it can

dimerize with p50, then translocate into the nucleus to trigger activation of the downstream canonical NF-κB pathway [206]. Beyond the traditional view of IκBα, recent reports have pointed out the existence of free IκBα in the cytoplasm as an intrinsic unstable molecule, which is important for activation of NF-κB itself and independent of IKK phosphorylation and ubiquitination [207]. This adds to the complexity of the NF-κB pathway and promotes the possibility of IκBα involvement in, not only the alternative NF-κB pathway, but also in an IKK kinase and p65-p50-independent manner. Degradation of IκBα releases NF-κB p65, which is a prerequisite for translocation of p65-p50. Although we showed that pro-inflammatory cytokines significantly upregulate IκBα degradation in both cell types, high glucose alone resulted in decreased IκBα levels in Müller cells. These results suggest that Müller cells may be a more active responder to high glucose conditions and perhaps have a larger role as a “decision maker” in regards to the resultant inflammatory response observed during DR.

It was observed that IL-4 suppressed phosphorylation at Thr-254, Ser-276, Ser-281 and Thr-435 in HREC and at Thr-254, Ser-276, Ser-281, Ser-468 and Thr-435 in Müller cells. Although it has been reported that IL-4 induces NF-κB p52 production by a PI3K and IκB kinase dependent pathway [208], to our knowledge, there is no established evidence regarding IL-4’s effect on regulating phosphorylation of NF-κB p65. The fact that IL-4 suppressed the phosphorylation of half of p65 sites in HREC and 5 out of 8 sites in Müller cells demonstrates its protective effect by directly suppressing NF-κB p65 activity in

high glucose. mRNA and protein results indicated that IL-4 effectively reduced IL-6, IL-8 and TNF-α in HREC and TNF-α, IFNγ, CXCL-11 and iNOS in Müller cells. Despite that this was somewhat expected since IL-4 significantly suppressed multiple NF-κB p65 phosphorylation sites, IL-4 treatment also upregulated IL-10 and COX-2. Whether this is a direct consequence of the inhibitory effect of IL-4 on p65 phosphorylation needs to be further tested, but it is thought that the regulatory effect of this anti-inflammatory cytokine on NF-κB works synergistically with its traditional PI3K and mitogenic signaling [209]. In Müller cells, IL-4 suppressed IL-8 protein levels, indicating that a translational effect as this was not reflected at the mRNA level. Previous results have indicated that IL-4 enhances IL-10 gene expression in the absence of TCR engagement [210]. In both types of cells, we demonstrated that IL-4 is capable of enhancing IL-10, possibly promoting an anti-inflammatory response in this situation. Since IL-10 has the ability to inhibit the expression of IL-8 [211] and regulates TNF-α signaling [212], it is possible that suppression of IL-8 and TNF-α by IL-4 is partially carried out by regulation of IL-10 through an autocrine manner in both cell types. This could be greatly beneficial as TNF-α is one of the major pro-inflammatory mediators during the pathogenesis of DR. This is also very comparable to our previous research indicating an anti-inflammatory effect of vasoactive intestinal peptide in suppressing intracellular TNF-α levels [213]. It is also important to note that IL-4 enhanced IκBα levels in Müller cells, which could also contribute to inhibition of NF-κB p65 phosphorylation and

subsequent expression of NF-κB target genes. In addition, the fact that IL-4 promoted expression of COX-2 in both cell types could be associated with COX-2 derived prostanoids, which are important at low levels in the development of a “healthy” immune response.

It is important to note that cytokine induced phosphorylation of NF-κB p65 was not always sustained, but instead fluctuated over time; some changes occurred earlier, while others happened later. For example, the suppressive effect of IL-4 regarding phosphorylation of NF-κB p65 in HREC were mostly observed at later time points (2 and 24 hours), whereas in Müller cells, some changes were observed early and lasted through 24 hours. Again, this could be partially derived from IL-4 induced upregulation of IL-10, which has been demonstrated to suppress NF-κB activity through regulation of p50 [214]. Upregulation of IκBα in Müller cells by IL-4 could possibly inhibit phosphorylation of p65, as well. In contrast, as expected, traditional canonical NF-κB pathway activators IL-1β and TNF-α evoked phosphorylation activity mostly at earlier time points.

The current study is the first one demonstrating the suppressive effect of IL-4 on the phosphorylation of NF-κB p65. Whether IL-4 is capable of inhibiting IL-1β and TNF-α evoked inflammatory response in high glucose cultured retinal residential cells warrants further investigation. Although the detailed regulatory chains between different NF-κB p65 sites and downstream genes and protein expression of related inflammatory mediators needs further study, the fact that

IL-4 not only suppressed NF-κB target inflammatory genes, but also inhibited phosphorylation of NF-κB at different sites in different cells provides us another avenue for treatment against the pathogenesis of DR. In addition, since NF-κB p65 phosphorylation is one of the most important events during high glucose induced pathogenic changes in the retina, in this study, it is important to understand how NF-κB p65 phosphorylation is promoted with high glucose versus cytokine exposure. Again, pro-inflammatory cytokines significantly exacerbated high glucose induced NF-κB activity, demonstrating their larger role in driving NF-κB phosphorylation and expression of target genes compared to high glucose conditions alone.

4.6 Conclusions

In summary, high glucose-induced site specific differential phosphorylation of NF-κB p65 in HREC and Müller cells were characterized and demonstrated the importance of pro-inflammatory cytokines in exacerbating the response. We also demonstrated that IL-4 effectively inhibited high glucose-induced NF-κB phosphorylation at multiple p65 sites in HREC and Müller cells. The current study is the first to characterize high glucose-induced NF-κB p65 phosphorylation and reveal IL-4's regulatory effect on this activity. As such, it underscores the importance of recognizing the differential influence of hyperglycemic conditions between multiple retinal cell types and that the observed effects go beyond a single phosphorylation site on NF-κB p65 subunit. These findings are important in understanding the pathologic events associated

with DR. NF-κB activation is a major event associated with high glucose-induced changes in the retina; however, we can conclude from this study that understanding how NF-κB is activated in each cell type is important since cell types and phosphorylation sites are differentially responsive to both high glucose and inflammatory mediators.

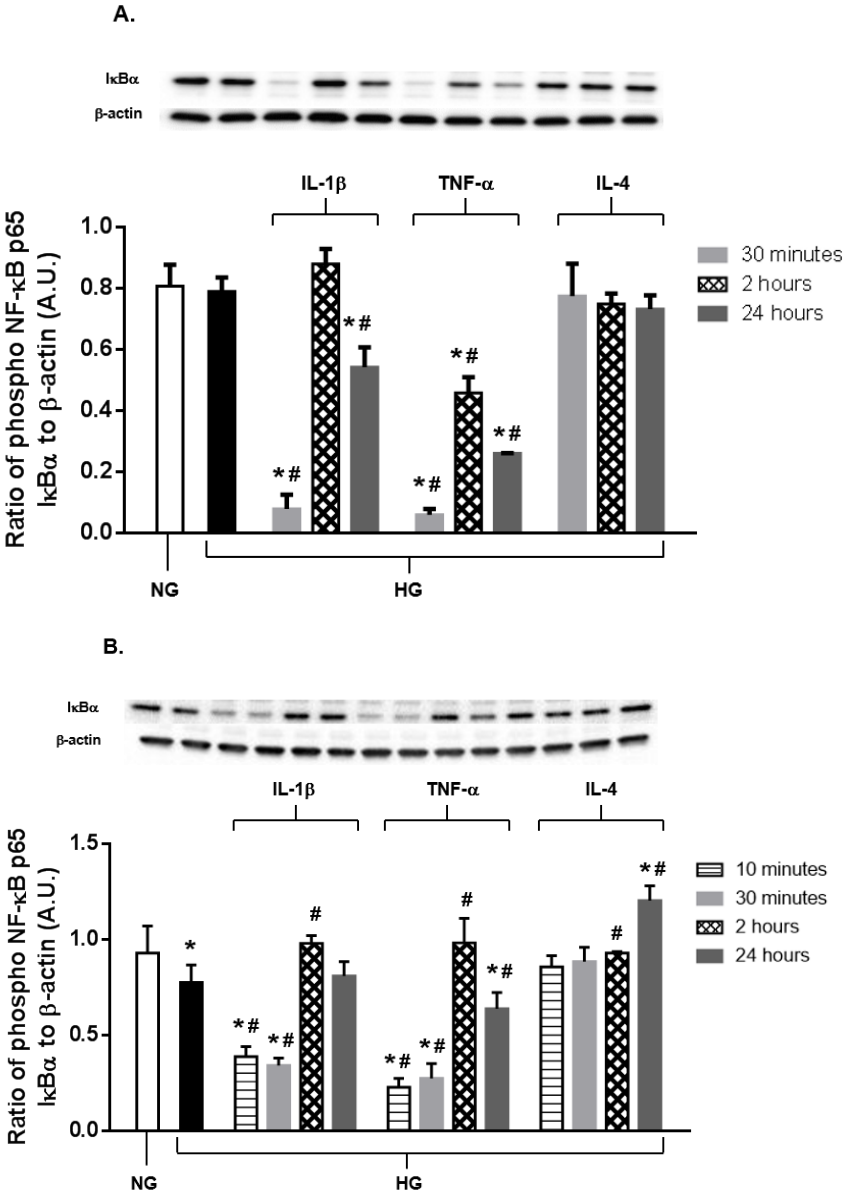

Figure 21. Degradation of IκBα in HREC vs Müller cells when cultured in high glucose with cytokine treatments. HREC and Müller cells were cultured under normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions followed by TNF-α, IL-1β versus IL-4 treatment for 10 minutes (Müller cells only), 30 minutes, 2 hours and 24 hours. Protein levels of IκBα in HREC (A) and IκBα in Müller cells (B) were detected by Western blot. Data shown are representative of 5 independent experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

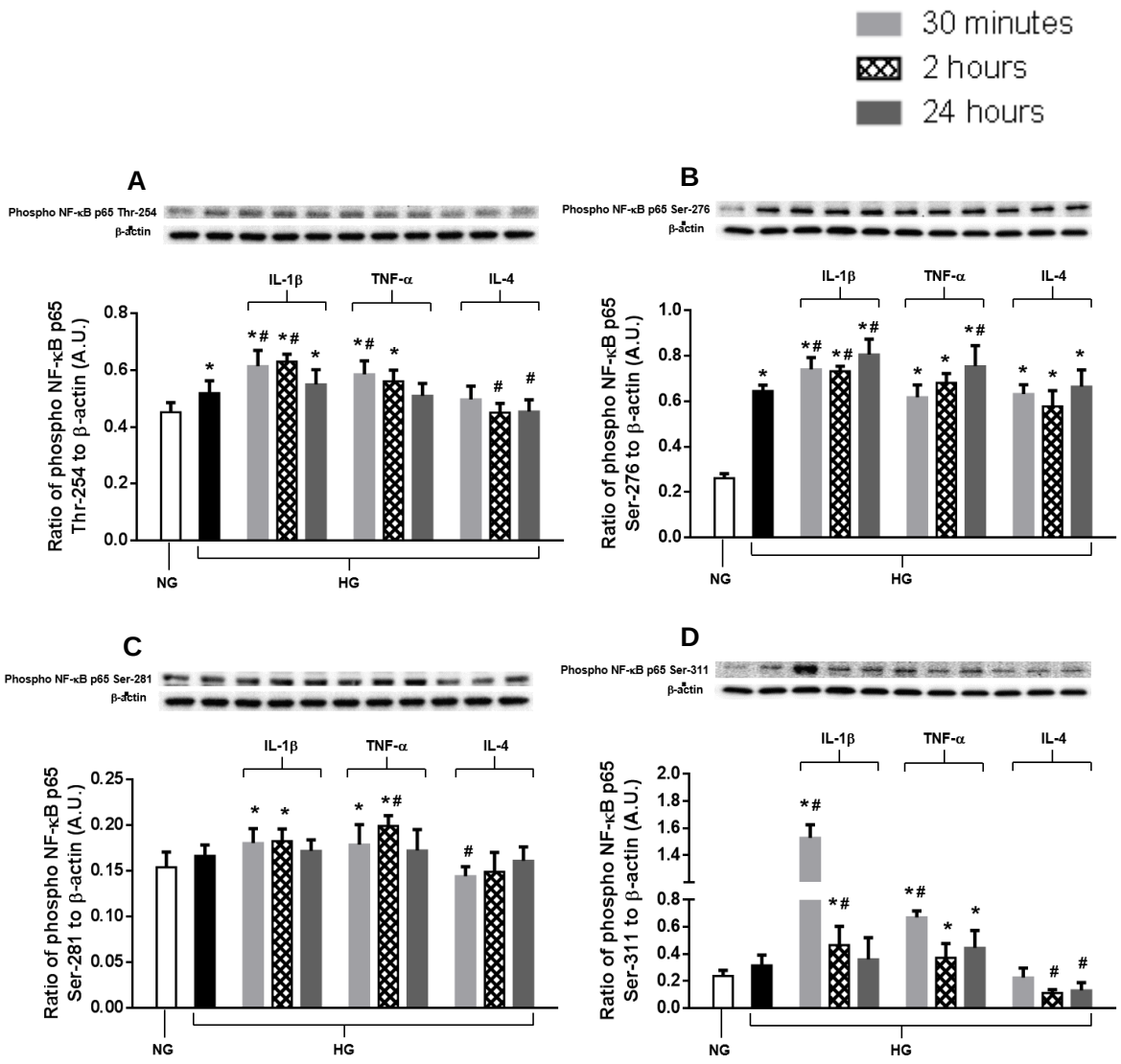

Figure 22-1. Differential site-specific phosphorylation of NF-κB p65 after high glucose exposure and pro- versus anti-inflammatory cytokine stimulation in HREC. HREC were cultured under normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions followed by TNF-α, IL-1β versus IL-4 treatment for 30 minutes, 2 hours and 24 hours. Protein levels of phosphorylated p65 Thr-254 (A), phosphorylated p65 Ser-276 (B), phosphorylated p65 Ser-281 (C) and phosphorylated p65 Ser-311 (D) were detected by Western blot. Data shown are representative of 5 independent experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

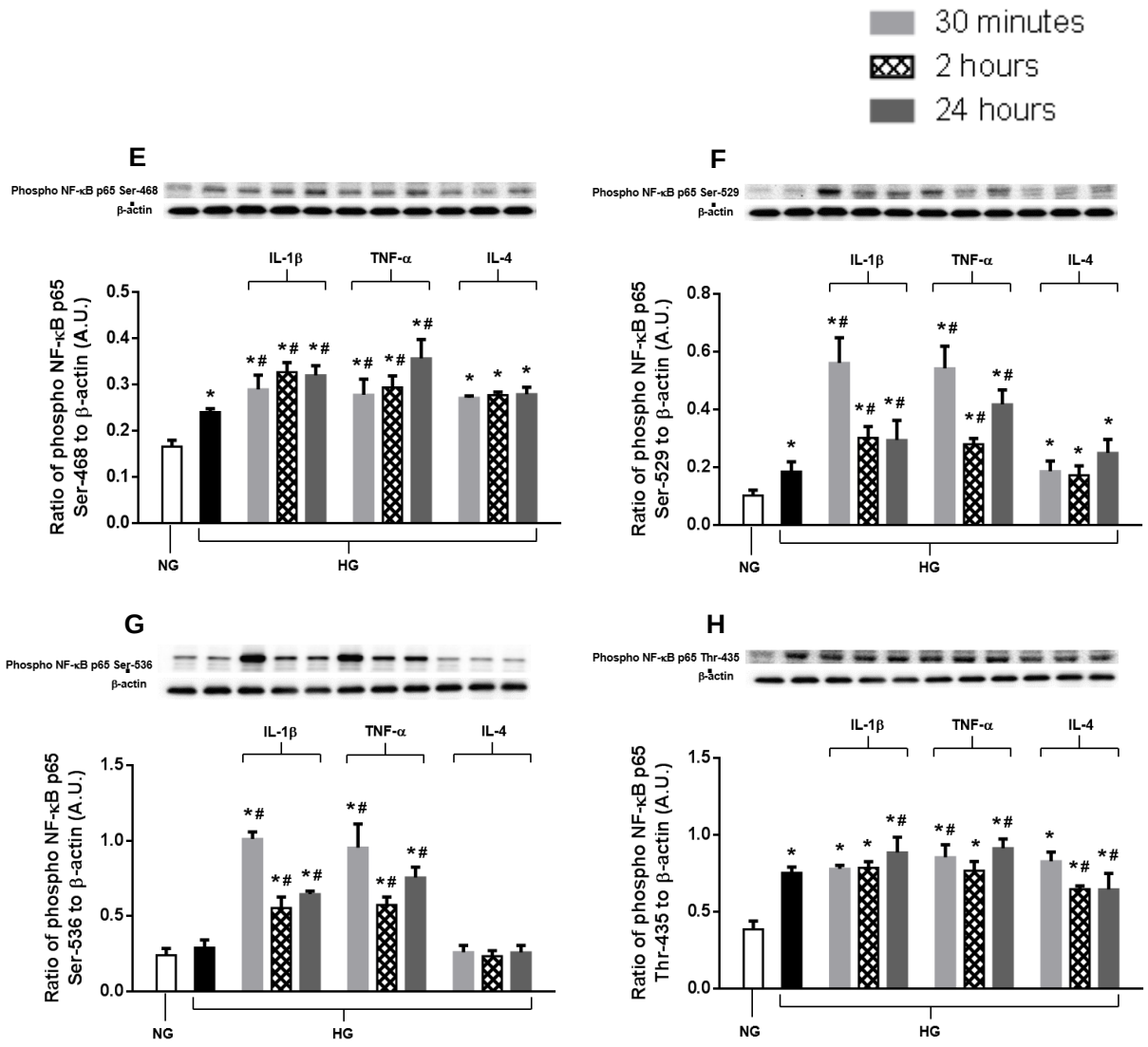

Figure 22-2. Differential site-specific phosphorylation of NF-κB p65 after high glucose exposure and pro- versus anti-inflammatory cytokine stimulation in HREC. HREC were cultured under normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions followed by TNF-α, IL-1β versus IL-4 treatment for 30 minutes, 2 hours and 24 hours. Protein levels of phosphorylated p65 phosphorylated p65 Ser-468 (E) and phosphorylated p65 Ser-529 (F), phosphorylated p65 Ser-536 (G) and phosphorylated p65 Thr-435 (H) were detected by Western blot. Data shown are representative of 5 independent experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

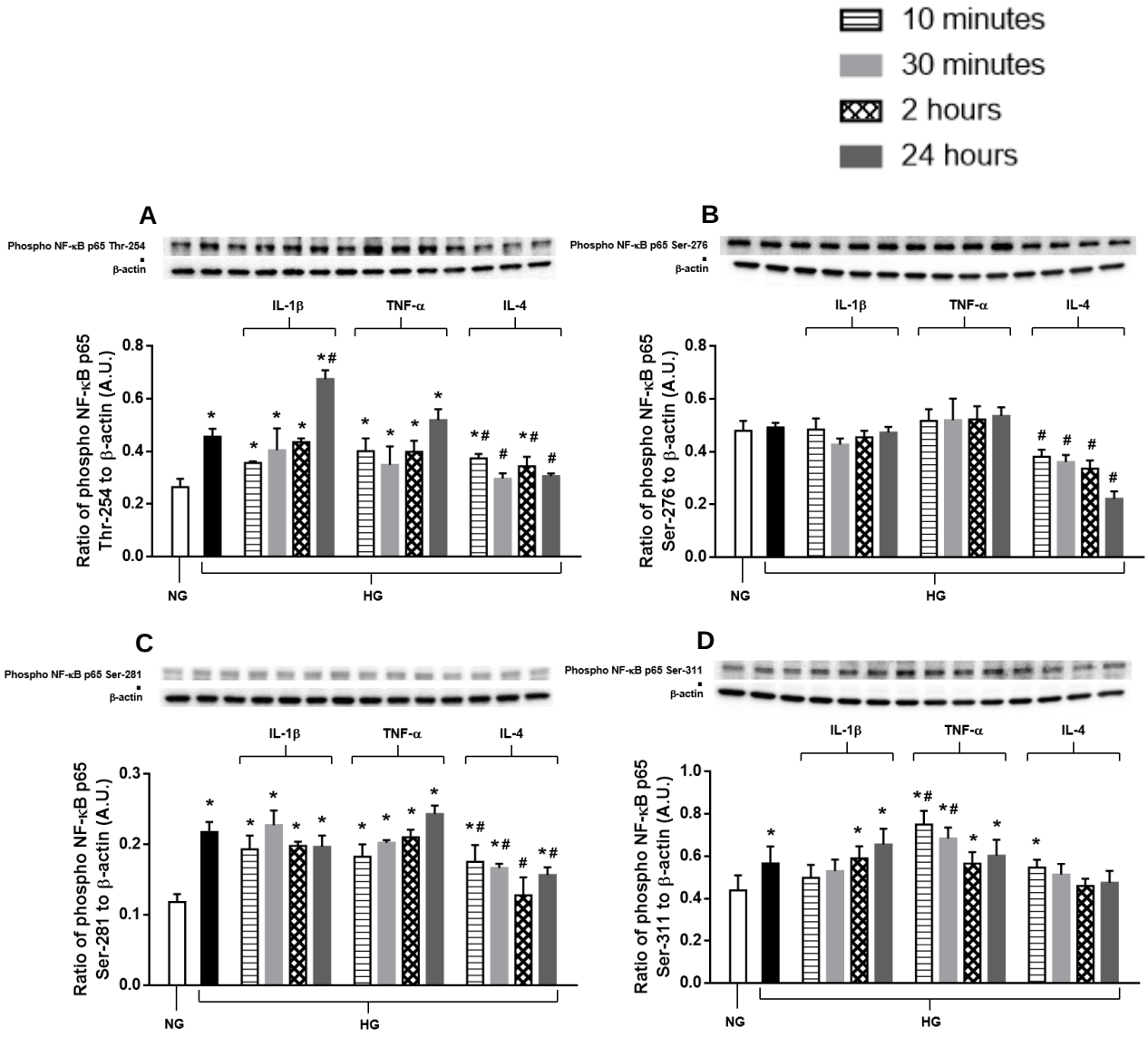

Figure 23-1. Site-specific differential phosphorylation of NF-κB p65 in Müller cells after high glucose exposure and pro- versus anti-inflammatory cytokine stimulation. Müller cells were cultured under normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions followed by TNF-α, IL-1β versus IL-4 treatment for 10 minutes, 30 minutes, 2 hours and 24 hours. Protein levels of phosphorylated p65 Thr-254 (A), phosphorylated p65 Ser-276 (B), phosphorylated p65 Ser-281 (C) and phosphorylated p65 Ser-311 (D) were detected by Western blot. Data shown are representative of 5 independent experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

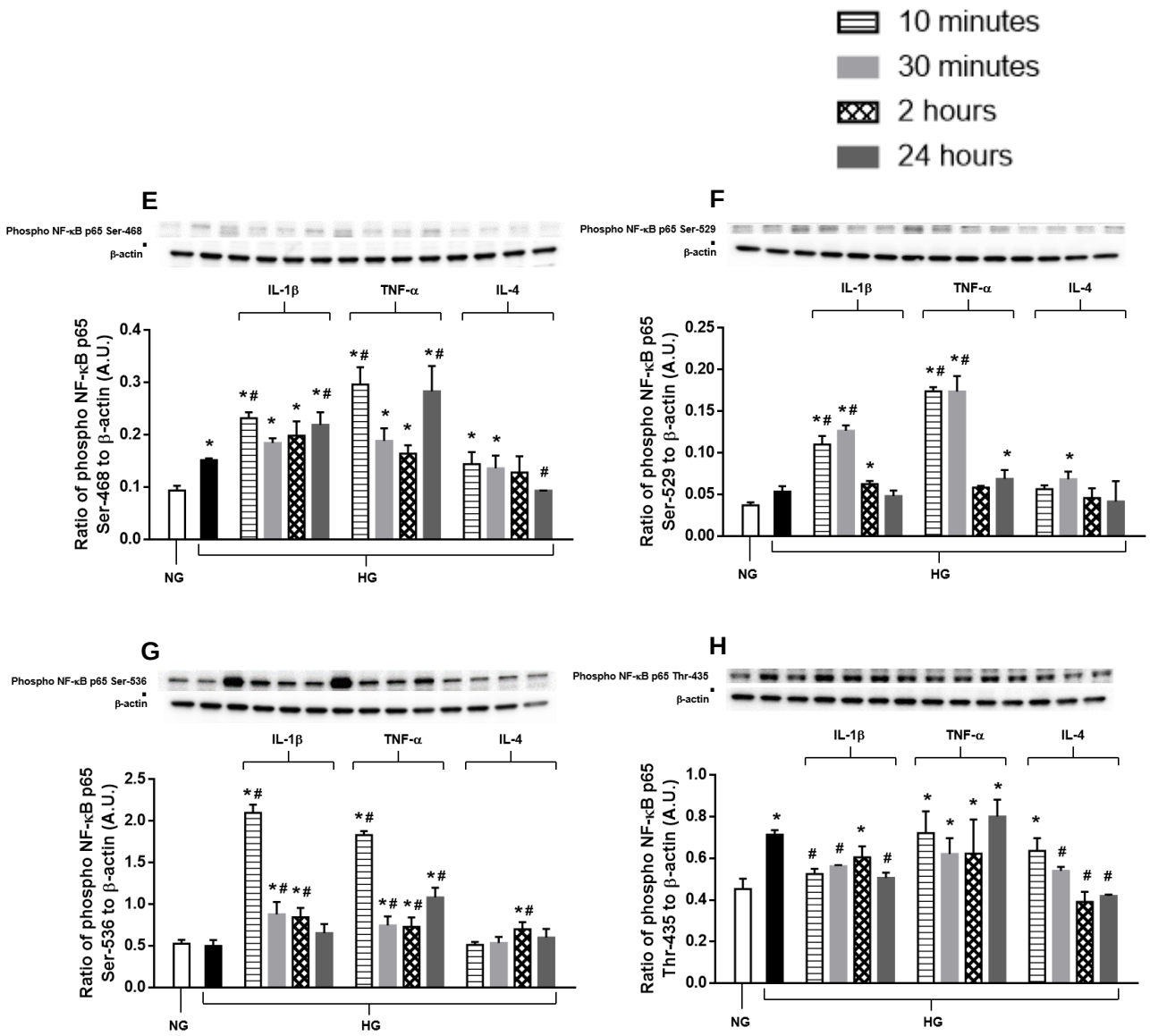

Figure 23-2. Site-specific differential phosphorylation of NF-κB p65 in Müller cells after high glucose exposure and pro- versus anti-inflammatory cytokine stimulation. Müller cells were cultured under normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions followed by TNF-α, IL-1β versus IL-4 treatment for 10 minutes, 30 minutes, 2 hours and 24 hours. Protein levels of phosphorylated p65 Ser-468 (E), phosphorylated p65 Ser-529 (F), phosphorylated p65 Ser-536 (G) and phosphorylated p65 Thr-435 (H) were detected by Western blot. Data shown are representative of 5 independent experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

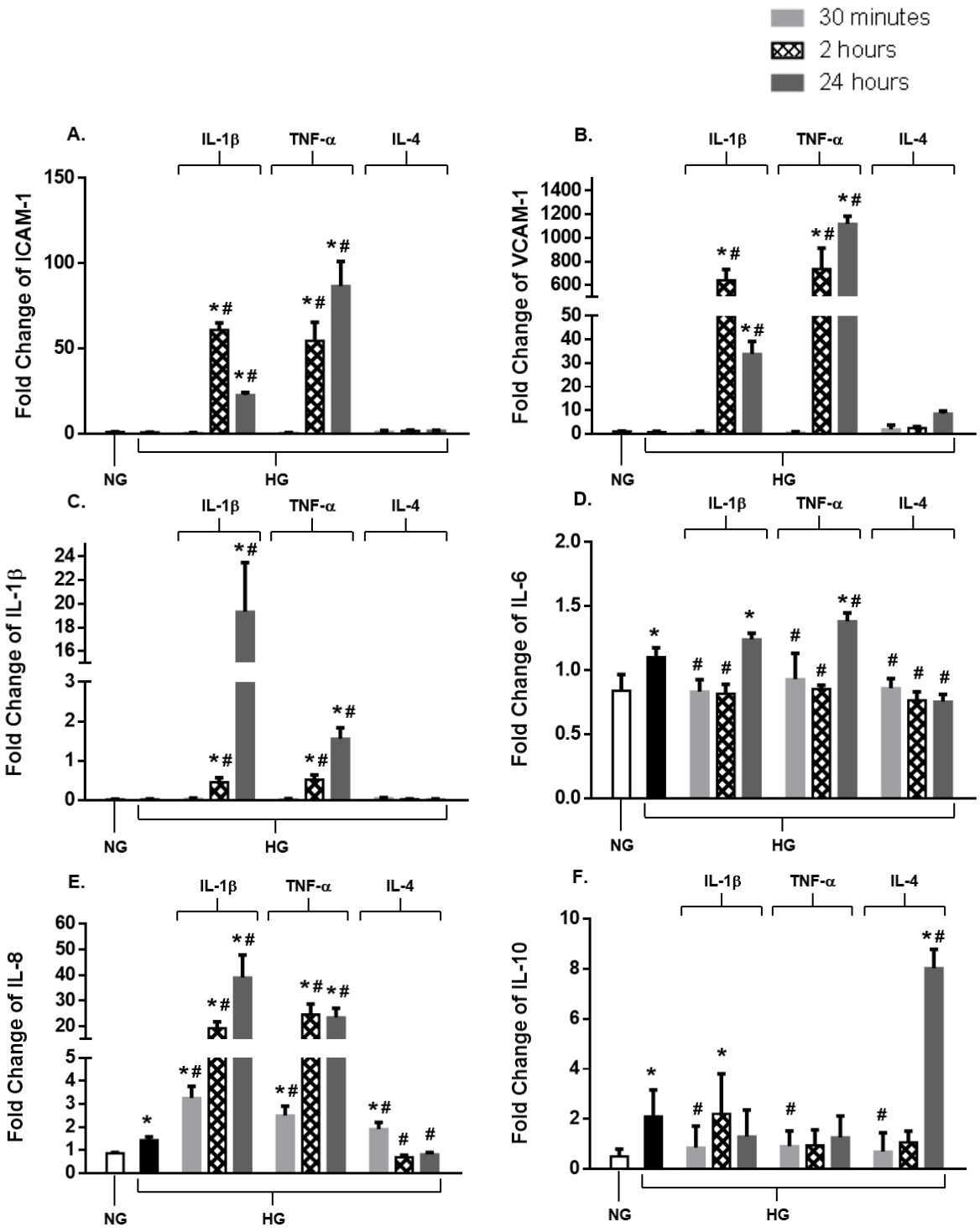

Figure 24-1. mRNA expression levels of NF-κB regulated pathogenic genes after high glucose exposure and pro- versus anti-inflammatory cytokine treatment in HREC. HREC were cultured under normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions followed by TNF-α, IL-1β versus IL-4 treatment over time. mRNA levels of ICAM-1 (A), VCAM-1 (B), IL-1β (C), IL-6 (D), IL-8 (E) and IL-10 (F) were detected by real-time RT-PCR. Data shown are representative of 5 independent experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

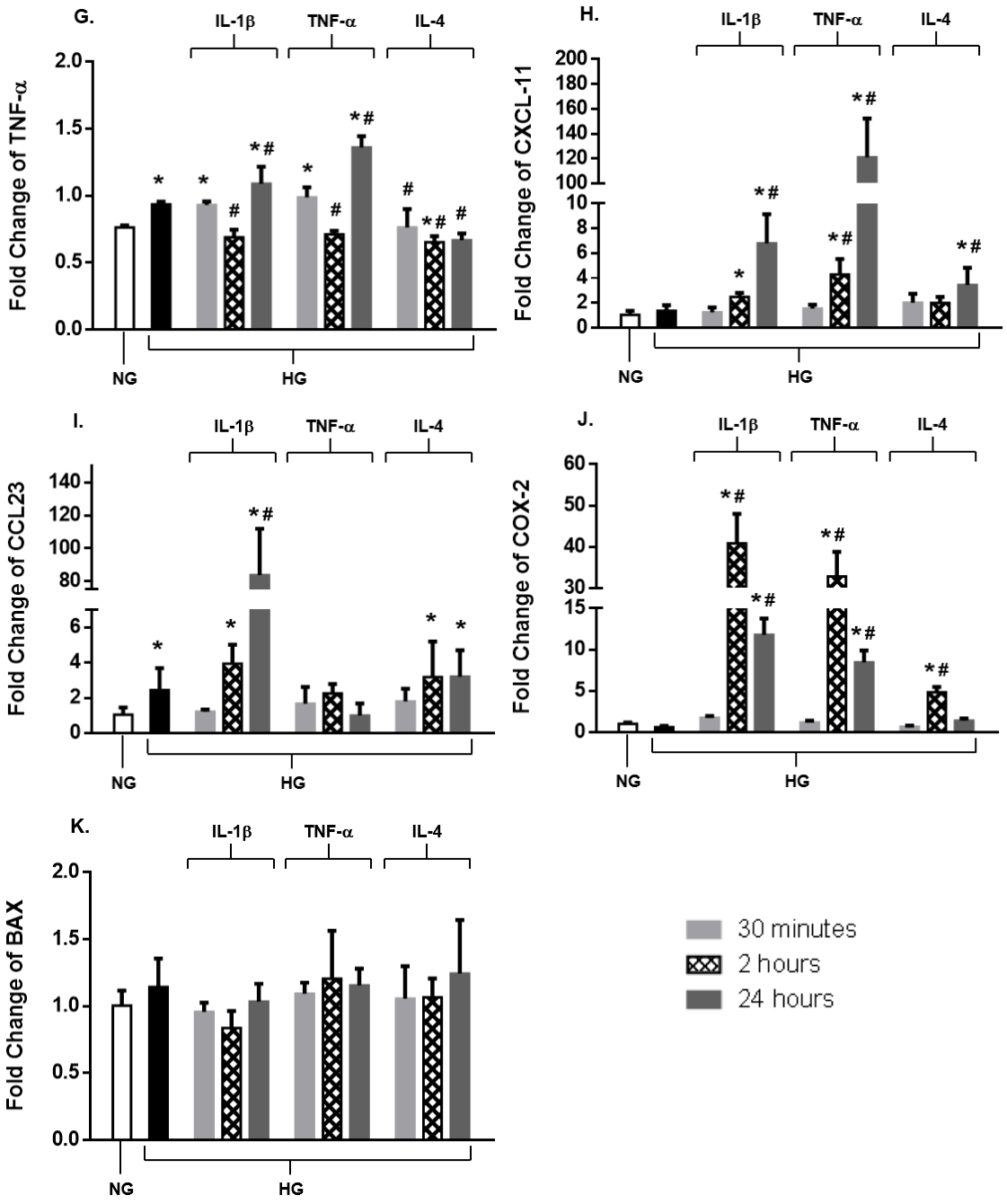

Figure 24-2. mRNA expression levels of NF-κB regulated pathogenic genes after high glucose exposure and pro- versus anti-inflammatory cytokine treatment in HREC. HREC were cultured under normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions followed by TNF-α, IL-1β versus IL-4 treatment over time. mRNA levels of TNF-α (G), CXCL-11 (H), CCL-23 (I), COX-2 (J) and BAX (K) were detected by real-time RT-PCR. Data shown are representative of 5 independent experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

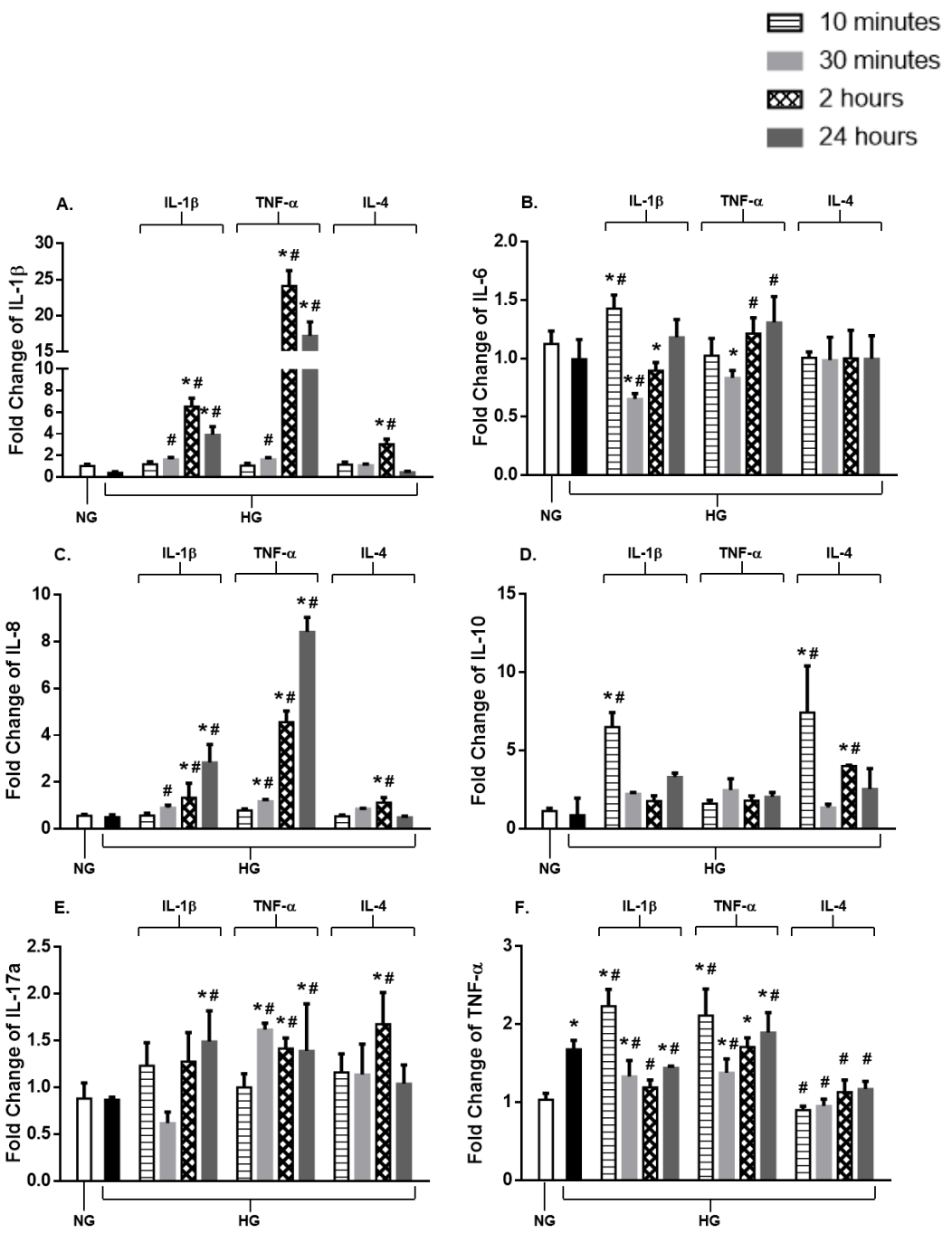

Figure 25-1. mRNA expression of NF-κB regulated pathogenic genes under high glucose conditions and cytokine polarization in Müller cells. Müller cells were cultured under normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions followed by TNF-α, IL-1β versus IL-4 treatment over time. mRNA levels of IL-1β (A), IL-6 (B), IL-8 (C), IL-10 (D), IL-17a (E) and TNF-α (F) were detected by real-time RT-PCR. Data shown are representative of 5 independent experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

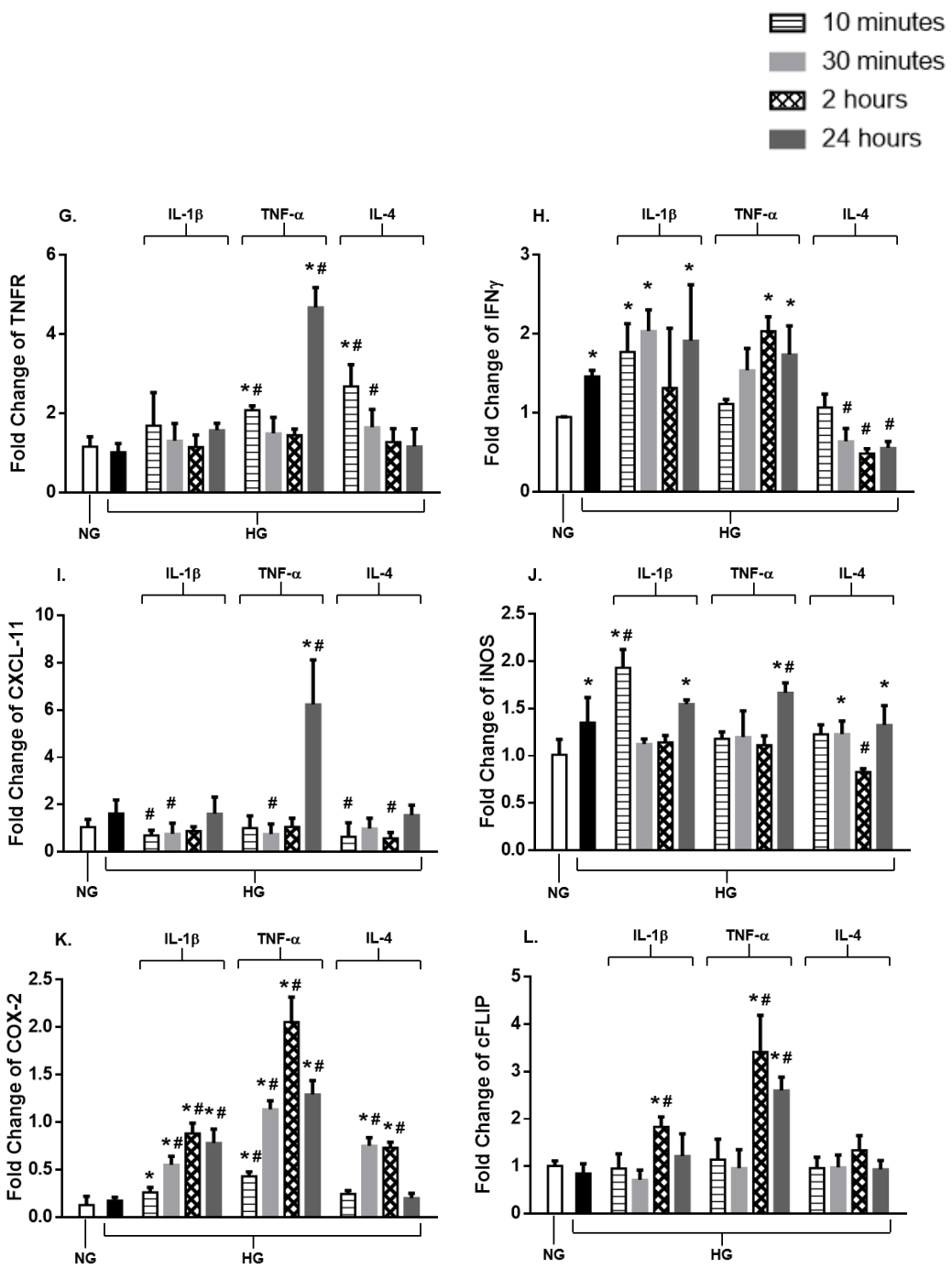

Figure 25-2. mRNA expression of NF-κB regulated pathogenic genes under high glucose conditions and cytokine polarization in Müller cells. Müller cells were cultured under normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions followed by TNF-α, IL-1β versus IL-4 treatment over time. mRNA levels of TNFR (G), IFNγ (H), CXCL-11 (I), iNOS (J), COX-2 (K) and cFLIP (L) were detected by real-time RT-PCR. Data shown are representative of 5 independent experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

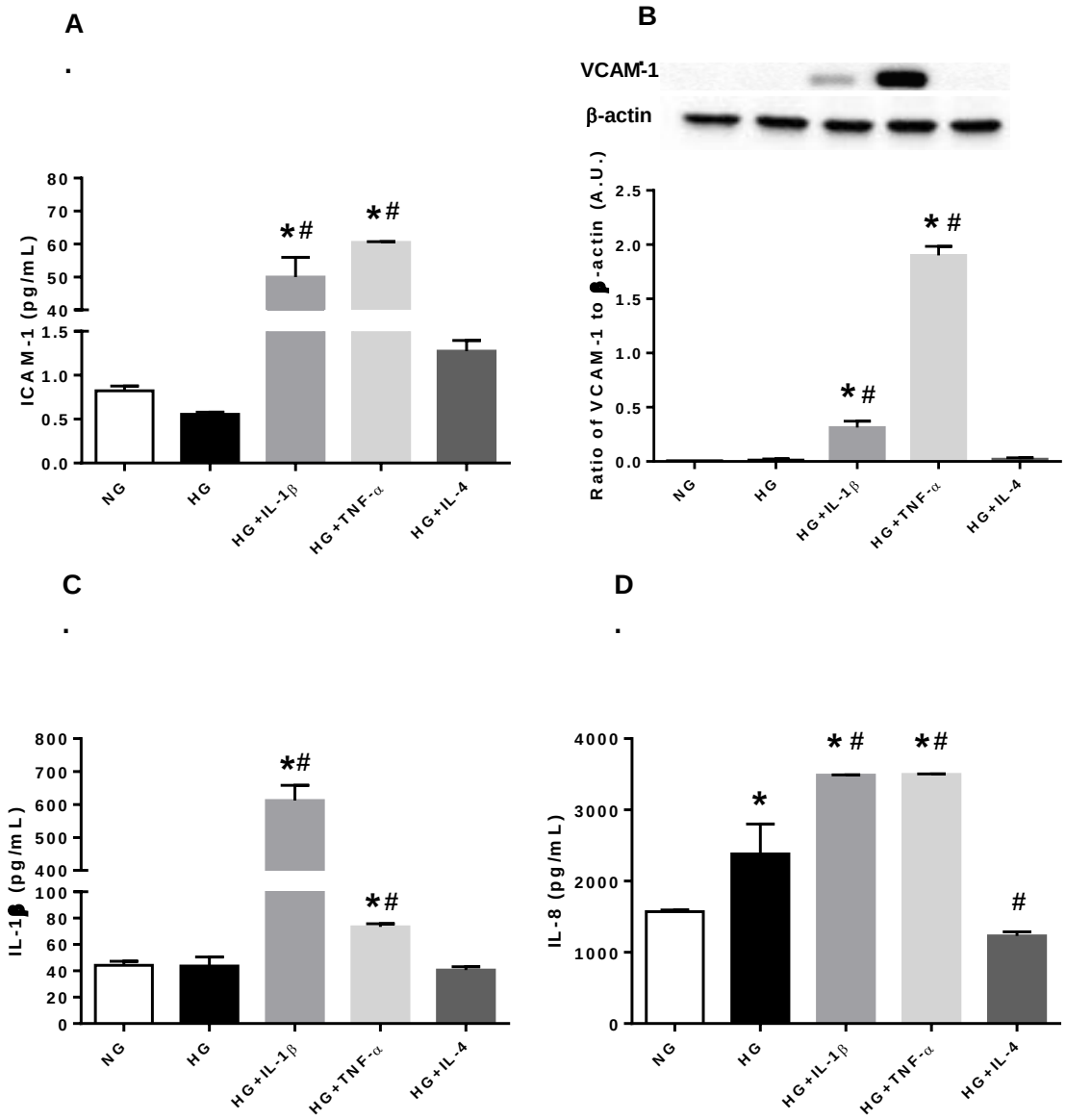

Figure 26-1. Protein levels of select NF-κB regulated inflammatory mediators in HREC after high glucose exposure and pro- versus anti-inflammatory cytokine polarization. HREC were cultured under normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions followed by TNF-α, IL-1β versus IL-4 treatment for 24 hours. Protein levels of ICAM-1 (A), VCAM-1 (B), IL-1β (C), IL-8 (D), IL-10 (E), TNF-α (F) and COX-2 (G) were detected by ELISA or Western-blot. Data shown are representative of 5 independent experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG..

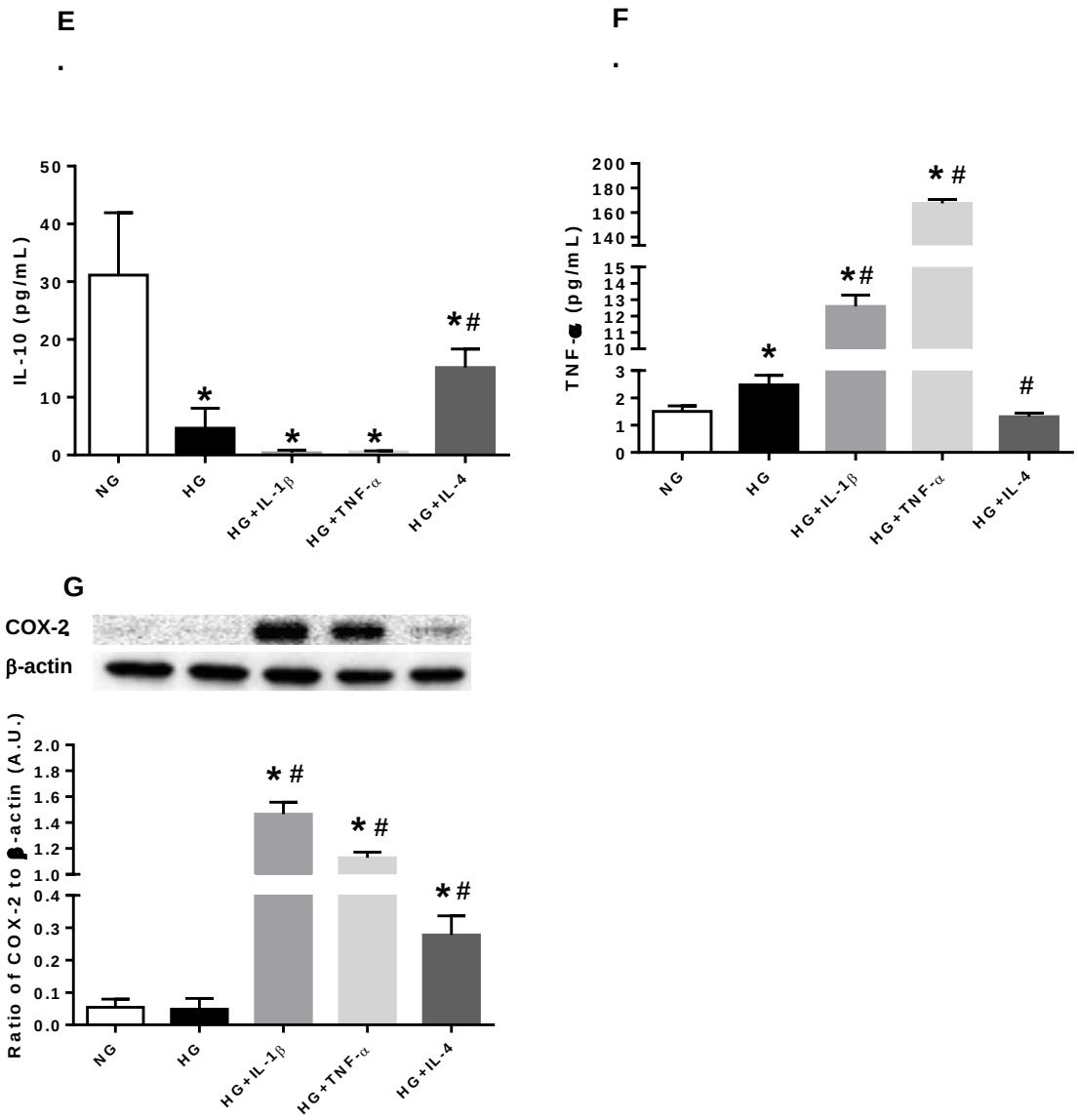

Figure 26-2. Protein levels of select NF-κB regulated inflammatory mediators in HREC after high glucose exposure and pro- versus anti-inflammatory cytokine polarization. HREC were cultured under normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions followed by TNF-α, IL-1β versus IL-4 treatment for 24 hours. Protein levels of IL-10 (E), TNF-α (F) and COX-2 (G) were detected by ELISA or Western-blot. Data shown are representative of 5 independent experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG..

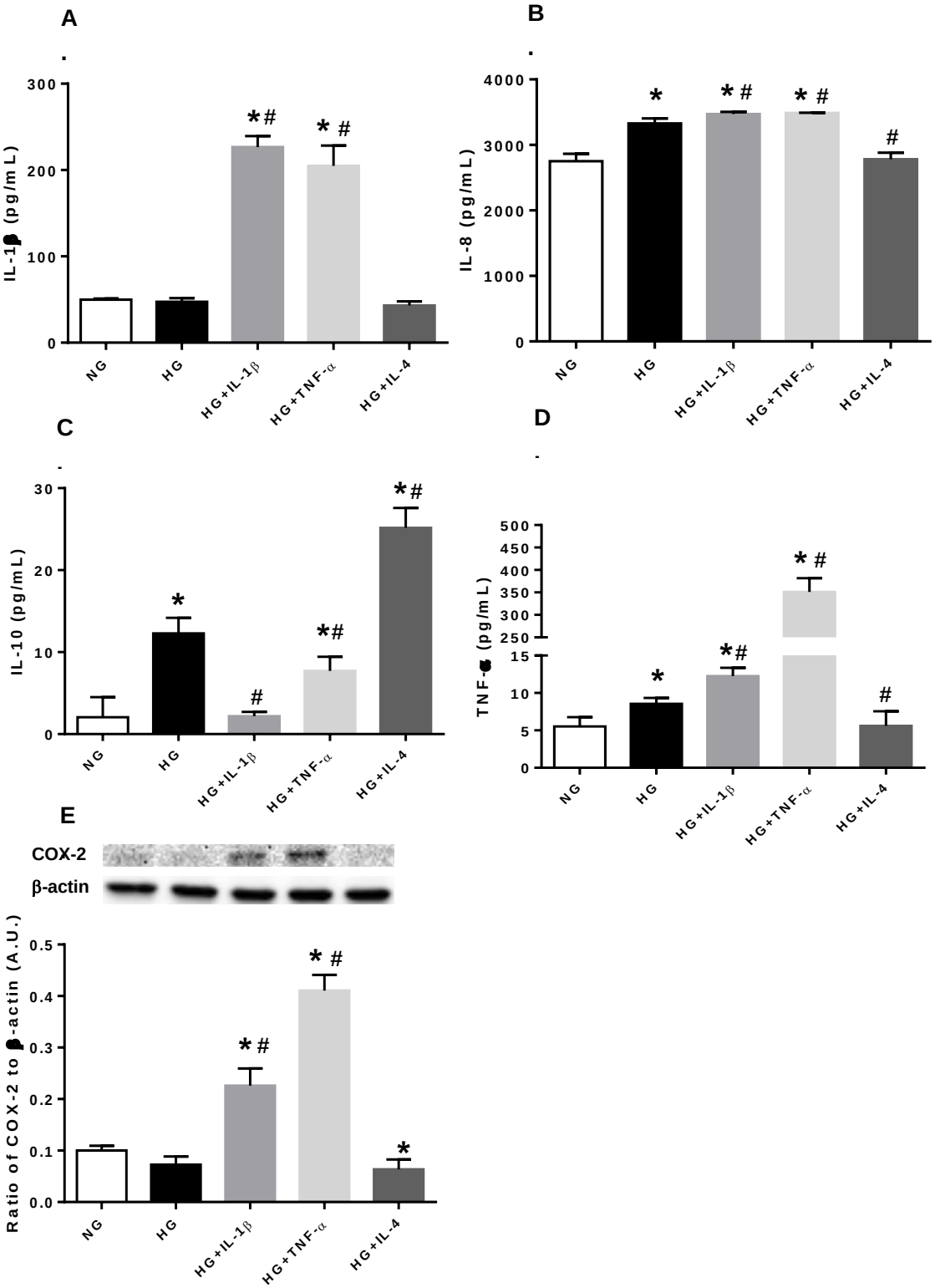

Figure 27. Protein levels of select NF-κB regulated inflammatory mediators in Müller cells exposed to high glucose with cytokine treatment. Müller cells were cultured under normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions followed by TNF-α, IL-1β versus IL-4 treatment for 24 hours. Protein levels of IL-1β (A), IL-8 (B), IL-10 (C), TNF-α (D) and COX-2 (E) were detected by ELISA or Western-blot. Data shown are representative of 5 independent experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

CHAPTER 5 “RETINAL ISCHEMIA-REPERFUSION INJURY MODEL: A CONTRAST IN PATHOGENIC RESPONSES BETWEEN C57BL/6J VERSUS BALB/cJ MICE”

5.1 Abstract

Retinal ischemia has been shown to contribute to the pathogenesis observed during diabetic retinopathy (DR). As a result, the retinal ischemia-reperfusion (I/R) injury model has been useful in studying neuronal and vascular damage to the retina – damage that is similarly observed during the development of DR. Notably, murine models of both I/R and DR tend to be carried out in C57BL/6J (B6) mice, which have been classified as type 1-dominant responders. In bacterial keratitis models, previous study has established that B6 mice are susceptible, while BALB/c mice (classified as type 2-dominant) exhibit a resistant phenotype [215]. Although the cornea and retina are quite different, we questioned whether the type 1/type 2 inflammation paradigm could be extrapolated to events associated with the pathogenesis of I/R and related DR. I/R injury was induced in both B6 and BALB/c mice by cannulating the anterior chamber of one eye of anesthetized animals. The other eye served as control. Retinas were collected after 2 days and stained by hematoxylin and eosin (H/E) to examine neuronal differences. After 10 days, retinal microvasculature was isolated from whole retinas to quantitate differences in degenerated capillaries. Retinal lysates were also processed for protein analyses of key inflammatory mediators known to play a role in the development of DR. Overall, strain specific differences were observed between

B6 and BALB/c mice in response to I/R injury. Although both strains showed signs of retinal injury, more damage was observed in B6 mice as detected by reduced retinal thickness, increased microvascular degeneration and elevated inflammatory mediators. These data suggest that B6 and BALB/c mice respond to I/R injury differently, which has implications not just for retinal ischemia, but for the development of DR. Elucidating these differences both offers potential therapeutic points of intervention and impacts the current modeling system used to study retinal diseases, such as DR.

5.2 Introduction

Ischemia-reperfusion (I/R) injury induces the production of free radicals resulting in local inflammation and tissue damage. At the reperfusion stage, restoration of the blood supply leads to accumulation of oxygen radicals, further causing oxidative stress such as lipid peroxidation [107, 108], damaging residential cells and stimulating secretion of pro-inflammatory cytokines. Production of IL-8 and adhesion molecules chemoattracts neutrophils and macrophages into the injured tissue. Accumulation of inflammatory cells at the I/R site elicits further production of reactive oxygen species (ROS), cytokines and chemokines, exacerbates local inflammation in the damaged tissue.

Regarding eyes, retinal I/R surgery, by increasing intra-ocular pressrure, was demonstrated to induce pathophysiological changes that are similarly observed in diabetic retinopathy (DR) and glaucoma [112]. The ROS scavenging system is severly impaired in DR, resulting in upregulated ROS

concentrations in the retina. This is comparable to the accumulation of ROS and its resultant upregulation of inflammation along with vascular damage after I/R. Earlier studies found that I/R promoted nitric oxide neurotoxicity, resulting in retinal neurodegeneration [113, 216]. This effect is also demonstrated by reduction of amplitude of a-wave and b-wave based on electroretinogram (ERG) [217]. In addition, studies in rodent models demonstrated vascular degeneration after retinal I/R surgery as detected by acellular retinal capilaries and inflammatory markers inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [114]. Retinal apoptosis, breakdown of the blood-retinal barrier (BRB), and excerbated retinal microvascular leakage were also recorded following induced intraocular pressure and reperfusion [218]. Thus, the retinal I/R model is a reliable tool to mimic neurodegeneration, microvasuclar degeneration and inflammation associated with DR.

In DR pathogenesis, upregulation of inflammatory markers such as iNOS, COX-2, intracellular adhesion molecule-1 (ICAM-1), vascular endothelial growth factor (VEGF), NF-κB, interleukin-1 beta (IL-1β) not only emphasizes the important role of inflammation, but also implies its crossing-talking with other pathogenic pathways [8]. Similarly, increased leukocyte rolling and accumulation at retinal microvasculature was observed in a rat model of I/R [219]. There is significant upregulation of inflammatory markers following retinal I/R surgery as well since it induces similar microvascular ischemia to DR; tumor

necrosis factor-alpha (TNF-α), IL-1β, ICAM-1 and interleukin-6 (IL-6) were all found to be upregulated in rats after retinal I/R surgery [220].

The type 1 versus type 2 inflammation paradigm emphasizes the cytokine function and denotes production by all cell types rather than CD4+ T Cells as the sole source of these mediators [221]. Type 1-dominance is usually characterized by strong pro-inflammatory activation, accompanied by elevated and sustained pro-inflammatory cytokine levels that exacerbate inflammation and can develop into chronic inflammation state, if left uncontrolled. In type 2 inflammation, anti-inflammatory response dominates, limiting inflammation with subsequent inflammation resolution by pro-resolving pathway. In this regard, the current study is focusing on the general inflammatory acitivties in the retina, in which residential retinal cells and inflammatory cells both partipate in by secreting cytokines that drive inflammation further. Despite all the effort has been put into studies of inflammation in DR, little is known regarding the infleuce of the type 1/type 2 balance on DR. BALB/c (resistant, type 2 dominant) and C57BL/6J (B6) (susceptable, type 1 dominant) type mice have proven as very suitable tools in investigating type 1/type 2 inflammation [40, 43, 222]. In this study, we sought to investigate the difference in the pathogenic response related to type 1 versus type 2 inflammation using a model of retinal I/R. We expect these findings will define a role for inflammation in the retinal response to ischemic conditions, which is a characteristic event associated with the development of DR.

5.3 Methods

Intraocular Ischemia-Reperfusion

All mouse experiments were approved by the Institutional Animal Care and Use Committee at Wayne State University (Protocol# 11-08-14). I/R was carried out in 7 weeks old male B6 and BALB/c mice purchased from Jackson Laboratories. The anterior chamber of one eye was cannulated with a 30-gauge needle attached to a line infusing normal saline. Intraocular pressure (IOP) was measured by a handheld tonometer (TONO Pen; Medtronic Solan, Jacksonville, FL) in mouse eyes, and pressure in the eye regulated to 80-90 mm Hg with a pressure infuser (Infu-surg; Ethox Corp., Buffalo, NY). The other eye of the same animal servedas a control. After 90 min of ischemia, the needle was withdrawn, IOP was normalized, and reflow of the retinal circulation was documented visually [114]. Ocular tissue was harvested at 2 and 10 days post-injury for analyses as described below.

Neuronal analyses

At 2 days post-I/R injury, whole eyes were removed, followed by a 30 min fixation in 4% paraformaldehyde. Ten-μm retinal sections were stained using for hematoxylin and eosin, then visualized by light microscopy for morphometry of retinal thickness. Photomicrographs were assessed for retinal thickness and the number of cells in the ganglion cell layer were quantitated. The thickness of the retina and the cell count was measured using Invitrogen EVOS FL Auto Cell Imaging System (Invitrogen, Carlsbad, CA).

Vascular analyses

Eyes were enucleated at 10 days after I/R exposure suspended in 10% buffered formalin for 5 days, and the retina was dissected in 40U/mL elastase solution (Merck Millipore, Burlington, MA). The retinal vascular tree was dried onto a glass slide and stained with hematoxylin-periodic acid-Schiff. Degenerate capillaries, identified as acellular capillary-sized tubes, were counted [223] in mid-retina in 5 fields. Degenerate capillaries were excluded if their average diameter was less than 20% of surrounding healthy capillaries [224].

ELISA

IL-1β and TNF-α ELISA kits (R&D Systems, Minneapolis, MN) were used to measure protein expression in whole retinal lysates. Retinal extracts were prepared by sonication. All samples were assayed in duplicate or triplicate per the manufacturer's instruction. Equal protein was loaded into all wells. The reported sensitivities of these assays are 4.8 pg/mL for IL-1β and 7.21 pg/mL for TNF-α.

Western Blotting

Retinal lysates were collected and processed as described above. Equal amount of total proteins were then separated on 4–20% tris-glycine gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. After blocking membranes in TBST (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) BSA at r.t. for 60 min, membranes were

incubated overnight at 4°C with antigen-specific primary antibodies. The primary antibodies were used as follows: anti-NF-κB p65 (phospho Ser-536), anti-ICAM-1 (Cell Signaling Technology, Danvers, MA); anti-VEGF (Abcam, Cambridge, United Kingdom); anti-COX-2, and anti-β-actin (Santa Cruz, Santa Cruz, CA). Blots were then incubated with species-specific HRP-conjugated secondary antibodies for 2h at r.t. Proteins were visualized by incubation with a chemiluminescence substrate kit (Thermo Fisher Scientific, Waltham, MA). Western blot images were collected (Azure Biosystem C500, Dublin, CA) and target protein expression was quantified (Image Studio Lite software) after normalizing to β-actin. One representative blot is shown.

Statistical analysis

All assays were carried out at least twice from five independent experiments and the data are presented as mean ± SD. Data were analyzed by the Analysis of variance (ANOVA) test following by Fisher's LSD test. $P < 0.05$ was considered to be statistically significant.

5.4 Results

Loss of retinal thickness and retinal ganglion cells as observed in BALB/c and B6 mice following I/R surgery

To begin investigating whether BALB/c mice are more resistant than B6 to retinal damage, we first measured I/R induced retinal neurodegenreration. Figure 28 shows significant retinal thining of retina in both B6 (B) and BALB/c mice (D), compared to non-injured control mice (A &C, respectively). Retinal

thickness loss is significantly less in BALB/c I/R mice compared to B6 I/R mice. This is supported by more ganglion cells observed in BALB/c I/R mice.

BALB/c mice exhibit reduced microvascular damage in response to I/R compared to B6

Next, we sought to find out if I/R-induced retinal microvascular damage could also be limited in these type 2-dominant mice. As shown in Figure 29, significantly fewer degenerated acellular capillaries were observed in BALB/c mice when compared to B6 mice following retinal I/R surgery.

Retinal I/R induced inflammatory activities are reduced in type 2 dominant BALB/c mice

It is important to determine whether retinal I/R induced local inflammation is different in type 2 dominant BALB/c mice versus type 1 dominant B6 mice. As shown in Figure 30, IL-1β (A), TNF-α (B), ICAM-1 (C), VEGF (D), phosphorylated NF-κB p65 at Ser-536 (F) have similar trends: I/R-induced upregulation of these molecules is significantly lower in BALB/c mice compared to B6 mice. Control mice of BALB/c have higher expression of COX-2 than that of B6 mice. I/R upreuglated levels of COX-2 in B6 mice yet downregulated it in BALB/c mice (E).

5.5 Discussion

For the recent decade, inflammation been indentified as one of the important therapeutic target for DR [116, 225, 226]. In vitreous fluid of eyes of patients with progressive diabetic retinopathy (PDR), elevated levels of

various cytokines have been detected; several of them are potent inflammatory mediators, including IL-1β, TNF-α, IL-6, IL-8 and macrophage chemotatic protein-1 (MCP-1) [227, 228]. As key players in local inflammation and homeostasis, the relative balance between type 1 pro-inflammatory cytokines (IFN-γ, TNF-α, IL1β) and type 2 anti-inflammatory cytokines (IL-4, IL-10) is expected to be essential in DR pathogenesis. The major goal of this study is to determine whether a predominately anti-inflammatory environment in type 2 models would build a “resistant” force against inflammatory mediators of DR. Using a Pseudomonas aeruginosa-induced bacterial keratisis model, previous work in our lab has demonstrated the benefits of type 2 BALB/c mice in terms of local homeostasis and reconsitution after infection [42]. Thus, we expected BALB/c mice to be at least partially resistant to I/R-induced DR-like inflammation, as well.

NF-κB is a pivotal regulator of general inflammation, and for the recent decade, was demonstrated to be a master transcription factor protein family to promote transcription of pathogenic molecules for DR as it could not only be upregulated by reactive oxygen species (ROS) [88] and advanced glycation endpucts (AGEs) [89, 90], but also further stimluates production of IL-1β, TNF-α, IL-6, IL-8, MCP-1, iNOS, COX-2, ICAM-1, vascular adhesion molecule-1 (VCAM-1), Fas and Fas ligand [91-99]. In type 2 dominant BALB/c mice, phosphorylation at NF-κB p65 ser-536 was significantly downregulated in comparison to B6 mice in current study. Since phosphorylation of NF-κB p65 is

prerequisite to its translocation and subsequent binding to target genes, and p65 is the most important activator in NF-κB family, this result implicates that, in type 2 dominant BALB/c mice, the ability of NF-κB to promote transcription of target genes is strongly compromised. This correlates with the observed changes in IL-1β, TNF-α and ICAM-1 protein levels since they are known to be regulated by NF-κB. Given IL-1β and TNF-α are hallmark inflammatory molecules in DR-induced inflammation and canonical activators of NF-κB, the fact that they were downregulated in I/R-induced type 2 dominant BALB/c mice indicates less extent of inflammatory activity. In a streptozotocin-induced DR model, inhibition of ICAM-1 decreased leukotasis and vascular leakage [22]. Thus, less expression of ICAM-1 in BALB/c mice compared to B6 mice suggests that these animals may have relatively fewer leukocyte docking and transmigration activities and reduced local inflammation in response to I/R injury. On the other hand, upregulation of COX-2 and its induced production of prostaglandins were reported in DR models [27, 229]. As the key enzyme for production of pro-inflammatory lipid mediators, COX-2 promotes pathogenesis of DR by producing prostaglandins. We observed a higher level of basal COX-2 in BALB/c mice, yet this level goes down with retinal injury. It is possible that decreased COX-2 expression will lead to compromised production of prostaglandins in BALB/c mice, although this needs further study to confirm.

The initial genesis of vision loss in DR has long-time been attributed to breakdown of blood-retinal barrier and increased retinal microvascular

permeability [230]. There are two major types of cells in the retinal microvascular endothelium: pericytes and endothelial cells. Based on animal study, pericytes begin to degenerate before the disappearance of endothelial cells in DR, leaving thin, acellular capillaries behind [231]. As a result, the ischemic retina starts to build more capillaries in an attempt to restore the blood supply, by secreting VEGF, which stimulates angiogenesis [232]. To this end, we observed fewer degenerated capillaries in the vascular analyses, indicating BALB/c mice are more resistant to microvascular degeneration. Additionally, BALB/c mice express lower levels of VEGF following I/R surgery, implying less pathogenesis of microvasculature [233]. While DR has been traditionally considered to be a microvascular eye complication, recently it is demonstrated to be more of a neurovascular event. Experts have concluded that neurodegeneration takes place very early during pathogenesis of DR, predicting and contributing to later microvascular changes [234]. As we observed much less neurodegeneration in BALB/c mice as well, it is concluded that BALB/c mice, with a type 2-dominant inflammatory response, are resistant to DR-like neurodegeneration and microvascular degeneration.

Even though steroids and nonsteroidal anti-inflammatory drug (NSAID) have been applied to treat ocular inflammatory coditions [235], very limited effort has been put into investigating anti-inflammatory and pro-resolving mechanism in DR disease progression. Current anti-inflammatory treatments are also expected to deliver unwanted consequence and may require

combination usage. The low efficacy of anti-inflammatory treatments might be attributed to insuffient resolution of inflammation. This study, in an effort to unravel more myth behind pathogenesis of inflammation in DR, demonstrates Type 2 dominant immune activity is able to resist I/R induced DR like inflammatory stimulation and neurovascular degeneration. Whether neurovascular changes in BALB/c mice is direct consequece of robust anti-inflammatory activities in Type 2 type immunity needs further study. However, the fact we observed significantly less expression of pro-inflammatory cytokines have provided another insight for the interventation point of treatments on DR.

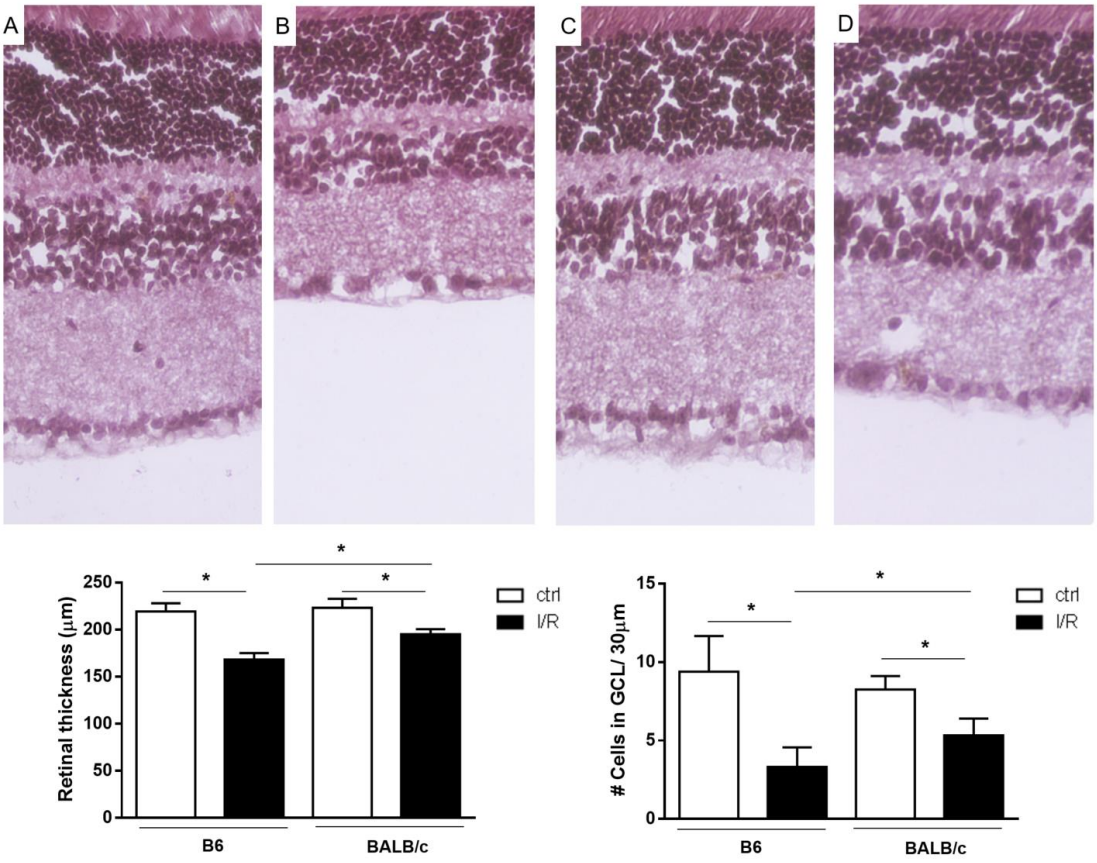

Figure 28. Neuronal analysis of BALB/c and B6 retinas after I/R. Panels A and C show the contralateral eyes (ctrl) of B6 and BALB/c mice, respectively. Panels B and D show eyes 2 days after I/R surgery in B6 and BALB/c, respectively. Bar graphs below quantitative changes in retinal thickness (left) and cell numbers (right). Data shown are representative of 5 independent experiments and are expressed as mean ± SD. *$P < 0.05$ indicates statistical significance.

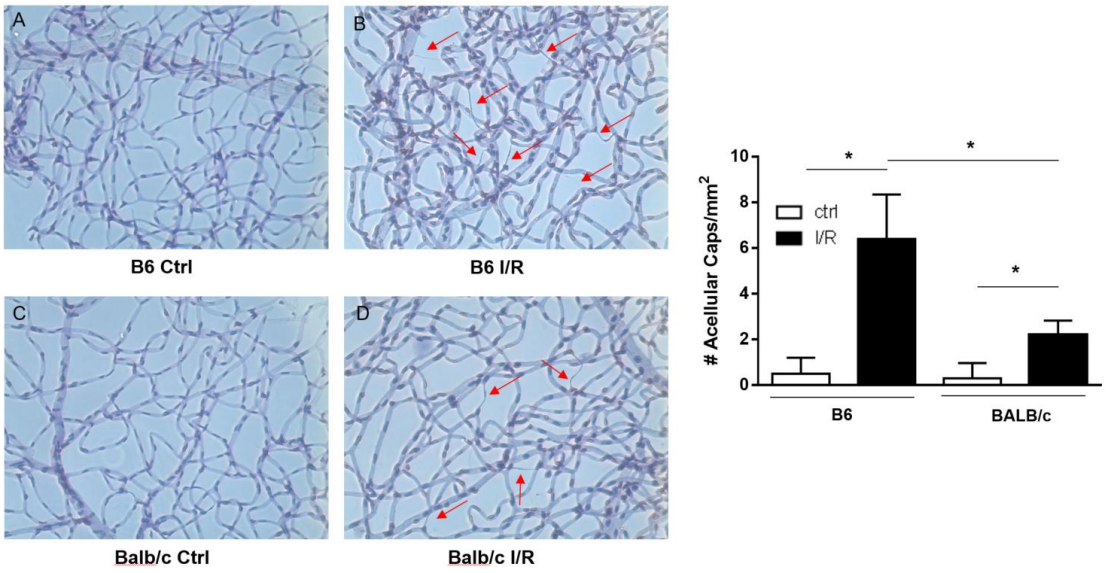

Figure 29. Vascular analysis of BALB/c and B6 mice after I/R. Panels A and C show the contralateral eyes (ctrl) of B6 and BALB/c mice, respectively. Panels B and D show eyes 10 days after I/R surgery in B6 and BALB/c, respectively. Quantification of acellular capillaries is presented in the bar graph. Data shown are representative of 5 independent experiments and are expressed as mean ± SD. *$P < 0.05$ indicates statistical significance.

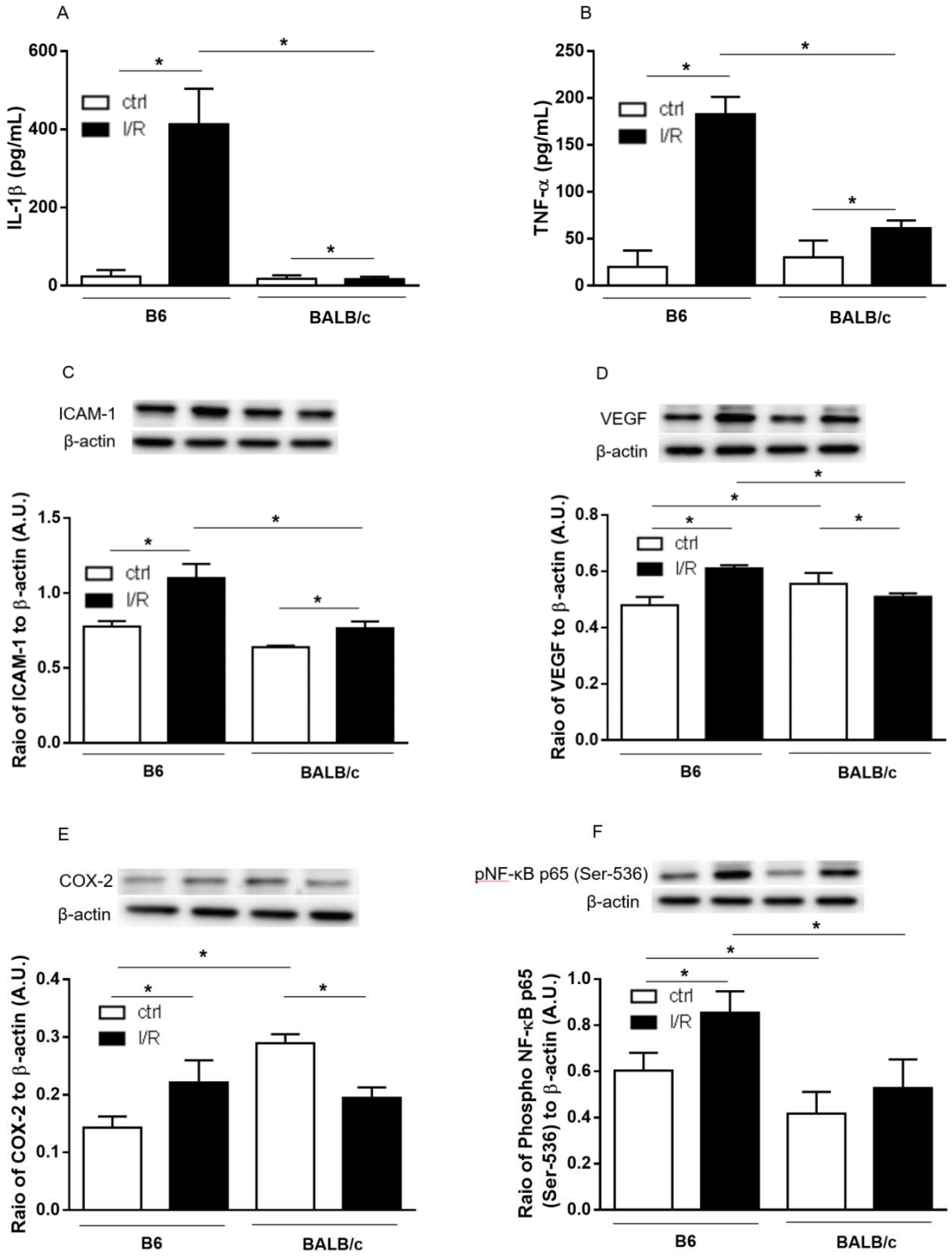

Figure 30. Selected inflammatory markers as detected in retinas of BALB/c and B6 mice in response to I/R. Retinal lysates were processed after 10 days of I/R injury. Protein levels of IL-1β (A), TNF-α (B), ICAM-1 (C), VEGF (D), COX-2 (E) and phosphorylated NF-κB at ser-536 were detected by ELISA or Western-blot. Data shown are representative of 5 independent experiments and are expressed as mean ± SD. *$P < 0.05$ indicates statistical significance.

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ABSTRACT

INFLAMMATION IN THE PATHOGENESIS OF DIABETIC RETINOPATHY

by

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Degree: Doctor of Philosophy

The general purpose of these studies is to investigate inflammation in diabetic retinopathy in an effort to identify key intervention points to develop as treatments. Firstly, we showed that the neuropeptide VIP displayed protective immunoregulatory effects on retinal endothelial cells cultured under high glucose conditions. This effect was carried out, in part through the VPAC2 receptor.

Next, we studied the β-adrenergic receptor agonist, Compound 49b, and its effect on the pro-resolving RvD1 pathway. Compound 49b was previously shown to suppress both inflammatory and apoptotic responses in DR. We demonstrated that Compound 49b rescued the high glucose-induced decrese in RvD1 and its receptors in diabetic animals and retinal endothelial cell culture, by upregulating 15-LOX enzyme expression.

We also studied the phosphorylation of NF-κB p65 in two retinal cell types exposed to high glucose. High glucose conditions stimulated phosphorylation

of NF-κB p65 at Thr-254, Ser-276, Ser-468, Ser-529, Thr-435 in retinal endothelial cells and Thr-254, Ser-281, Ser-311, Ser-468, Thr-435 in Müller cells. IL-4, an anti-inflammatory cytokine, suppressed phosphorylation at Thr-254, Ser-311, Thr-435 in retinal endothelial cells and Thr-254, Ser-276, Ser-281, Thr-435 in Müller cells. Futhermore, IL-4 also reduced related downstream NF-κB regulated molecules IL-8, TNF-α, and upregulated IL-10.

The influence of type 1 vs type 2 immune backgrounds on DR-related damage using a model of retinal ischemia-reperfusion was studied in C57BL/6 and BALB/c mice. Notably, both neuronal and vascular degeneration were significantly less in BALB/c compared to B6 mice. Furthermore, key inflammatory molecules IL-1β, TNF-α, NF-κB, ICAM-1 and VEGF were downregulated in BLAB/c mice, as well.

Collectively, we have shown the extensive role that inflammation plays in diabetic retinopathy pathogenesis. More importantly, the innate type 1/type 2 paradigm suggests that the potential of anti-inflammatory treamtents and pro-resolving lipid mediators in suppressing pathogenesis of DR. We expect our findings in pathogenesis of inflammation to contibute to development of anti-inflammatory and pro-resolving treatments for diabetic retinopathy.

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