THE ROLES OF ARACHIDONIC ACID- AND DOCOSAHEXAENOIC ACID-DERIVED EPOXIDES AND THEIR ENDOCANNABINOID DERIVATIVES IN DIABETES-INDUCED RETINAL VASCULAR INFLAMMATION

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CHAPTER I

INTRODUCTION

The studies described in this dissertation sought to better understand the role of vascular inflammation in the pathogenesis of blinding retinal disease, and explored routes to mitigate this inflammation in the pursuit of therapeutic intervention. Our lab is particularly interested in understanding the mechanisms underlying the early stages of diabetic retinopathy (DR), which is a visually destructive secondary complication of diabetes mellitus. DR is the leading cause of vision loss among working age Americans, and effects roughly 10 million people in the US alone[1,2]. Current therapeutic interventions target late-stage DR after the appearance of irreparable damage; thus, we aim to fuel the development of novel therapeutic targets that would intervene in earlier stages of the condition, before irreversible vision loss occurs. Herein, our studies focused on the anti-inflammatory potential of arachidonic acid- and docosahexaenoic acid-derived epoxides and their endocannabinoid derivatives to mitigate diabetes-induced retinal vascular inflammation. This work constitutes the first comprehensive evaluation of the role of these lipids in, and their potential benefits to, DR. We employed a balance of *in vitro* and *in vivo* models, first to identify relevant and druggable targets in monocultures of specific retinal cells, and then to validate the targets and employ the therapeutic objectives in reliable animal models of DR. Ultimately, this dissertation's primary focus is to contribute to the development of effective treatments for DR by evaluating the efficacy of pharmacotherapeutic strategies in relevant pre-clinical models.

Diabetic Retinopathy Background

Insulin:

Insulin is a polypeptide hormone produced by beta cells in pancreatic islets and is largely responsible for regulating glucose metabolism in the body. When food is consumed, it is broken down into a variety of molecules necessary for physiologic function. Among these

molecules is glucose, which is released into the bloodstream. The increase in blood sugar after eating prompts the production and release of insulin from pancreatic beta cells, which in turn facilitates the uptake of glucose by liver, muscle, and adipose tissue. Insulin promotes the conversion of glucose into glycogen for storage in the liver and muscles, and the synthesis of fats in adipose tissue. This effectively removes glucose from the bloodstream, lowering blood glucose levels back to baseline or homeostatic levels. Insulin is therefore crucial for blood sugar regulation, in particular the prevention of excess glucose accumulation in the blood, while also ensuring a stable and optimal energy supply to cells[3].

Diabetes:

Insulin is essential for maintaining overall metabolic health and the body's ability to efficiently utilize and store energy. Insulin influences a wide range of physiological processes, therefore functional deviation may result in the emergence of chronic disease, notably diabetes mellitus[3]. Diabetes mellitus is a systemic metabolic disease that occurs when the pancreas fails to produce adequate amounts of insulin, or when the body cannot effectively use the insulin that it does produce. Diabetes mellitus is characterized by abnormally elevated blood glucose levels, known as *hyperglycemia*, and is categorized into two main subsets: type 1 and type 2. Type 1 diabetes (T1D) typically develops in childhood or early adolescence and is classified as an autoimmune disorder. In T1D, pancreatic beta cells are destroyed by T-cell-mediated autoimmunity, resulting in insulin deficiency and a subsequent exogenous insulin-dependence. Exogenous insulin administration is therefore crucial in the management of T1D, because without insulin, cells would be unable to take up and utilize glucose as an energy source, leading to overwhelming glucose concentrations in the blood[3,4]. Type 2 diabetes (T2D) is characterized by insulin resistance, in which the body stops appropriately responding to insulin regardless of endogenous production[4]. Insulin resistance, islet dysfunction, reduced insulin secretion, and decreased glucose utilization are all contributing factors to the onset and

progression of hyperglycemia in T2D[3]. Prolonged hyperglycemia via inadequate disease management can lead to serious health complications, such as irreparable damage to nerves and blood vessels of some tissues[5].

Complications:

Prolonged exposure to diabetes can lead to many secondary macrovascular and microvascular complications. *Macrovascular complications* are those that cause damage to the larger blood vessels in the body. The central pathological mechanism in macrovascular disease is atherosclerosis, which involves the buildup of plaque in major arteries, narrowing arterial lumena. Atherosclerotic plaque formation in diabetes is profoundly associated with the development of cardiovascular diseases, as people with diabetes have increased risk of coronary artery disease, peripheral arterial disease, and stroke. Macrovascular complications from diabetes can be severe and life-threatening, with cardiovascular disease being the primary cause of death in people with either T1D or T2D[5]. In contrast*, microvascular complications* are those that cause damage to the smaller blood vessels of the body. The three main microvascular complications associated with diabetes are diabetic nephropathy, diabetic neuropathy, and diabetic retinopathy. Diabetic nephropathy refers to diabetes-induced damage to blood vessels of the kidneys, and it is the leading cause of renal failure in the United States. Diabetic neuropathy refers to the peripheral nerve dysfunction seen in diabetic patients, and increases the risk of sensation loss, pain, foot ulcerations and amputation. Diabetic retinopathy may be the most common microvascular complication of diabetes, with damage to the blood vessels of the eye resulting in ~10,000 new cases of blindness every year in the US alone[5].

Diabetic Retinopathy:

Diabetic retinopathy (DR) is a complication of diabetes-associated metabolic dysfunction that causes damage to the retinal vasculature. Although recent therapeutic advances have been

achieved, DR continues to be the leading cause of irreversible vision loss in working-age adults in the US[6,7]. Individuals with T1D and T2D have a 95% and 60% chance, respectively, of developing some degree of DR 25 years post-diabetes diagnosis[8,9]. Unfortunately, the increasing prevalence of DR worldwide continues unabated. In 2020, it was estimated that 103 million adults worldwide lived with DR; by 2045, that number is projected to increase to 161 million[10]. DR pathology presents in two clinically distinct stages: early-stage non-proliferative diabetic retinopathy (NPDR), exhibiting fundus abnormalities such as microaneurysms, hemorrhages, and vasoregression; and late-stage proliferative diabetic retinopathy (PDR), exhibiting pre-retinal neovascularization[1,11,12]. Additionally, fluid accumulation via retinal vessel leakage in either stage can cause swelling of the macula known as diabetic macular edema (DME), which expands vision loss associated with diabetes[12].

Risk Factors:

Many risk factors contribute to the development and progression of DR, with the greatest risk factor being the duration of disease. In the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR), the prevalence of retinopathy in T1D patients was 25% at five years and 80% at 15 years of disease, with PDR prevalence being 0% at three years and 25% at 15 years of disease[13,14]. The longer an individual has diabetes, the higher the risk of developing DR, especially if the disease is poorly controlled. Prolonged high blood sugar or fluctuations in blood sugar can increase the risk of retinal damage, therefore stable blood sugar is an important goal in the prevention of DR[15,16]. In the Diabetes Control and Complications Trial (DCCT), intensive blood glucose control was found to reduce the risk of retinopathy by 76% and reduce the risk of DR progression by 54%[13,17]. Similarly, intensive blood glucose control was found to reduce the risk of DME by 46% at the end of the trial, and 58% four years later[15,17]. Worldwide population studies support the notion of a continuous relationship between fasting blood glucose levels and

retinopathy risk, therefore tight glycemic control is a widely accepted preventative measure to stave off DR[17,18].

Of note, DR may develop independent of tight glycemic control. According to the DCCT, glycated-hemoglobin only accounted for 11% of the risk of DR in their studies[12,17], therefore other contributing maladies of diabetes cannot be dismissed. Dyslipidemia is a major risk factor in developing DR, as elevated levels of cholesterol and lipids in the blood can contribute to the development and progression of retinal damage[19]. For example, in 2013, the MADIABETES Study found high serum levels of low-density lipoprotein (LDL) cholesterol to be a significant risk factor in DR incidence[20]. Additionally, Report 22 of the Early Treatment Diabetic Retinopathy Study (ETDRS) found that patients with elevated LDL cholesterol and lipid levels in the serum were twice as likely to develop vision-threatening hard exudates in DR[21]. In response to this risk factor, it has been found that patients on long-term lipid-lowering therapy showed a significantly lower risk of DR progression. According to the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study, patients experienced a one-third reduction in the need for their first DR treatment after five years of taking the PPARalpha agonist fenofibrate[22]. Additionally in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial, daily doses of simvastatin with fenofibrate after four years resulted in a 44% reduction in the progression of DR, from 6.5% with dyslipidemia therapy, to 10.2% without[23].

Diabetic Retinopathy Characteristics and Mechanisms

Characteristics of Diabetic Retinopathy:

The transparency of anterior eye tissues allows for the non-invasive observation of the retina to examine the emergence and progression of disease features. Via fundus examinations, clinicians are able to comprehensively view the retinal surface through the pupil. The clinically observable signs of DR are predominately vascular, and begin with the appearance of tiny red dots along the retinal vasculature known as *microaneurysms*[1,12] (**Fig.1**). Microaneurysms are the

ballooning of blood vessels in response to wall weakness, and their turnover rate is indicative of DR progression[1,24]. Next, the retina will progress to exudative changes, such as *hemorrhages* and *hard exudates*. A hemorrhage can occur when a microaneurysm ruptures, resulting in the blood vessel bleeding into the retinal tissue or into the vitreous cavity. Lipids can also leak from these damaged blood vessels, resulting in yellowish deposits known as *hard exudates*. In patients with early NPDR, vascular hyperpermeability and leakage primarily result from microaneurysms and can lead to retinal *edema* – thickening of the tissue due to fluid excess. Macular edema is present in 25% of diabetic patients and is the clinical feature most closely tied to vision loss in DR[1,12]. Edema can also be caused by leakage from the growth of new abnormal blood vessels, a process termed *neovascularization*. When seen through fundus imaging, these nascent abnormal vessels are the tell-tale sign that DR has progressed to late-stage PDR. Neovascularization can cause severe complications including retinal detachment and vitreous hemorrhage, potentially causing vision loss[1,12]. Continued tissue deteriorations will result in patients experiencing declines in visual acuity, contrast sensitivity, and visual field area, which can affect all aspects of daily life, including the management of the disease[12].

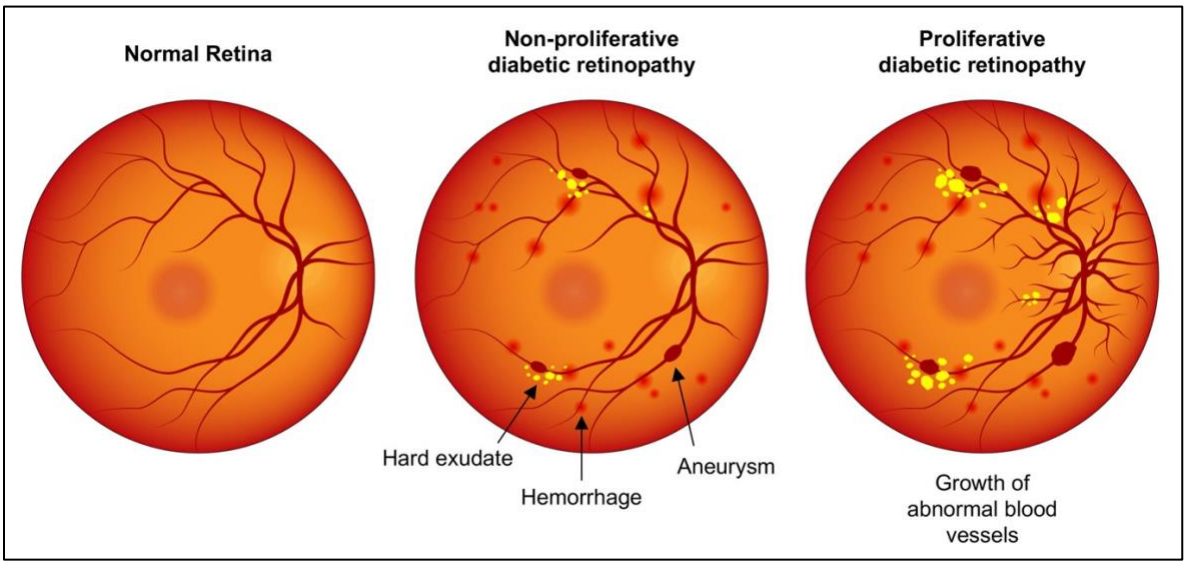


Figure 1. Representative fundus schematic of retinal degeneration in diabetic retinopathy progression. Image credited to AntlerA Therapeutics, created by artist Allison Bruce, scientific illustrator[25].

Other important pathological signs of DR are not observable upon fundus examination, but are critical to the progression of the condition and the characterization of its pathology. Retinal vascular *basement membrane (BM) thickening* is a structural abnormality of DR observed on histologic examination of retinal tissue that can develop before or coincident with other morphological lesions. It occurs as a result of the increased synthesis of BM material, such as fibronectin, type IV collagen, and laminin, by pericytes and endothelial cells that comprise the BM. This is believed to cause structural and permeability changes in the retinal capillaries[26]. The terms *vasoregression* or *capillary dropout* are used to describe the atrophy of retinal capillaries in DR. Vasoregression is a critical morphologic manifestation of DR, and is hypothesized to begin with pericyte loss, followed by a subsequent loss of endothelial cells, and a compromise of the blood-retina barrier[27]. Endothelial cell dysfunction is associated with other consequences as well. In DR, endothelial cells comprising retinal capillary walls more readily bind circulating leukocytes, an event termed *leukostasis.* Leukostasis is a prominent cellular feature in DR that impedes proper functioning of the capillary network via occlusion, leading to nonperfusion, ischemia and subsequent neovascularization[1,28,29]. Therefore, in addition to fundus examinations, clinicians use other diagnostic tools such as optical coherence tomography (OCT) and fluorescein angiography (FA) to diagnose and stage DR. OCT imaging allows the determination of retinal thickness, indicating the presence of fluid leakage and edema of the retinal tissue. FA involves the intravenous delivery of a fluorescent dye to the circulation. As the dye circulates, retinal arterial, capillary and venous filling can be observed, and structural and functional abnormalities such as vasoregression, occlusion or leakage can be assessed. Therefore, in addition to fundus exams, OCT and FA are important methodologies to aid DR diagnosis as well as monitor its progression[30].

Diabetic Retinopathy Mechanisms and the Inflammation Hypothesis:

Inflammation is a result of the unspecific activation of the innate immune system in response to injury or infection, and while inflammation is instrumental to immune protection in an acute setting, it can become destructive if left unresolved[1]. Systemic inflammation is an inherent response to diabetes, and the chronic nature of diabetes means that its associated inflammation can become chronic as well[12,31]. In 2002, Doganay et al. found that patients with DR had elevated levels of inflammatory cytokines in the bloodstream, and that serum cytokine concentrations increased in correlation with disease escalation from NPDR to PDR[32]. Still, while systemic inflammation has been reported consistently in animals with experimental diabetes and in diabetic patients, the production of local ocular inflammation may be more critical in the pathogenesis of DR. In animal models[33–36] and patients with DR[33,34,37–40], increased retinal, vitreous, or aqueous levels of pro-inflammatory cytokines are observed early in DR progression, and are experimentally linked to several hallmark DR features. Diabetes-linked hyperglycemia and dyslipidemia are the proposed cause of this inflammation, as extensive *in vitro* evidence shows that direct stimulation of retinal cells with high glucose and/or fatty acids triggers their production of inflammatory cytokines[1,41–49]. Hypothetically, when retinal levels of inflammatory cytokines from this process of local amplification increase beyond a certain threshold, they may assume a dominant role in DR progression as compared to that of systemic metabolic dysfunction.

When pattern recognition receptors of the immune system like Toll-like receptors (TLRs) are engaged, their activation triggers an increased production of inflammatory cytokines. This is achieved through proinflammatory transcription factors such as Nuclear Factor-κB (NF-κB), that regulate the synthesis of many cytokines at the transcription level. NF-κB has been strongly implicated in contributing to retinal inflammation in DR, and is known to transcribe many notable cytokines including interleukin-1β and tumor necrosis factor-α[1]. Interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), interleukin-8 (IL-8) and interleukin-6 (IL-6) are inflammatory cytokines

that have garnered considerable attention in DR pathogenesis. Elevated levels of these cytokines are observed in the serum and vitreous humor of diabetic patients and correlate with DR severity and progression[1,32,34,37–40,50–55]. As increased inflammation is experimentally linked to several DR morbidities, these inflammatory cytokines may assume a causative role in experimental DR.

Both TNFα and IL-1β activate NF-κB-dependent transcription, creating a positive feedback loop of inflammatory cytokine production, as well as producing other pro-inflammatory factors, such as leukocyte adhesion molecules[1,56,57]. Leukocyte adhesion molecules are proteins expressed by endothelial cells of the retinal vasculature and contribute to the capture and adherence of leukocytes to the vessel wall. Leukocytes are circulating cells that are recruited to inflamed tissues during an immune response, and contribute to tissue repair by clearing away foreign invading agents, degrading necrotized tissue components, etc[58]. The chronic inflammatory environment in the diabetic retina causes endothelial cells to produce increased leukocyte adhesion molecules, resulting in increased immobilization of leukocytes to retinal capillary walls, known as *leukostasis* (**Fig. 2**)[1,58,59]. First, the selectin family of adhesion molecules facilitates the capture of leukocytes from circulating blood and their subsequent rolling along the capillary wall. Next, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) slow the rolling velocity of tethered leukocytes and anchor them to endothelial cells. Though recruitment and adhesion of leukocytes is beneficial in an acute response, the accumulation of leukocytes during prolonged inflammation ultimately can result in tissue damage[1,58].

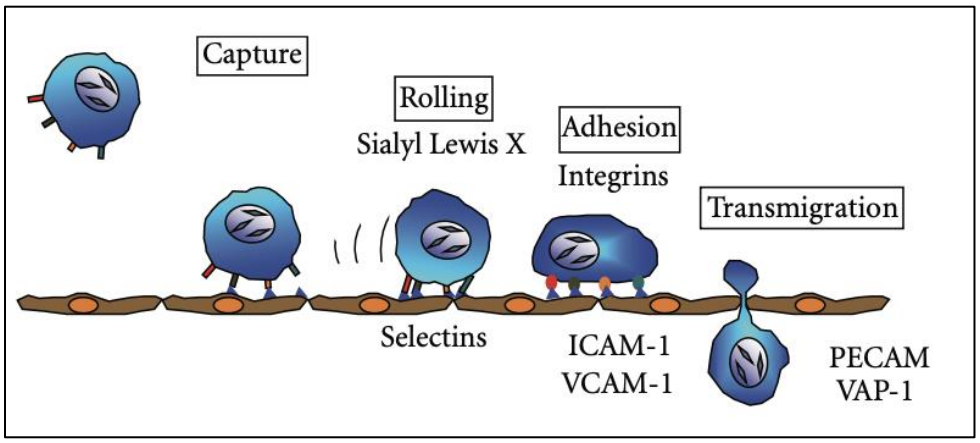


Figure 2. Leukocyte recruitment to vessel wall. From Noda et al. (2012)[58], this diagram depicts the process of leukostasis as it occurs in the retinal vasculature.

Abundant data suggest that increased retinal leukostasis is linked to the development of several hallmark DR pathologies[1,60]. For example, adherent leukocytes may elaborate reactive oxygen species and cytokines, triggering autocrine and paracrine signaling cascades that amplify inflammatory retinal cytokines to levels that cause the death of retinal neurons, the breakdown of the blood-retinal barrier, and functional deficits[1,61,62]. In some cases, leukostasis may cause physical capillary occlusion, which results in downstream ischemia, hypoxia, and capillary death[1,29]. Hypoxia upregulates the release of vascular endothelial growth factor (VEGF), which is the primary factor responsible for neovascularization in PDR. Though VEGF promotes new blood vessel growth in an attempt to re-oxygenate damaged areas, the nascent vessels are abnormal, fragile, and leak, resulting in microhemorrhages and edema[1].

Important Retinal Cells in Diabetic Retinopathy:

Many retinal cell types are negatively impacted by diabetes, and their aggravation contributes to DR progression. This passage will discuss three categories of cells in the retina and how their dysregulation promotes disease progression: retinal neurons, glial cells, and vascular cells.

The retina uses complex cellular circuitry to transduce light into a signal that moves from

the back of the retina to the axons that comprise the optic nerve. The cells that are responsible for generating and propagating those neural signals are called *neurons*, and the retina has five types of neuronal cells: photoreceptors, bipolar cells, ganglion cells, amacrine cells, and horizontal cells. Photoreceptors are comprised of rod and cone cells, and these are activated by the absorption of light photons. Photoreceptor activation results in a cascade of altered membrane potential that travels through bipolar cells to ganglion cells, whose axons comprise the optic nerve[63]. This network of neurons is highly active and sensitive to aberrant conditions, and patients with DR experience gradual yet consistent neuronal apoptosis. This chronic neurodegeneration is irreversible, precedes vascular lesions, and leads to a degradation of visual function[64].

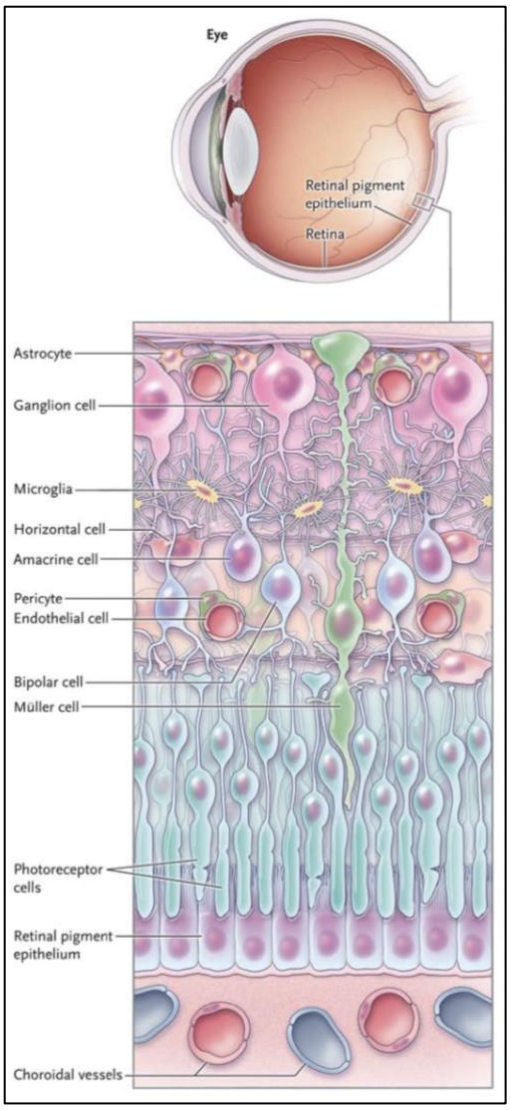


Figure 3. Retinal cells and their alterations in diabetic retinopathy. From Antonetti et al. (2012)[12], this is a cartoon of a retinal cross section, identifying the cells of the retina and their relation to one another. Reproduced with permission from New England Journal of Medicine, Copyright Massachusetts Medical Society.

Glial cells provide support to neurons by maintaining a homeostatic environment, regulating nutrients, and offering structural stability. There are three kinds of glial cells in the human retina, broken into two main categories: macroglia, which are composed of Müller cells and astrocytes, and microglia[65]. Müller cells (MC) are the primary glial cell of the retina and maintain retinal homeostasis through a variety of functions including neurotransmitter recycling,

ion and nutrient regulation, and photoreceptor maintenance. MC are unique in that they are the only cell type that spans the entire retina, allowing them to maintain retinal health and function through direct contact with almost every other cell type. Their spatial distribution and high sensitivity and responsivity to various pathogenic stimuli, however, cause MC to be particularly vulnerable to disturbances in the retina, including the influence of diabetes. Evidence suggests that diabetes-induced inflammation causes MC dysfunction, which in turn causes MC to become destructive to the retina and promote DR pathogenesis[66]. MC are potent initiators and propagators of inflammation, and MC changes have been observed prior to the appearance of overt vascular pathology in DR[66–68]. Retinal MC elevate and amplify inflammatory cytokines, and are the largest producer of VEGF in the retina[1]. MC activation can be monitored through glial fibrillary activated protein (GFAP), which is elevated in the MC of experimental diabetes models and from patients with DR[66–69]. Additionally, the ability of MC to remove glutamate from its surroundings is diminished in experimental models of DR, causing neurotoxicity and neuronal death[70–72]. Likewise, microglia demonstrate high sensitivity to various pathogenic stimuli and become activated in diabetic conditions[73]. Their activation results in the production of pro-inflammatory cytokines that can elicit pro-inflammatory responses from other cells[1]. Finally, astrocytes envelop blood vessels, are crucial for the development of the retina, and the integrity of the blood-retinal barrier[74].

Retinal microvascular endothelial cells (RMEC) comprise the blood vessels of the retina, and the junctional complexes between neighboring RMEC maintain the blood-retinal barrier. This barrier serves to protect the retina from circulating toxins and inflammatory cells that could be potentially damaging, while allowing the selective passage of nutrients and metabolites required for healthy retinal function. Endothelial cells are tasked with meeting oxygen and nutrient demands and removing waste products while protecting retinal tissue that is crucial for vision[75]. In the case of DR, RMEC respond to their disease environment with the increased expression of chemokines and leukocyte adhesion molecules[46,76]. RMEC dysfunction in DR

results in pathologies such as leukocyte adhesion, compromise of the blood-retinal barrier causing vascular leakage into the retina, and vasoregression leading to nascent blood vessel proliferation[27]. Pericytes are vascular cells that envelope endothelial cells and are crucial for the maintenance of the blood-retinal barrier. Perhaps owing to their close spatial association to RMEC, neither is spared dysfunction, and pericyte death is a characteristic feature of DR. Pericyte death is believed to promote vasoregression and loss of barrier function[27,77,78].

Current Therapeutic Approaches

Two major therapeutic strategies exist for the treatment of diabetic retinopathy: laser photocoagulation and anti-VEGF medications. Laser photocoagulation is a medical procedure that uses a focused beam of coherent light to manage abnormal blood vessel growth in the retina. This approach can be used focally to cauterize leaky blood vessels, which will in turn prevent further fluid accumulation in the retina, reducing retinal swelling and edema. Additionally, this approach can be applied in a panretinal grid pattern to ablate peripheral cells of the retina in an attempt to preserve high acuity central vision. Ablation of cells in the retinal periphery decreases oxygen demand and the production of angiogenic factors like VEGF. While laser photocoagulation can be effective in stabilizing and preventing further deterioration of vision, it comes with disadvantages, including permanent retinal scarring and the loss of peripheral and/or night vision[79].

More recent DR therapies largely focus on inhibiting the activity of VEGF, which is responsible for promoting neovascularization, blood vessel permeability, and the subsequent irreparable retinal damage seen in PDR[1]. Anti-VEGF therapies attempt to mitigate hyperpermeability and inhibit the growth of abnormal blood vessels, which would in turn reduce the risk of bleeding, leakage, and other retinal disease complications of DR. There are several anti-VEGF drugs approved for the treatment of diabetic retinopathy, including bevacizumab, ranibizumab, and aflibercept. Anti-VEGF drugs are administered through intravitreal injection

and require intermittent injections over time for effect and therapeutic maintenance. While anti-VEGF therapy has been shown to be effective in stabilizing diabetic retinopathy progression, it is not effective in all patients, and its invasive nature can result in poor patient compliance. Additionally, though rare, repeated intravitreal injections may result in *endophthalmitis*, an inflammatory and potentially blinding infection that can occur following repeated globe puncture. Still, anti-VEGF therapy is currently the standard of care for diabetic retinopathy (and diabetic macular edema) management, providing a targeted and often successful approach to preserving vision[8,80].

Both use of laser photocoagulation and anti-VEGF therapies are not fully effective at preventing the patient population from transitioning to PDR, where neovascularization has the potential to cause irreversible vision loss. This therefore underscores the need to investigate novel therapeutic alternatives that target factors pertinent to initial stages of NPDR. Doing so may intercept disease onset and progression, preventing the development of PDR and its irreparable and vision-threatening consequences.

Epoxygenated Fatty Acid Background

Diabetic dyslipidemia is an underappreciated influence in the onset and progression of DR, and an underappreciated aspect of diabetic dyslipidemia is an elevation of FFA in the bloodstream[19,81,82]. An abundance of data suggests that lipid mediators derived from ω-6 and ω-3 fatty acids regulate diabetes-induced retinal inflammation[1,83,84]. Arachidonic acid (AA; ω-6) and docosahexaenoic acid (DHA; ω-3) are polyunsaturated fatty acids found more abundantly in the retina than any other tissue in the body, making their metabolites of particular importance to retinal vascular homeostasis[85–87]. Polyunsaturated fatty acids are metabolized through the cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 epoxygenase (CYP) pathways. AA is metabolized by the COX and LOX pathways to yield prostaglandins, leukotrienes, and hydroxyeicosatetraenoic acids (HETEs) – oxygenated metabolites that are

primarily pro-inflammatory[1,88]. Consequently, classic COX inhibitors such as aspirin and NSAIDS have been studied for their potential to reduce DR associated inflammation in the eye, though no consensus on their efficacy has been established[1,89–92]. DHA, on the other hand, yields COX and LOX metabolites that are largely anti-inflammatory, among them resolvins and protectins[88]. For example, it was found that streptozocin-induced diabetic rats that received intravitreal injections of resolvin D1 demonstrated reduced levels of retinal IL-1β and NF-κB activity[93]. Recently, there has been growing interest in the epoxygenation of ω-6 and ω-3 fatty acids by CYPs. CYPs are endoplasmic reticulum membrane-bound monooxygenases that act on a variety of substrates to yield epoxygenated lipid metabolites that demonstrate potent anti-inflammatory activities in a variety of biological systems[56].

In response to inflammation, phospholipase A_2 (PLA_2) liberates AA and DHA from the plasma membrane[88]. CYP enzymes such as CYP2C8, CYP2C9 and CYP2J2 epoxygenate AA and DHA to produce the epoxides, epoxyeicosatrienoic acid (EET) and epoxydocosapentaenoic acid (EDP), respectively. CYP enzymatic activity yields four EET regioisomers (5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET) and five EDP regioisomers (7,8-EDP, 10,11-EDP, 13,14-EDP, 16,17-EDP, 19,20-EDP)[56]. These epoxygenated fatty acid products, or “epoxides”, have demonstrated potent anti-inflammatory and anti-apoptotic activities in various physiological contexts, and elevating their levels in tissue can reduce diabetes-related pathology[94–99].

Soluble epoxide hydrolase (sEH) hydrolyzes EET and EDP to less biologically active diol products, dihydroxyeicosatrienoic acid (DHET) and dihydroxydocosapentaenoic acid (DHDP) respectively[100,101]. sEH thereby reduces the half-life of these epoxides, decreasing their abundance in tissues and the potency of their anti-inflammatory and anti-apoptotic effects. Therefore, sEH inhibition represents a rational therapeutic method to elevate epoxide levels, preserving their anti-inflammatory bioactivities and reducing inflammation (**Appendix A**). Its inhibition is currently being clinically tested in multiple disease contexts[101–105].

The specific receptor(s) that EET and EDP activate remains unknown, though

mechanistically epoxides are believed to act through multiple well-characterized pathways. EETs inhibit NF-κB nuclear translocation and reporter activity in multiple cell types, reducing expression of inflammatory markers[97,106]. Additionally, EETs and EDPs activate PPARα and PPARγ[95,107–109], which are anti-inflammatory and anti-apoptotic in DR[110–112]. Finally, EETs have been shown to inhibit apoptosis by Akt activation, which in turn inhibits the pro-apoptotic transcription factor FOXO1[113].

Vitreous humor levels of EETs are significantly reduced in patients with both NPDR and PDR relative to non-diabetic patients, suggesting diabetic dysregulation[37]. In support, studies have found that CYP levels are decreased, and sEH levels are increased, in many inflammatory[114–116] or diabetic states[117–122] (discussed in greater detail in future paragraphs). Thus, restoring epoxide balance may be a valid and worthy pursuit. The 11,12- and 14,15-EET isomers and the 19,20-EDP isomer are the most abundant in the retina[123,124], and these regioisomers have well-characterized anti-inflammatory activities[97,106], justifying their examination as a potential therapeutic strategy in DR.

Cannabinoid and Endocannabinoid Background and History

Cannabinoids:

Cannabinoids (CB) are compounds found in cannabis, such as tetrahydrocannabinol (THC) or cannabidiol (CBD). The earliest evidence of medicinal cannabis use dates back to 2700 BCE, when it was recorded in Sheng-nung Pen-ts'ao Ching, the earliest Chinese materia medica book and pharmacopoeia[125,126]. There, cannabis was recognized in the treatment of over 100 conditions, including gout, malaria and inflammatory pain[125,127]. The popularity of cannabis reached India by 1000 BCE. There it was used in religious practices and medically as an anesthetic, anti-inflammatory, antibiotic, anticonvulsant and analgesic. Thus, it was great benefit in treating aliments such as rheumatism, anxiety, and epilepsy. Arab traders took cannabis from India to Africa in the 1300s, where it was used to treat asthma, malaria, fever,

and dysentery. Cannabis reached South America via Brazil in the 1500s, and from Brazil to Mexico later that century. The first recorded use of cannabis in Western medicine was in 1839 by Irish physician William O'Shaughnessy after he observed its use in India. Its efficacy as a therapeutic agent by O'Shaughnessy and other Western physicians caused it to spread through Europe and arrive in North America[125,128]. In 1860, the Ohio State Medical Society reported its therapeutic benefits in numerous diseases, and it was available in various forms over-the-counter in the US in the early 1900s. Cannabis was documented by US physicians to be effective in migraines, inflammation, pain, coughing, tetanus and rabies[125,129]. With time, the popularity of cannabis as a therapeutic began to wane. Vaccines circumvented the need to treat diseases like tetanus, new analgesics like aspirin were invented to relieve pain and inflammation, and opiates could now be administered intravenously due to the invention of the hypodermic needle[125,128].

With its diminished importance in medicine, its recreational use became the subject of political restriction. Despite the American Medical Association's advice to preserve it as a medical agent, cannabis was restricted by federal law in 1937, and removed from the American pharmacopeia in 1941[125,128,129]. Scientific interest in cannabis became revitalized in the 1960s with its recreational reemergence and the discovery of its active compound, delta-9-tetrahydrocannabinol (Δ^9-THC) by Israeli chemist Raphael Mechoulam in 1964. Cannabis publications ramped up in the early 1970's, and again energized in the 1990s following the discovery and cloning of cannabinoid receptors[128]. Using modern day laboratory approaches, cannabis is being revisited as a therapeutic agent. Today, the medicinal benefits of cannabis are confirmed in many aliments, including decreasing intraocular pressure in glaucoma, spasticity symptoms in multiple sclerosis, and symptoms of Parkinson's disease[130,131]. There are currently two synthetic derivatives of THC that are FDA approved: (1) nabilone, used to manage chemotherapy-induced nausea and vomiting and (2) dronabinol, used to stimulate appetite in HIV/AIDS anorexia[132–134]. Additionally, the FDA has approved one synthetic derivative of CBD,

Epidiolex, for the treatment of seizures associated with epilepsy[135]. Still, the natural cannabis plant and its constituents continue to be stigmatized, highly regulated, and even criminalized, resulting in a challenging climate for studying and harnessing its full medicinal and humanitarian potential.

Endocannabinoids:

In addition to exogenous cannabinoids derived from plant sources, a broad array of endogenous bioactive cannabinoid ligands has been identified, starting with anandamide in 1992[136]. These endogenous lipids (that activate the same system that phytocannabinoids do) are collectively referred to as endocannabinoids (eCB). The body's endocannabinoid system (ECS) is a physiologic system comprised of (1) eCB, (2) the receptors through which they exert their bioactivities, and (3) the enzymes that synthesize and degrade them[137]. eCB include the ethanolamide (EA) and glycerol derivatives of arachidonic (ω-6) and docosahexaenoic (ω-3) acids, including arachidonyl EA (anandamide, or AEA), docosahexaenoyl EA (synaptamide, or DHEA), 2-arachidonyl-glycerol (2-AG), and 2-docosahexaenoyl-glycerol (2-DHG)[83,138–140]. Later studies revealed that some CYPs convert AEA into 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid ethanolamides (EET-EA), and DHEA into a similar array of regioisomeric epoxydocosapentaenoic acid ethanolamides (EDP-EA)[83,141–143] (**Fig. 4**). With the CYP-mediated addition of an epoxide moiety, these eCB products, EET-EA and EDP-EA, display negligible/reduced psychotropic activity but enhanced anti-inflammatory activity, resulting in therapeutic interest[83,141,144,145]. Conversely, EET-EAs and EDP-EAs may be hydrolyzed by fatty acid amide hydrolase (FAAH) to yield ω-6 or ω-3 fatty acids and ethanolamine, or by sEH to yield the corresponding vicinal diols[83,141].

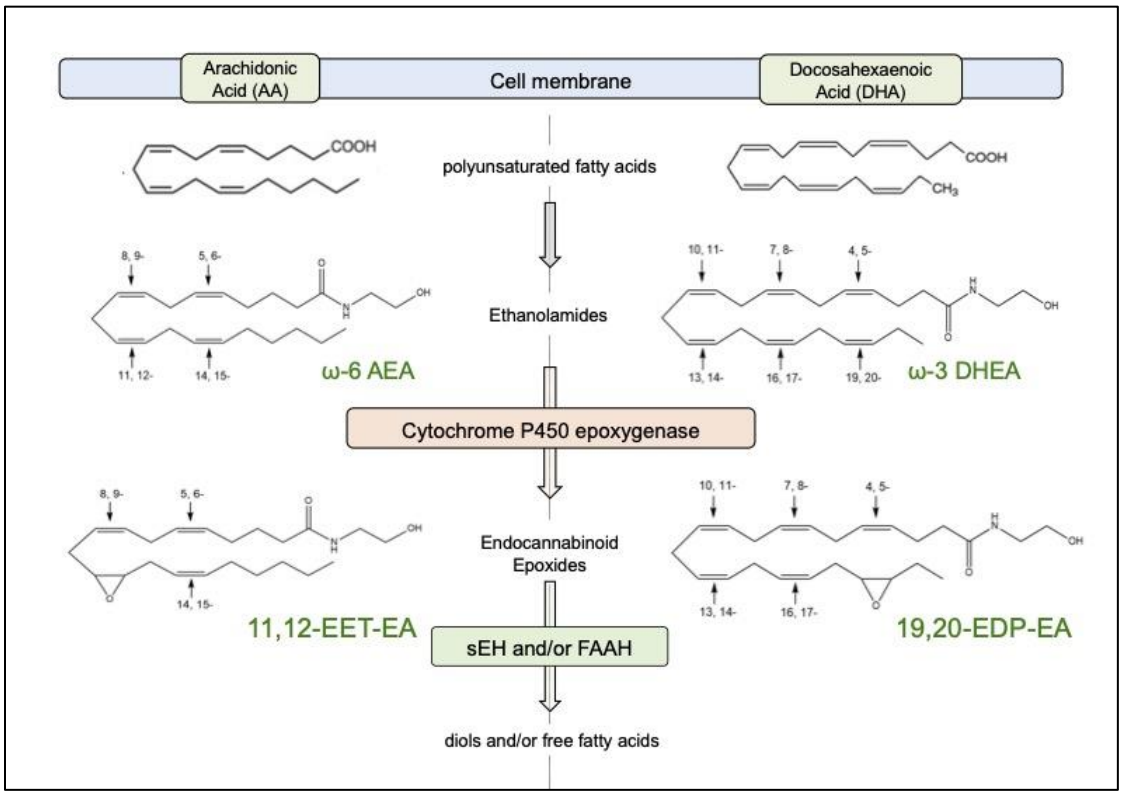


Figure 4. Overview of the metabolism of eCB. Both ω-6 (AA) and ω-3 (DHA) fatty acids are stored in the plasma membrane and can be converted to the eCB AEA and DHEA, respectively. CYP epoxygenases convert AEA and DHEA to EET-EA and EDP-EA, respectively. The eCB epoxide regioisomers of greatest interest are shown, and other possible epoxides at each double bond are indicated by the numbering system.

Receptors:

The discovery of THC lead to a search for the receptor(s) through which it exerted its bioactivities. In 1988, cannabinoid receptor type 1 (CB1) was discovered, and in 1993, a second cannabinoid receptor was discovered, cannabinoid receptor type 2 (CB2)[125,146]. CB and eCB work predominately through these two receptors, CB1 and CB2, which are both seven-transmembrane, G protein-coupled receptors[137]. CB1 is found in the central nervous system and is responsible for psychotropic effects induced by cannabis, while CB2 is found more broadly in the body and is believed to be responsible for cannabis's immunomodulatory effects[147–149]. CB2 agonism is immunomodulatory, anti-inflammatory, neuroprotective, and anti-nociceptive in numerous animal models and disease contexts and is devoid of psychotropic bioactivities[147,150].

In non-ocular inflammation models, CB2 agonism has been shown to decrease leukocyte recruitment and adherence, inflammatory cytokine production, barrier function deficits, and apoptosis, events that are, as discussed above, all pathogenic components of early DR progression[147,151,152]. Additionally, CB2 expression and protein levels increase in brain microglia and EC under inflammatory conditions[151,153,154]. Therefore, CB2 activation has excellent therapeutic potential for ocular inflammatory diseases, and testing the effectiveness of CB2 agonists in experimental models of ocular disease is both rational and compelling. While little is known about the potential effects of CB2 agonism in the context of DR, results from non-ocular and non-diabetic ocular inflammation models set a firm precedent for testing CB2 agonism as a therapeutic strategy to address DR.

Cannabinoids and Endocannabinoids in Inflammation and Diabetes Homeostasis:

Inflammation plays an important role in the pathogenesis of diabetic complications and has been associated with the dysregulation of the body's ECS – specifically, the decreased synthesis and increased hydrolysis, of eCB, resulting in a down-regulation of CB2 activation[155]. Accordingly, pharmacologic strategies have been developed that attempt to restore this homeostatic balance and increase CB2 activation. As described below, these strategies include: 1) increased expression or activation of eCB-synthesizing enzymes (CYPs) to increase eCB production, 2) inhibition of eCB-hydrolyzing enzymes (FAAH, sEH) to prolong eCB half-life[156–158], and 3) treatment with exogenous eCB or synthetic CB2 agonists to activate CB2 directly.

1) A subset of CYP enzymes act on a variety of substrates to yield epoxygenated lipid metabolites[56], and are responsible for the production of CB2-specific endocannabinoid epoxides, EET-EA and EDP-EA[83,141,143]. CYP3A4, CYP2D6, CYP4F2, and CYP2J2 have been shown to produce EET-EA and EDP-EA in various physiological contexts[83,141,143], and the literature suggests that CYP activity and expression may be negatively impacted in many

disease states. Diabetic conditions, such as high glucose, are found to suppress CYP2C9 activity in coronary artery endothelial cells (EC), subsequently decreasing the production of CYP metabolites[118]. Direct cytokine stimulation of hepatocytes has also been shown to directly down-regulate CYPs[159]. Diabetic patients have reduced CYP3A4 activity and protein levels in liver[160,161] and, specific to the eye, vitreous samples from patients with DR were found to have reduced levels of anti-inflammatory CYP products compared to non-diabetic controls[37]. Studies have shown that the remediation of CYP to homeostatic levels may have beneficial outcomes. For example, in diabetic mice, CYP2J2 global over expression, and the resulting increase in its products, reduced the production of proinflammatory cytokines in liver, decreased the activation of NF-κB in hepatocytes, and attenuated insulin resistance *in vivo*[95]. CYP2J2 overexpression was also found to attenuate nonalcoholic fatty liver disease induced in high fat diet mice by decreasing plasma triglyceride levels, reducing inflammatory responses, decreasing NF-κB pathway activation, and reducing oxidative stress[162].

2) EET-EA and EDP-EA contain epoxide and ethanolamide motifs, making them susceptible to degradation by sEH and FAAH. This renders EET-EA and EDP-EA relatively short-lived lipid mediators[83]. sEH hydrolyzes EET-EA and EDP-EA to their less biologically active vicinal diols, dihydroxyeicosatrienoic acid ethanolamide (DHET-EA) and dihydroxydocosapentaenoic acid ethanolamide (DHDP-EA), respectively[83,163]. FAAH hydrolyzes EET-EA and EDP-EA to their corresponding epoxides, EET and EDP, respectively[83,141]. sEH/FAAH greatly reduce the half-life of eCB, decreasing eCB abundance in tissues and the potency of their anti-inflammatory activities.

Studies have shown that sEH expression and activity are elevated in diabetic murine retinas, as well as diabetic human retinas and vitreous. The sEH hydrolysis product of DHA, diol 19,20-dihydroxydocosapentaenoic acid (19,20-DHDP), was found in elevated amounts in diabetic ocular environments, and seen to compromise pericyte-endothelial cell and inter-endothelial cell junctions by means of disturbed cholesterol-binding protein localization[164]. In

murine models of DR, retinal sEH expression and 19,20-DHDP levels increased in correlation with disease progression, with normal mice having half the retinal sEH expression of diabetic animals at 12 months. It was similarly found that non-diabetic mice engineered to overexpress sEH exhibited abnormalities comparable to those seen in DR mouse models[164]. sEH inhibition was shown to prevent vascular permeability and pericyte death in murine models of DR[164]. These results suggest that sEH is a contributor to DR pathology progression and represents a rational target for therapeutic intervention. sEH inhibitors (sEHi) are currently being tested in animal models of inflammatory disease to mitigate inflammation via CYP product stabilization. For example, sEH inhibition was shown to attenuate high blood pressure and glucose fluctuations in murine models of obesity and hypertension respectively[165]. Promising results in these studies have led to clinical trials testing sEH inhibition in diabetes-relevant pathologies, such as pulmonary disease in obese male smokers and hypertension and glucose tolerance in diabetic patients[166,167].

In studies of high intraocular pressure-induced ischemia, FAAH activity (assayed as hydrolysis rate of AEA to AA in rat retinas) was increased while endogenous AEA levels and CB1 expression declined[168]. In this context, systemic administration of a FAAH inhibitor was found to mitigate the damage caused by the ischemic model of acute glaucoma, including reducing cell loss[168]. In an *in vivo* model of optic nerve axotomy, FAAH inhibition was likewise found to be neuroprotective to retinal ganglion cells in young and aged rats, again confirming the therapeutic potential of maintaining the neuroprotective effects of eCB through the inhibition of their hydrolysis[169]. The efficacy of FAAH inhibitors (FAAHi) has also been tested in clinical trials for conditions such as major depressive disorder, Post Traumatic Stress Disorder, and osteoarthritis, but efficacy has yet to be evaluated in DR[170,171]. Therefore sEH/FAAH inhibition presents a rational therapeutic method to prolong eCB half-life, thereby elevating eCB levels, preserving anti-inflammatory bioactivities and reducing inflammation in the retina.

3) There is strong interest in the therapeutic potential of the endocannabinoid epoxides EET-EA and EDP-EA because they are anti-inflammatory lipid mediators that preferentially bind and activate CB2[83,141]. It is proposed that these eCB, as stated above, may be diminished in disease states, allowing for inflammatory responses that further propagate ECS dysfunction and sustain tissue inflammation. Therefore, the exogenous addition of either CB2-selective eCB or CB2-selective agonists may increase CB2 activation to homeostatic levels, returning the tissue environment to a non-disease state. For example, 19,20-EDP-EA addition was shown to decrease proinflammatory cytokine expression and increase anti-inflammatory cytokines in microglial mouse cells when studied in the context of neurodegenerative disease[83]. Additionally, several natural eCB ligands have been shown to elicit anti-inflammatory cellular responses in non-ocular disease models, such as suppressed L-homocysteine-induced IL-1β and caspase-1 activity and NLRP3 inflammasome activation in cultured podocytes[172], and reduced inflammatory and increased anti-inflammatory cytokine levels in LPS-treated microglia[83]. In our own preliminary experiments, we found similar protective properties were replicated by exogenous eCB in retinal cells (**Appendix B**).

While exogenous eCB addition holds therapeutic promise via the activation of CB2, this thesis work chose to focus on CB2 synthetic agonism for three reasons: 1) certain CB2 receptor agonists have been identified that are a thousand-fold more selective for CB2 over CB1, eliminating concerns about psychotropic side effects induced by some eCB; 2) CB2-specific agonists have extremely low affinity for other eCB receptors (e.g., transient receptor potential vanilloid 1 and 2 [TRPV1, 2]; other G protein-coupled receptors [GPR18, 55, 119]) that might otherwise confound mechanism of action; and 3) notably, CB2 agonists have already been tested in clinical trials for non-ocular inflammatory pathologies, lowering the bar for their clinical translation to DR treatments[173]. In both mouse and rat models of sepsis, synthetic CB2 agonists have demonstrated significant anti-inflammatory activity[174,175]. In rat models of Parkinson's disease, synthetic CB2 agonists showed neuroprotective and anti-inflammatory activities[155,176].

In mouse models of diabetes, synthetic CB2 agonists reduced blood plasma glucose and cytokine levels, and increased insulin secretion[155,177,178]. CB2 null mice demonstrate inflammatory phenotypes consistent with those produced by pharmacologic inhibition of CB2[179]. Collectively, this evidence strongly supports the idea that CB2 activation strategies can be harnessed to mitigate tissue inflammation and thus effectively target the retinal inflammation occurring secondary to diabetes.

Cannabinoid Receptor 2 Selective Agonists

We chose two highly CB2-selective, commercially available agonists for use in our studies: HU-308 and CB65. HU-308 and CB65 are both at least three orders of magnitude more selective for CB2 over CB1[180,181], allowing for CB2-specific activation at appropriate concentrations and avoiding off-target effects of CB1 activation. We also chose these agonists because they are structurally distinct from one another and are provided by different vendors (Cayman Chemical and Santa Cruz, respectively), allowing us to better substantiate our results and attribute our findings to the effects of CB2 signaling.

Previous literature contains references to HU-308 being therapeutic in ocular contexts. HU-308 has been described as having anti-inflammatory effects in endotoxin-induced uveitis, reducing pro-inflammatory mediators such as TNFα and IL-1β, and reducing pro-inflammatory transcription factors such as NF-κB and AP-1[182]. HU-308 was also therapeutic in an experimental model of proliferative vitreoretinopathy, decreasing mean histopathological scores, number of microglia, and leukocyte adhesion[183]. Finally, HU-308 was shown to reduce corneal inflammation in mouse models of corneal hyperalgesia[184]. The characterization of CB65 is less extensive in the literature, though studies in rat retinal ganglion cells show that HU-308 and CB65 suppressed T-type Ca^{2+} channels, showing their influence in regulating neuronal activity in the eye and their potential role in protecting RGCs against injury[185]. We anticipated the absence of adverse effects from this therapeutic strategy because the activation of CB2 is

ideally a modification of the ECS back to homeostasis. Consequently, this treatment approach is promising for transition to clinical applications.

NF-κB Signaling

CB2 is a G protein-coupled receptor that primarily couples to $G\alpha_{i/o}$ proteins, leading to the inhibition of adenylate cyclase and decreased cellular cAMP levels[186–188]. Under inflammatory stimulation, CB2-G protein-coupling has been observed to suppress ERK1/2 and p38 MAPK activation [189,190]. For example, IL-1β-induced activation of ERK1/2 and p38 was suppressed by HU-308, a potent and highly specific CB2 agonist, in fibroblast-like synoviocytes[190]. CB2 signaling through these combined pathways induced CREB-dependent transcription of anti-inflammatory IL-10[188]. Additionally, CB2-dependent downstream inhibition of the pro-inflammatory stress-activated protein kinase c-Jun NH2-terminal kinase (JNK) in LPS-treated monocytes has been reported[191]. In summary, these data suggest that CB2 signaling can be leveraged to control inflammation via the regulation of kinase signaling cascades.

The NF-κB pathway has long been recognized for its role in the development of inflammation, including the elaboration of inflammatory cytokines and leukocyte recruitment molecules[1,192,193]. Notably, each of the CB2-dependent ERK1/2, p38, and JNK pathways is known to activate NF-κB and AP-1[194–197], which may then engage in synergistic pro-inflammatory activities. Thus, based solely on what is known currently about CB2 signal transduction, it can be concluded that modulation of these signaling intermediates by CB2 can both elevate tissue levels of anti-inflammatory cytokines (e.g., IL-4, IL-10) and suppress levels of pro-inflammatory cytokines (e.g., TNFα, IL-1β). Furthermore, there is also direct evidence that CB2 agonism suppresses NF-κB-dependent inflammatory events. For example, both HU-308 and JWH-133, another CB2-selective agonist, inhibited TNFα-induced *ICAM-1* and *VCAM-1* expression, NF-κB nuclear translocation, and RhoA activation in coronary artery EC[193]. In another study, 2-AG, a non-selective CB2 ligand, inhibited induction of IL-2 by PMA/ionomycin

in mouse splenocytes and decreased NFATc and NF-κB promoter binding and activity[198]. While it is clear that CB2 activation mitigates inflammation via cell- and tissue-specific signaling, there remain significant gaps in our understanding of the precise signaling pathways that regulate this control.

Thesis Work Overview

We and others hypothesize that in the earliest stages of DR pathogenesis, initial insults to the retina are those imposed by metabolic dysfunction such as elevated glucose or free fatty acids in the bloodstream. Cells affected by these metabolic stimuli increase their expression and secretion of inflammatory cytokines, which become the dominant source of DR-associated local inflammation. It is well established that inflammation induces pathogenic behaviors in RMEC early in DR, and previous findings served as examples that CB2 activation suppresses analogous behaviors in non-ocular cells and tissues. For example, when hRMEC are activated by certain paracrine cytokines, they respond with increased expression and secretion of the same (auto-amplification) and other cytokines that further contribute to DR-related inflammation. Remarkably, Persidsky, et al. demonstrated that CB2 agonists were able to diminish the expression of 32 of 33 pro-inflammatory genes that were upregulated in brain microvascular EC treated with TNFα[199]. Additionally, inflammatory cytokines have been shown to induce leukostasis, stimulating expression of E-selectin which promotes leukocyte tethering, and VCAM-1 and ICAM-1 which induce firm adhesion to RMEC[56,58,200]. CB2 agonism prevents inflammation-induced adhesion molecule expression, monocyte adhesion, and/or monocyte extravasation in a variety of non-ocular EC types[151,193,201]. Here, we hypothesize that CB2 activation will mitigate these RMEC pathogenic and pathologic behaviors in the context of DR, and we demonstrate for the first time that CB2 agonism has therapeutic influence in the context of early DR intervention targeting leukostasis.

CHAPTER II

METHODS

The experiments described in this dissertation were conducted using both *in vitro* and *in vivo* methods. Our lab isolates and cultures a variety of primary retinal cell types, including Müller cells and vascular endothelial cells, increasing the relevance and accuracy of our *in vitro* disease models. Additionally, our *in vitro* results are further complemented and validated by work conducted in *in vivo* mouse models that recapitulate DR characteristics. Together, we believe that our models faithfully simulate a variety of DR features, and thus our methods appropriately test the potential efficacy of our proposed therapeutic strategies in DR.

Human Müller Cell Culture

Human Müller cells (hMC) were isolated from human donor tissue (NDRI; Philadelphia, PA) within 24 hours postmortem. The retinas were dissected from the eyecups and dissociated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies; Carlsbad, CA) containing 0.1% trypsin and 70U/mL collagenase (Worthington Biochemical Corp; Lakewood, NJ). Following incubation in dissociation medium, cells were grown in DMEM containing 10% fetal bovine serum (FBS; R&D Systems; Minneapolis, MN) and 1X antibiotic/antimycotic solution (Thermo Fisher Scientific LLC; Asheville, NC). Cells were incubated at 37°C, 5% CO_2, 20.9% O_2, and 95% relative humidity. Collectively, these conditions favor the survival of MC over other retinal cell types[202]. If needed, cultures were policed for removal of non-MC or colonies of pure MC were sub-cloned into a new dish. The final MC purity of cultures was > 97% and was determined by immunohistochemistry (IHC) with antibodies against cellular retinaldehyde-binding protein (CRALBP), glutathione synthetase (GS), and glial fibrillary acidic protein (GFAP). Passages 4-6 were used for all experiments.

Human Retinal Microvascular Endothelial Cell Culture

Primary human retinal microvascular endothelial cells (hRMEC) were purchased from Cell Systems (Kirkland, WA). Cells were validated using immunofluorescence, stained for the presence of cytoplasmic VWF/Factor VIII, cytoplasmic uptake of Di-I-Ac-LDL, cytoplasmic CD31, and absence of GS, GFAP, Neural/Glial Antigen 2 (NG2), and Platelet Derived Growth Factor Receptor beta (PDGFR-beta). hRMEC were cultured on Attachment Factor™ (Cell Systems)-coated culture dishes in Endothelial Basal Medium (EBM; Cell Signaling; Danvers, MA) with 10% FBS and EGM™ SingleQuots™ (Lonza, Inc.; Allendale, NJ). Cultures were incubated at 37°C, 5% CO_2, 20.9% O_2, and 95% relative humidity. hRMEC were used at passages 6-8.

Mouse Retinal Microvascular Endothelial Cell Culture

Male and female founder *Cnr2*$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME; B6.129P2-*Cnr2*^{tm1Dgen}/J; JAX® Strain No. 005786) and were bred to establish a *Cnr2*$^{-/-}$ colony. Mouse *Cnr2*$^{-/-}$-derived RMEC (mRMEC) were isolated using an adaptation of the Miltenyi Biotec protocol to isolate mouse brain microvascular endothelial cells[203]. Cells were validated using immunofluorescence, stained for the presence of CD31. mRMEC were cultured on Attachment Factor™-coated culture dishes in EBM (Cell Signaling) with 10% FBS and EGM™ SingleQuots™ (Lonza, Inc.). Cultures were incubated at 37°C, 5% CO_2, 20.9% O_2, and 95% relative humidity. mRMEC are used at passages 4-6.

Human Müller cell Treatment for Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

In preparation for treatment, hMC were seeded in 6-well plates and grown to 70% confluence using 10% FBS-containing DMEM. Culture media were changed to serum-reduced conditions

(2% FBS) for 12 hours before treatment. Cells were treated with SKF-525a (5.0μM; Enzo Life Sciences; Farmingdale, NY, USA) or vehicle for 24 hours. *Experiments using PA as a stimulus are described as follows.* Cells were treated for 24 hours in 2% FBS medium with BSA-bound palmitic acid (PA; 250μM; Sigma-Aldrich; St Louis, MO) or fatty acid-free BSA vehicle (100mg/mL in PBS; Sigma-Aldrich). BSA-bound PA was prepared by dissolving PA in EtOH at 200mM. This PA/EtOH solution was mixed for 2 hours at 37°C with 100mg/mL BSA in PBS to yield 5mM PA before dilution to the final concentration of 250μM in culture media. hMC treated with BSA-bound PA were co-treated during the final 3 hours of the 24-hour PA treatment with the sEH inhibitor GSK2256294 (0.1nM, 1.0nM, or 10nM; Axon Medchem LLC; Reston, VA); 11,12-EET (0.5μM; Cayman Chemical; Ann Arbor, MI); or 19,20-EDP (0.5μM; Cayman Chemical). *Experiments using IL-1β as a stimulus are described as follows.* Cells were treated for 8 hours in 2% FBS-containing DMEM supplemented with human recombinant protein IL-1β (1.0ng/mL; R&D Systems) and GSK2256294 (0.1nM, 1.0nM, or 10nM); 11,12-EET (0.5μM); or 19,20-EDP (0.5μM). In experiments using GSK2256294, cells were pre-treated with corresponding concentrations for 2 hours before treatment with IL-1β. In all experiments, epoxygenated fatty acid concentrations (0.5μM) were based on our previously published studies and literature precedents.

Human Retinal Microvascular Endothelial Cell Treatment for qRT-PCR

hRMEC were seeded in 12-well plates coated with Attachment Factor™ and treated at 90-100% confluency. hRMEC were stimulated with TNFα (0.1ng/mL; Sigma-Aldrich), or IL-1β (0.1ng/mL) and co-treated with HU-308 (1.0μM; Cayman Chemical), or CB65 (1.0μM; Tocris; Bristol, UK) for 4 hours.

qRT-PCR

After treatment, cells were washed twice with cold PBS, lysed with RNeasy Lysis Buffer (RLT; Qiagen; Germantown, MD), and total RNA was isolated using a RNeasy Mini kit (Qiagen). RNA was reverse transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems; Troy, NY). qRT-PCR was performed by co-amplification of target transcription vs. *18S* or *TBP* (TATA-binding protein), as normalization controls, using gene-specific TaqMan™ Gene Expression Assays (Applied Biosystems). The delta delta Ct method was used to determine the relative expression of the targeted mRNA normalized to *18S* or *TBP* levels. These commercial assays were performed according to the Applied Biosystems' protocol.

Soluble Protein Quantification

hMC were seeded in 24-well plates and grown to 70% confluence using 10% FBS-containing DMEM. Culture media were changed to serum-reduced conditions (2% FBS) for 12 hours before treatment. Cells were pretreated for 2 hours with GSK2256294 (10nM) and 11,12-EET (0.5μM) or 19,20-EDP (0.5μM). Media were removed and cells were treated with IL-1β (1.0ng/mL), GSK2256294, and 11,12-EET or 19,20-EDP for 8 hours. As controls, media were removed, and cells were treated similarly with GSK2256294 and 11,12-EET or 19,20-EDP for 8 hours in the absence of IL-1β. After the 8-hour treatment, culture media were collected and assayed for secreted IL-1β, IL-6, and IL-8 using the IL-1 beta Human ELISA Kit, High Sensitivity (Thermo Fischer Scientific) and IL-6 or IL-8 (human) AlphaLISA® Detection Kit (PerkinElmer, Inc.; Waltham, MA). Cells were washed with cold PBS and lysed using radio-immunoprecipitation assay buffer (RIPA buffer; Qiagen), and the concentration of cell lysates was determined using a bicinchoninic acid assay (BCA assay; Pierce; Rockford, IL). Secreted protein concentrations were normalized to total protein of corresponding cell lysates and reported as pg/mL.

Western Blot

hRMEC were seeded in 6-well plates and treated with TNFα or IL-1β (0.1ng/mL), in the presence or absence of HU-308 or CB65 (1.0μM) for 8 hours. A 30-minute pre-treatment of CB2 agonist (1.0μM) was used when hRMEC were stimulated with IL-1β. Post-treatment, cells were washed twice in cold PBS and lysed using RIPA buffer (Qiagen) with cOmplete™ protease inhibitors (Roche; Basel, Switzerland). The total protein concentration of each sample was determined using a Pierce BCA assay, and 20μg of each sample was migrated in 4-20% SDS-PAGE gel (Mini-PROTEAN® TGX™ Gels; Bio-Rad; Hercules, CA). Proteins were transferred to nitrocellulose membranes using the iBlot system (Thermo Fisher; Waltham, MA). Membranes were blocked and probed in TBS Intercept® Block Buffer (LI-COR Biosciences; Lincoln, NE) + 0.2% Tween 20 (Sigma-Aldrich) for ICAM-1 (1:1,000; Santa Cruz Biotechnology; Dallas, TX), VCAM-1 (1:1,000; Abcam; Cambridge, UK) and β-actin (1:1,000; Cell Signaling). Blots were labeled with IRDye® secondary antibodies (1:10,000; LI-COR Biosciences). β-actin was used as a loading control. Blots were quantified as a ratio of target to β-actin band density using ImageJ software.

Immunocytochemistry

hRMEC were seeded with Attachment Factor™ onto 15mm plastic coverslips (Thermo Scientific) placed in 24-well plates. Cells were treated with TNFα or IL-1β (0.1ng/mL), in the presence of HU-308 or CB65 (1.0μM) for 30 minutes. Cells were then fixed with 4% paraformaldehyde (PFA; Electron Microscopy Studies; Hatfield, PA), permeabilized with 0.3% Triton X-100™ (Sigma-Aldrich), blocked with nonfat dry milk (Blotting-Grade Blocker; Bio-Rad), and probed for NF-κB-P65 (1:200; Santa Cruz Biotechnology) and VE-cadherin (1:400; Abcam). Alexa Fluor secondary antibodies (Invitrogen; Waltham, MA) were added along with DAPI (1:1,000; Tocris), and slides were imaged using a Zeiss LSM 710 confocal microscope.

NF-κB Activity Assay

hMC experiments: hMC were seeded on 96-well black-walled, clear bottom plates. Each well was transfected with 200 ng NF-κB-luciferase promoter-reporter from the Cignal NFκB Reporter Assay (Qiagen) for 12 hours. Cells were then washed with PBS and fresh 10% FBS DMEM was added for 12 hours. At 24 hours post-transfection, cells were treated with vehicle, IL-1β (1.0ng/mL) or PA (250μM) in the presence or absence of 11,12-EET (0.5μM) or 19,20-EDP (0.5μM) for 4 hours and 8 hours respectively. Firefly and Renilla luciferase activity was quantified using the Dual-Glo® Luciferase Assay System (Promega; Madison, WI), according to the manufacturer's protocol. Data were reported as the relative ratio of Firefly-to-Renilla luciferase activity. *hRMEC experiments:* hRMEC were seeded in 48-well plates using Attachment Factor™ Each well was transfected with 200ng NF-κB-luciferase promoter-reporter from the Cignal NFκB Reporter Assay for 12 hours. Cells were then washed with PBS and fresh 10% FBS EBM was added for 12 hours. At 24 hours post-transfection, cells were treated with vehicle, TNFα (1.5ng/mL) for 8 hours or IL-1β (1.0ng/mL) for 6 hours, in the presence or absence of HU-308 or CB65 (1.0μM). Firefly and Renilla luciferase activity was quantified using the Dual-Glo® Luciferase Assay System, according to the manufacturer's protocol. Data were reported as the relative ratio of Firefly-to-Renilla luciferase activity.

Static Adhesion Assay

hRMEC were seeded in 24-well plates coated with Attachment Factor™ and treated with TNFα or IL-1β (0.1ng/mL), in the presence or absence of HU-308 or CB65 (1μM) for 4 and 8 hours, respectively. Human peripheral blood mononuclear cells (PBMC; Precision for Medicine; Fredrick, MD) were stained with NucBlue™ (Thermo Fisher) for 20 minutes at 37°C, and added to each well at a concentration of 250,000 cells/well. PBMCs were allowed to settle on hRMEC monolayers for 30 minutes at 37°C, after which monolayers were gently washed three times

with warm PBS to remove non-adherent cells. Monolayers were fixed with 4% PFA for 10 minutes at 37°C and washed once more with warm PBS. Four fields were selected per well and adherent PBMC were counted. Each data point represents the average number of adherent leukocytes for the four captured fields per well and were reported as adherent cells per mm^2.

In vivo Model Generation for Retinal Leukostasis Assay

All experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Acute Inflammation Model: Six-week-old C57BL/6J mice (Charles River; Wilmington, MA) received daily intraperitoneal injections of vehicle (0.1% DMSO and 0.3% EtOH), or HU-308 (5mg/kg) for one week, then received an intravitreal injection of TNFα (25ng/mL). The leukostasis assay was performed 12 hours post-TNFα injection.

*STZ Model***:** Six-week-old C57BL/6J mice (Charles Rivers) were given streptozotocin (STZ; Selleck Chemical; Houston, TX) for 5 consecutive days. For intraperitoneal (IP) administration, mice were given daily IP injections of vehicle or HU-308 (5mg/kg) for 4 weeks, 6 weeks post-STZ injections. For intravitreal (IVIT) administration, mice were given a single IVIT injection of HU-308 (5μM) the day before leukostasis readings. Leukostasis was performed 10 weeks post-STZ injections.

Retinal Leukostasis Assay

As adapted from Bretz et al.[200], mice were anesthetized with ketamine and xylazine and perfused with 0.9% saline for 2.5 minutes, followed by an infusion of FITC-conjugated concanavalin-A (40μg/mL in 2.5 mL PBS; Vector Laboratories; Burlingame, CA) to label the retinal vasculature and leukocytes. Mice were then perfused with saline again for 2.5 minutes to remove any non-adherent leukocytes. Retinas were immediately dissected into 4% PFA, flat-

mounted, and images were captured with an AX70 upright microscope (Olympus; Tokyo, Japan) and DP71 digital camera (Olympus) at 4x magnification. Adherent leukocytes in the vasculature were counted and reported per mm^2.

Statistical Analysis

Data were analyzed using Prism software (GraphPad; La Jolla, CA). T-tests and analyses of variance (ANOVAs) with Tukey's multiple comparisons post-hoc test were used to evaluate significant differences among treatment groups. Values of $p<0.05$ were considered statistically significant.

CHAPTER III

CYTOCHROME P450-EPOXYGENATED FATTY ACIDS INHIBIT MÜLLER GLIAL INFLAMMATION

From: Ontko CD, Capozzi ME, Kim MJ, McCollum GW, Penn JS. Cytochrome P450-epoxygenated fatty acids inhibit Müller glial inflammation. Sci Rep. 2021 May 6;11(1):9677. PMCID: PMC8102485.

Introduction

DR progression is associated with systemic dyslipidemia, and circulating free fatty acids (FFAs) are known to initiate inflammatory cytokine release[22,23,204]. Diabetic mice have over three times the retinal fatty acid content of healthy controls, and palmitic acid (PA) is elevated above other FFAs in the circulation and tissues of diabetic patients and experimental models of diabetes[45,205–209]. Müller cells (MC) are particularly responsive to PA and other FFA[44,45]. RNA sequencing has shown that PA stimulates a variety of DR-relevant pathways in MC – including NF-κB signaling and inflammation, intracellular lipid signaling, angiogenesis, and MAPK signaling – that are not altered by elevated glucose stimulation alone[45]. It is proposed that diabetes-related dysregulation of PA and other FFAs inflame MC, resulting in their production of inflammatory retinal cytokines[1,44,45]. One such cytokine, interleukin-1β (IL-1β), is purported to be a “master regulator” of cytokine-induced inflammation[43,210]. IL-1β is elevated in DR and induces MC to produce and release itself (auto-amplification) and other inflammatory cytokines. When MC become activated in response to diabetes-related metabolic dysfunction, they divert from their homeostatic functions and contribute to DR pathogenesis and progression[45,65,211]. The foregoing, along with other MC-dependent behaviors, suggests that therapies targeting MC inflammation in DR could potentially preempt PDR and its vision threatening consequences.

Ample data suggest that lipid mediators derived from arachidonic acid (AA; ω-6) and

docosahexaenoic acid (DHA; ω-3) regulate diabetes-induced retinal inflammation[1,83,84]. AA is metabolized by the cyclooxygenase (COX) and lipoxygenase (LOX) pathways to yield pro-inflammatory metabolites such as leukotrienes, HETEs and prostaglandins[1,88]. Conversely, DHA yields COX and LOX metabolites that are largely anti-inflammatory such as “resolvins” and “protectins”[88]. The epoxygenation of ω-6 and ω-3 fatty acids by the cytochrome P450 epoxygenase (CYP) pathway remains less explored. CYPs are endoplasmic reticulum membrane-bound monooxygenases that act on a variety of substrates to yield epoxygenated lipid metabolites that demonstrate potent anti-inflammatory activities in a variety of biological systems[56]. CYP2C8, CYP2C9, and CYP2J2 enzymes epoxygenate AA and DHA to epoxyeicosatrienoic acid (EET) and epoxydocosapentaenoic acid (EDP), respectively[56,83,212]. CYP levels are suppressed in diabetic conditions, and reduced levels of EETs are observed in the vitreous of patients with NPDR and PDR[37,118]. Additionally, soluble epoxide hydrolase (sEH), an enzyme that degrades epoxides, exhibits increased activity in response to diabetic conditions, contributing to lower epoxygenated fatty acids levels, and creating conditions permissive to inflammation[121,213]. Thus, pharmacologic manipulations that elevate epoxygenated fatty acids might constitute a rational strategy to reduce retinal inflammation in DR. We have previously shown that 11,12-EET and 19,20-EDP mitigate the pathologic responses of TNFα-stimulated human retinal microvascular endothelial cells (hRMEC)[56]. We wished to also determine if similar therapeutic effects manifest in cytokine-stimulated human MC. In this study, we tested the efficacy of 11,12-EET and 19,20-EDP to mitigate PA- and IL-1β-induced expression of inflammatory cytokines in primary cultures of human Müller cells (hMC) via exogenous epoxide addition and epoxide hydrolysis inhibition.

Results

Before testing the effects of increasing epoxygenated fatty acids levels in hMC, we first investigated the effects of their depletion. CYP epoxygenase activity is responsible for

converting AA and DHA to regioisomeric EETs and EDPs respectively[56]. In order to prove the concept that epoxide bioactivity is an anti-inflammatory influence, hMC were treated with the broad-spectrum CYP inhibitor SKF-525a to reduce intracellular EET/EDP levels. In the presence of SKF-525a we observed significant increases in the expression levels of the DR-relevant inflammatory cytokines TNFα, IL-1β, IL-6 and IL-8. Others have shown that the proinflammatory effects of SKF-525a, confirming the inhibition of epoxide formation and showing increased prostaglandin production under LPS-stimulated conditions[214,215]. Combined, these observations support our hypothesis that epoxide depletion, such that is hypothesized to occur in DR, promotes hMC inflammation. Herein, we show that exogenous addition of 11,12-EET, 19,20-EDP, and the sEH inhibitor GSK2256294, demonstrated a potent capacity to reduce inflammatory cytokine expression in hMC activated by PA and IL-1β. While previously demonstrated in hRMEC, this is the first report of the anti-inflammatory potential of these agents in retinal glia.

The CYP epoxygenase inhibitor SKF-525a promotes inflammatory cytokine expression in hMC.

hMC were treated with the CYP epoxygenase inhibitor SKF-525a or vehicle and inflammatory cytokine expression was assayed via qRT-PCR. SKF-525a increased expression of the following DR-relevant cytokines: *TNF* (5.13 fold; p=0.057), *IL1B* (3.92 fold; p<0.0001)*, IL6* (2.38 fold; p=0.0001), and *CXCL8* (2.90 fold; p=0.033). Only *TNF* did not achieve statistical significance (**Fig. 5**).

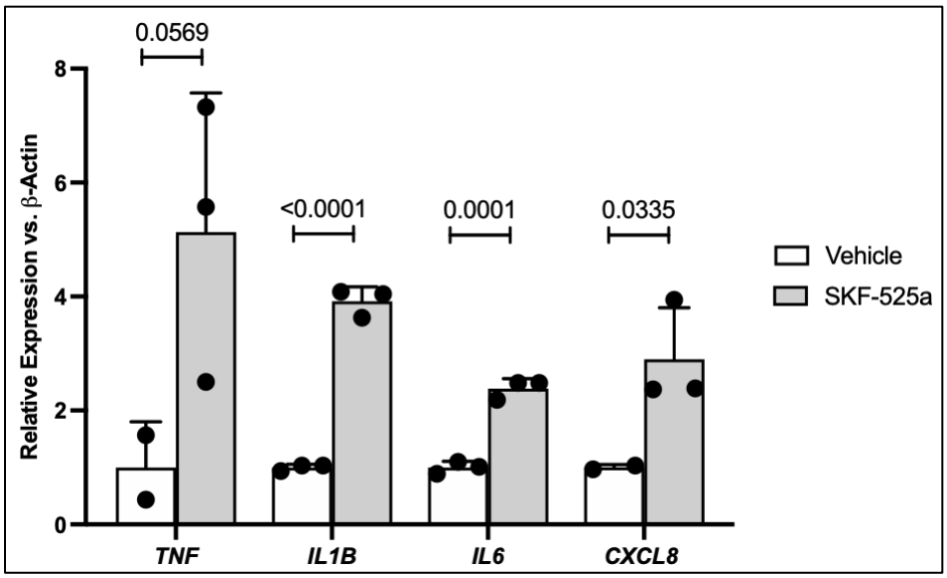


Figure 5. The effect of CYP epoxygenase inhibitor SKF-525a on Müller cell inflammatory cytokine expression. Human Müller cells were treated with SKF-525a (5.0μM) for 24 hours. After treatment, total RNA was isolated and inflammatory cytokine expression was assayed by qRT-PCR. *TNF*, *IL1B*, *IL6* and *CXCL8* expression was increased by SKF-525a, though statistical significance was not achieved for *TNF*. Data are displayed as mean ± SD (n=2 or 3).

11,12-EET, 19,20-EDP or the sEH inhibitor GSK2256294 reduces PA-stimulated inflammatory cytokine expression.

The epoxygenated fatty acids 11,12-EET, 19,20-EDP or the sEH inhibitor, GSK2256294, were tested against PA-induced inflammatory cytokine expression in hMC. hMC were treated with PA in the presence or absence of 11,12-EET, 19,20-EDP or GSK2256294, and inflammatory cytokine expression was assessed by qRT-PCR. 11,12-EET significantly reduced PA-stimulated expression of *TNF* by 84.67%, *IL1B* by 68.72% and *IL6* by 58.54% (**Fig. 6a-c**. p=0.0099, p<0.0001, p=0.0008, respectively). 19,20-EDP significantly reduced PA-stimulated expression of *TNF* by 63.67%, *IL1B* by 56.76%, and *IL6* by 56.19% (**Fig. 6a-c**. p=0.0434, p=0.0001, p=0.0011, respectively). 11,12-EET and 19,20-EDP reduced PA-stimulated *CXCL8* expression by 26.70% and 28.59%, though results were not significant. (**Fig. 6d**). A range of GSK2256294 concentrations were tested (0.1nM, 1.0nM, and 10nM) and GSK2256294 significantly reduced PA-stimulated hMC cytokine expression at each concentration. At the

lowest concentration tested, 0.1nM, GSK2256294 reduced PA-stimulated expression of *TNF* by 90.94%, *IL1B* by 67.31%, *IL6* by 60.86%, and *CXCL8* by 47.02% in hMC (**Fig. 7a-d**. p=0.0017, p<0.0001, p=0.0003, p<0.0001, respectively). At 1nM, GSK2256294 reduced PA-stimulated expression of *TNF* by 91.64%, *IL1B* by 70.39%, *IL6* by 62.13%, and *CXCL8* by 58.74% in hMC (**Fig. 7a-d**. p=0.0016, p<0.0001, p=0.0002, p<0.0001, respectively). At 10nM, GSK2256294 reduced PA-stimulated expression of *TNF* by 94.65%, *IL1B* by 79.87%, *IL6* by 75.36%, and *CXCL8* by 58.83% in hMC (**Fig. 7a-d**. p=0.0012, p<0.0001, p<0.0001, p<0.0001, respectively).

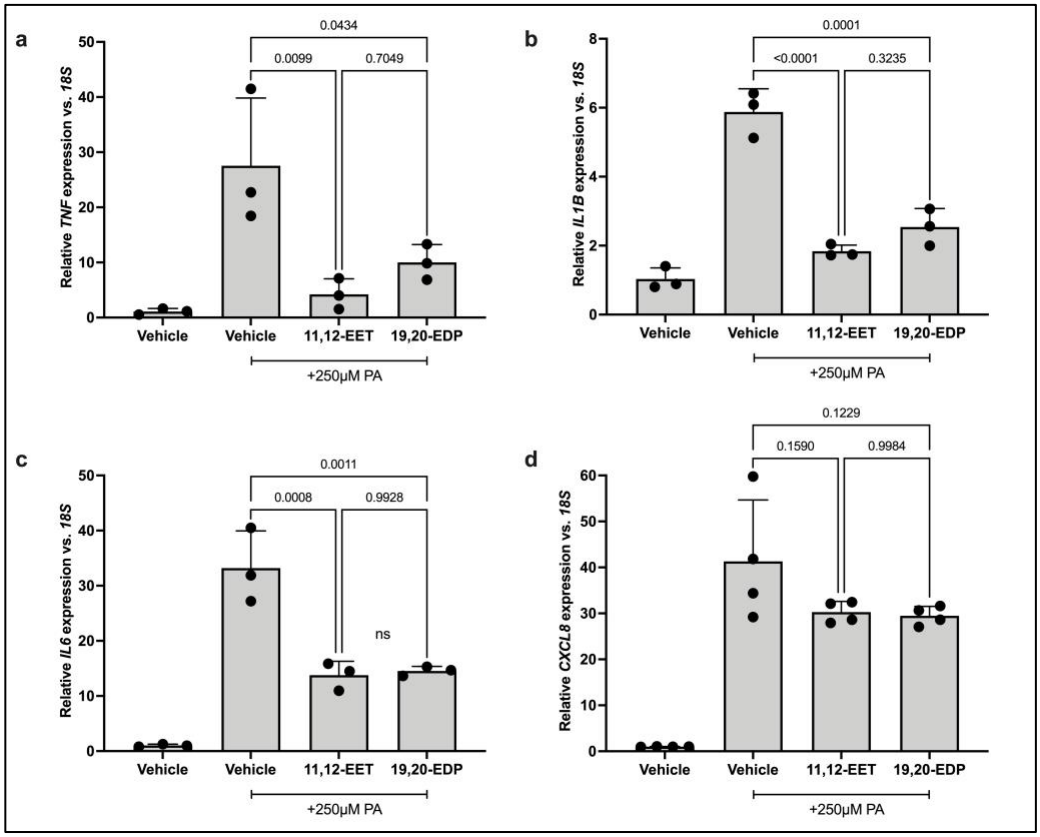


Figure 6. The effect of 11,12-EET and 19,20-EDP on PA-induced inflammatory mediator expression by Müller cells. Human Müller cells were treated with PA (250μM) for 24 hours. 11,12-EET (0.5μM) or 19,20-EDP (0.5μM) was added during the final 3 hours of treatment. After 24 hours, total RNA was isolated and expression was assayed by qRT-PCR. (a) *TNF* (b) *IL1B*, and (c) *IL6* expression was significantly decreased by both epoxygenated fatty acids. (d) *CXCL8* expression was reduced but statistical significance was not achieved. Results depicted are representative of three separate experiments. Data are displayed as mean ± SD (n=3 or 4 for each experiment).

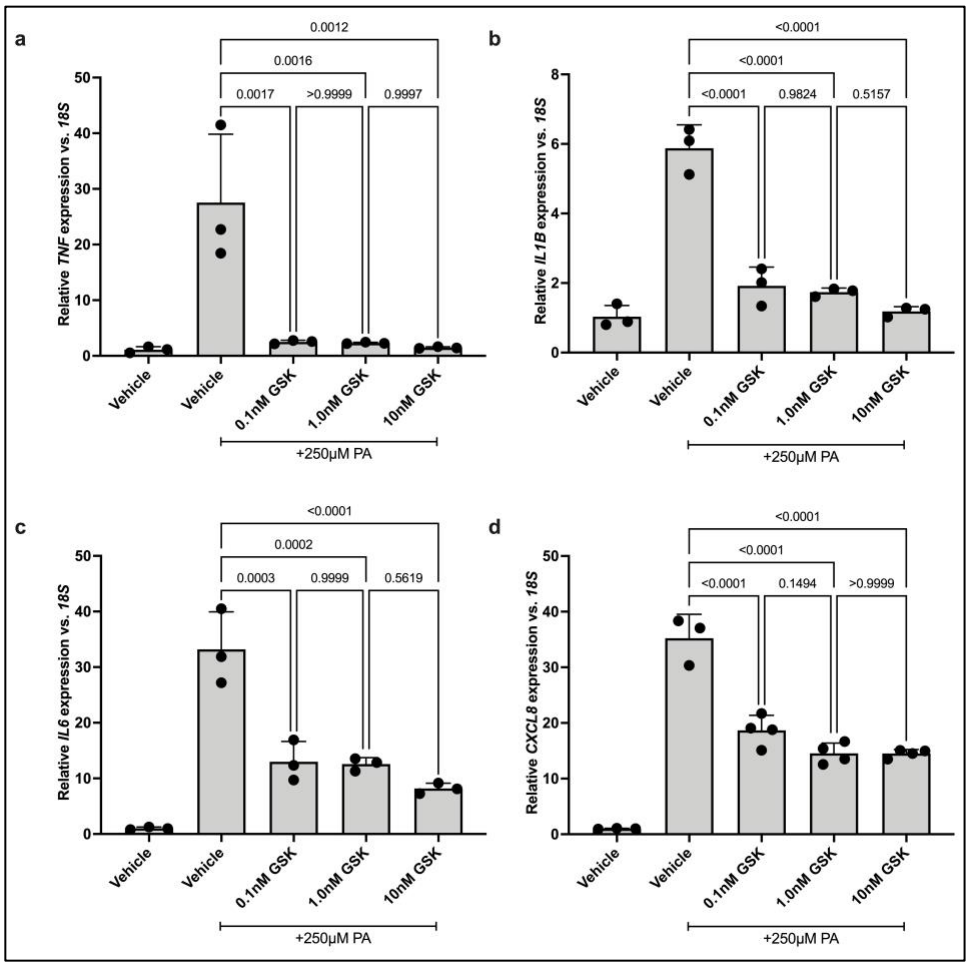


Figure 7. The effect of sEH inhibitor GSK2256294 on PA-induced inflammatory mediator expression by Müller cells. Human Müller cells were treated with PA (250μM) or PA plus 0.1nM, 1.0nM or 10nM GSK2256294 (sEH inhibitor). After 24 hours, total RNA was isolated, and expression was analyzed by qRT-PCR. (a) *TNF*, (b) *IL1B*, (c) *IL6*, and (d) *CXCL8* expression was significantly decreased by the addition of the sEH inhibitor at each of the concentrations tested. Results depicted are representative of three separate experiments. Data are displayed as mean ± SD (n=3 or 4 for each experiment).

11,12-EET, 19,20-EDP or the sEH inhibitor GSK2256294 reduces IL-1β-stimulated inflammatory cytokine expression.

hMC were treated with IL-1β in the presence or absence of 11,12-EET, 19,20-EDP or GSK2256294 to test the effect of each on IL-1β-induced inflammatory cytokine expression. Total RNA was isolated, and inflammatory cytokine expression was assessed by qRT-PCR. 11,12-EET significantly reduced IL-1β-stimulated expression of *IL1B* by 35.65%, *IL6* by 30.06%, and

CXCL8 by 27.26% in hMC (**Fig. 8b-d**. p=0.0036, p=0.0125, p=0.0184, respectively). 19,20-EDP significantly reduced IL-1β-stimulated expression of *IL1B* by 22.88%, *IL6* by 37.18%, and *CXCL8* by 24.10% in hMC (**Fig. 8b-d**. p=0.0414, p=0.0036, p=0.0342, respectively). *TNF* expression, however, was significantly increased by both epoxygenated fatty acids (**Fig. 8a**. p=0.0026, p=0.0136). sEH inhibition was tested at a range of GSK2256294 concentrations (0.1nM, 1.0nM, and 10nM) and at each concentration IL-1β-stimulated cytokine expression was significantly reduced. At the lowest concentration tested, 0.1nM, GSK2256294 reduced IL-1β-stimulated expression of *TNF* by 25.11%, *IL1B* by 40.78%, *IL6* by 29.05%, and *CXCL8* by 36.37% in hMC (**Fig. 9a-d**. p=0.0003, p<0.0001, p<0.0001, p<0.0001, respectively). At 1nM, GSK2256294 reduced IL-1β-stimulated expression of *TNF* by 38.56%, *IL1B* by 48.33%, *IL6* by 42.56%, and *CXCL8* by 44.91% in hMC (**Fig. 9a-d**. p<0.0001, p<0.0001, p<0.0001, p<0.0001, respectively). At 10nM, GSK2256294 reduced IL-1β -stimulated expression of *TNF* by 79.45%, *IL1B* by 79.96%, *IL6* by 62.26%, and *CXCL8* by 78.05% in hMC (**Fig. 9a-d**. p<0.0001, p<0.0001, p<0.0001, p<0.0001, respectively).

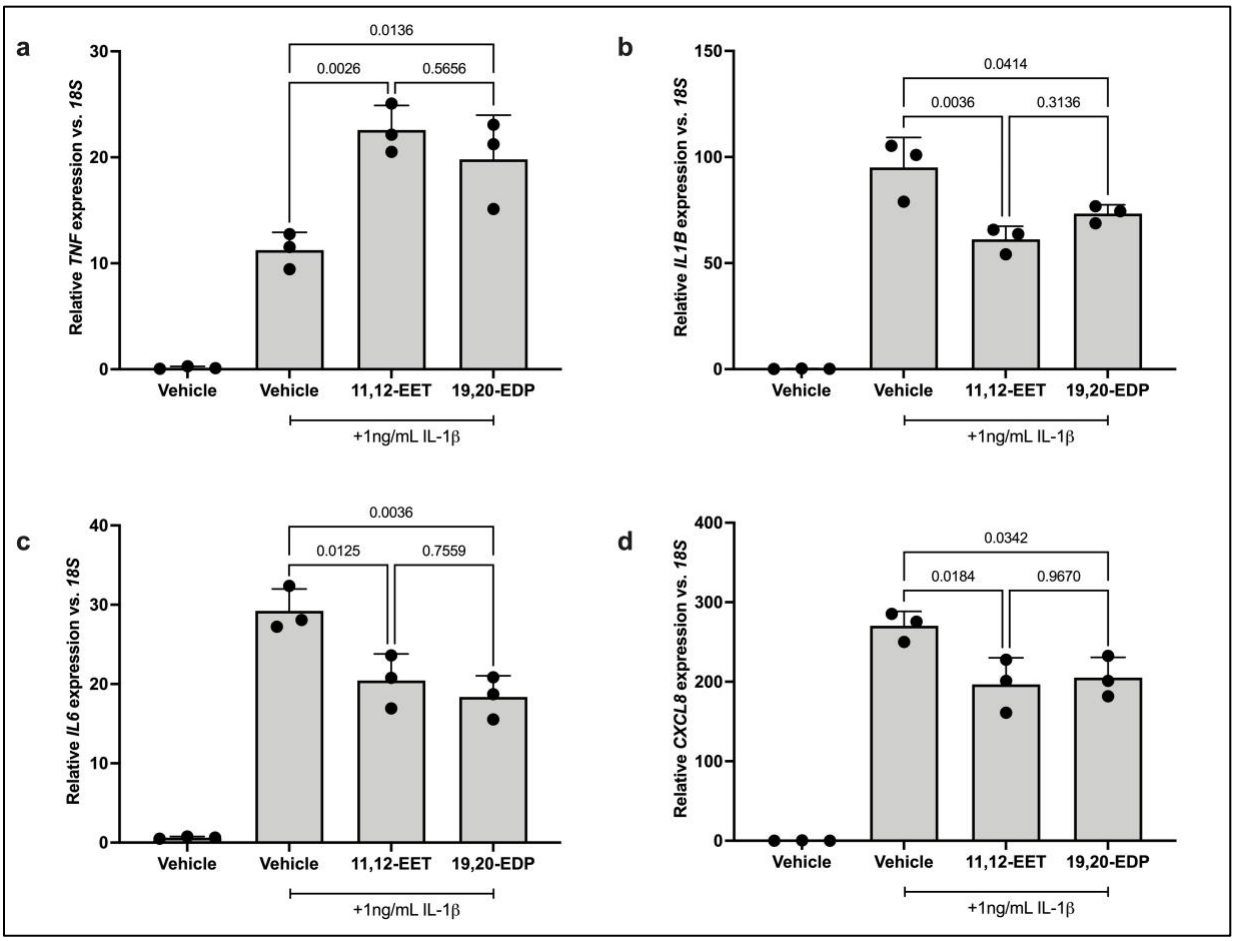


Figure 8. The effect of 11,12-EET and 19,20-EDP on IL-1β-induced inflammatory cytokine expression by Müller cells. Human Müller cells were treated with IL-1β (1.0ng/mL) or IL-1β plus 11,12-EET (0.5μM) or 19,20-EDP (0.5μM) for 8 hours. Total RNA was isolated and cytokine expression was assayed by qRT-PCR. (a) *TNF* expression was significantly elevated while (b) *IL1B*, (c) *IL6*, and (d) *CXCL8* expression was significantly decreased by the addition of both exogenous epoxygenated fatty acids. Results depicted are representative of three separate experiments. These data are normalized to induction levels illustrated in **Figure 9**. Data are displayed as mean ± SD (n=3).

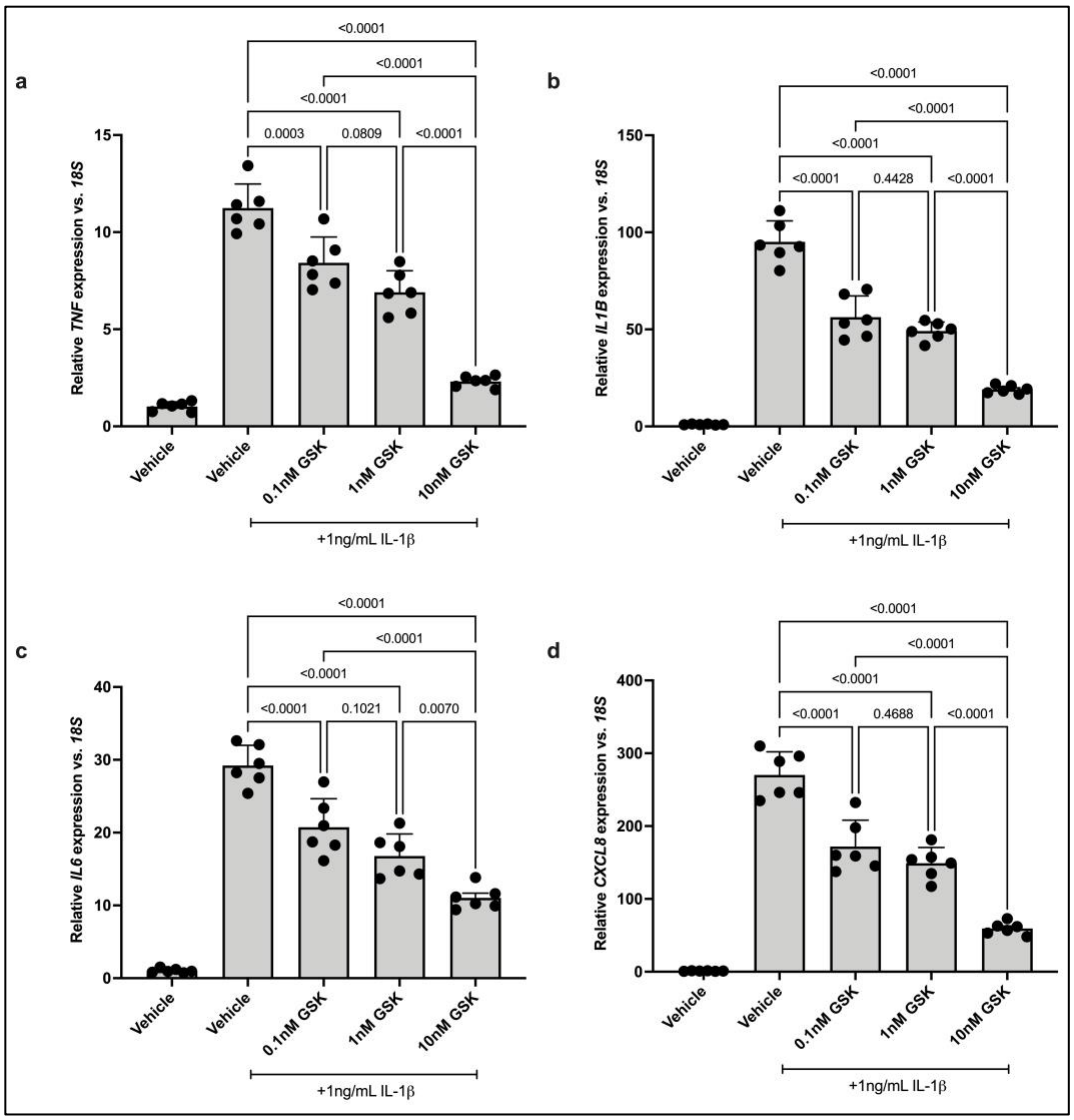


Figure 9. The effect of sEH inhibitor GSK2256294 on IL-1β-induced inflammatory mediator expression by Müller cells. Human Müller cells were treated with IL-1β (1.0ng/mL) or IL-1β plus 0.1nM, 1.0nM, or 10nM GSK2256294 (sEH inhibitor) for 8 hours. Total RNA was isolated and cytokine expression was assayed by qRT-PCR. (a) *TNF,* (b) *IL1B*, (c) *IL6*, and (d) *CXCL8* expression was significantly decreased at each sEH inhibitor concentration tested. Bars represent mean ± SD (n=6).

11,12-EET or 19,20-EDP reduces PA- and IL-1β-induced NF-κB promoter activity.

hMC were transfected with a NF-κB-luciferase promoter-reporter construct and treated with PA or IL-1β in the presence or absence of 11,12-EET or 19,20-EDP. As shown in **Figure 10**, 11,12-EET and 19,20-EDP decreased both PA and IL-1β-induced NF-κB-dependent luciferase

activity. 11,12-EET and 19,20-EDP decreased PA-induced reporter activity by 49.2% and 57.3%, respectively (**Fig. 10a**. p<0.0001, p<0.0001), and they decreased IL-1β-induced reporter activity by 23.6% and 17.2%, respectively (**Fig. 10b**. p=0.0006, p=0.0116).

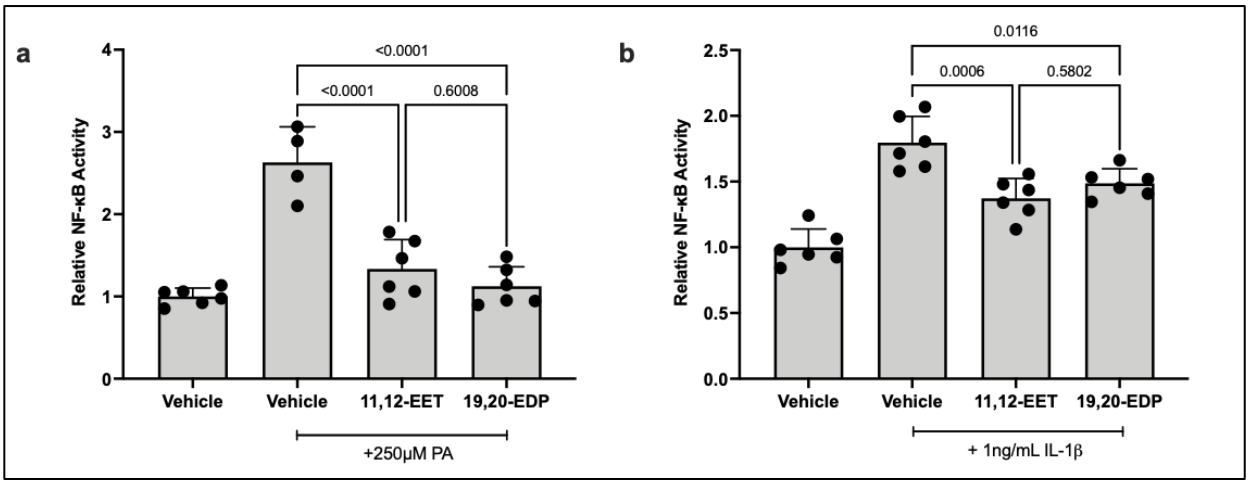


Figure 10. The effect of 11,12-EET or 19,20-EDP on PA or IL-1β-induced NF-κB promoter activity. hMC were transfected with a NF-κB-luciferase reporter construct and treated with (a) PA (b) IL-1β or in the presence or absence of 11,12-EET (0.5μM) or 19,20-EDP (0.5μM). NF-κB activity was determined by measuring the ratio of firefly-to-renilla luciferase luminescence activity. Each bar represents the mean ± SD (n=4-6).

Conclusions

EET or EDP in combination with sEHi were previously shown to mitigate several DR-relevant experimental endpoints including: the expression of leukocyte adhesion proteins by hRMEC, peripheral blood monocyte (PBMC) adhesion to hRMEC monolayers, and TNFα-induced retinal leukostasis in mice[56]. In these studies, epoxygenated fatty acids were determined to act through NF-κB-dependent signaling[56]. The anti-inflammatory potency of these lipid mediators in hRMEC raised speculation of their efficacy in other retinal cell types that are known to contribute to inflammatory conditions, such as glia. MC serve as a functional link between the neuronal and vascular components of the vertebrate retina and are known to initiate and sustain retinal inflammation in a variety of pathologic conditions[66]. MC span nearly the entire thickness of the retina and control retinal homeostasis including recycling

neurotransmitters, maintaining the inner blood-retinal barrier, regulating retinal cation flux, and maintaining photoreceptor function[66]. MC function in innate immunity[216] and it is hypothesized that diabetes-induced inflammation leads to MC dysfunction, resulting in a destructive MC phenotype that promotes DR pathogenesis[66]. Additionally, changes in MC have been observed prior to the appearance of overt vascular pathology in DR[66–68]. Accordingly, therapeutics that block diabetes-related MC inflammation could prevent or slow the onset and progression of early DR.

11,12-EET and 19,20-EDP were selected for these experiments because both demonstrated efficacy in previous studies[56,97]. Additionally, 19,20-EDP is the most highly abundant regioisomer in because CYP epoxygenases preferentially mono-oxygenate the terminal double bond of DHA, and sEH hydrolysis of 19,20-EDP is slower compared to the other regioisomers[83,123,124]. Therefore, 19,20-EDP turnover is presumably lower, enhancing efficacy through increased bioavailability. We chose 11,12-EET because it was one of the most abundant EET regioisomers in tissues and demonstrated potent anti-inflammatory activities in previous studies[97,106]. Notably, compared to other regioisomers, 11,12-EET and 19,20-EDP were also found in higher concentrations in MC-conditioned medium as determined by our mass spectrometric analysis (**Supp. Fig. 1, Appendix**). It is important to note that EET and EDP are degraded relatively rapidly *in situ*, and thus sEH inhibitors are a rational therapeutic route to extend epoxide half-lives, and these are currently under development for human use[165–167]. Administration of sEH inhibitors would be expected to extend the biological half-lives and activities of all epoxygenated fatty acids, meaning that any efficacy observed against inflammation would presumably result from a summed response on all regioisomers, potentially maximizing the effect.

We hypothesize that in the earliest stages of DR pathogenesis, the predominant stimuli are those imposed by metabolic dysfunction such as elevated glucose and/or free fatty acids in the bloodstream and ocular tissues. Abnormal levels of glucose and/or FFA may cause damage

to retinal cells that respond by producing and releasing inflammatory cytokines. These cytokines amplify through autocrine and paracrine mechanisms and become the dominant inflammatory stimulus in late-stage DR. Accordingly, we purposefully chose two stimuli, one from each of these stages, to test whether epoxides could continuously intervene, as the weights of these respective stimuli shift along the temporal axis of DR pathogenesis. Past experiments show that, among non-neuronal retinal cells, MC demonstrated the greatest increases in expression and secretion of inflammatory mediators in response to metabolic stimuli[44]. Accordingly, we believe that MC act as the primary driving force of chronic inflammation in DR through their synthesis, release and auto-amplification of inflammatory cytokines that propagate inflammation in neighboring vascular and neuronal cells. While elevated glucose is commonly used to simulate diabetic conditions *in vitro*, we found that elevated glucose yields little to no response when studying many primary human retinal cells. However, FFAs reliably and consistently induce inflammation in these cells, which is consistent with DR[44,45]. Thus, we studied the response of primary human retinal cells to a free fatty acid, PA, that plausibly models the influence of diabetes-associated dyslipidemia. We specifically demonstrated the effectiveness of PA as a DR-appropriate stimulus for human Müller cells. We chose to use 250μM PA because it is physiologically relevant. Analysis of plasma levels of free fatty acids determined PA to be at a concentration of 234.9 +/- 58.1μmol/L in obese diabetic individuals fasted overnight[207]. Similar studies aiming to create comprehensive profiles of fatty acids in the plasma of type 2 diabetics have substantiated this finding[217]. Furthermore, this concentration is within ranges used in studies of other retinal cell behaviors, as well as other *in vitro* studies of diabetes[218–220]. We chose to use 1ng/mL IL-1β empirically, because this concentration promoted elevated gene expression of the cytokines TNFα, IL-1β, IL-6, and IL-8 in hMC cultures, like that observed in the vitreous of diabetic patients and retina of experimental diabetes models[1,34,36–38,40,52,54,221]. Likewise, *in vivo,* cytokine-producing Müller cells are juxtaposed to vascular and neuronal responder cells, causing local concentrations at surface receptors that are higher than those

measured in ocular fluids, retinal lysates and sera. Our chosen concentration of IL-1β is well within the range of those concentrations tested in several published studies[43,222] mimicking cytokine amplification via autocrine and paracrine mechanisms. Finally, while lower IL-1β concentrations could also be relevant to DR inflammation, EET/EDPs and sEH inhibition proved efficacious when tested against the severe inflammation induced by 1ng/mL, suggesting that this therapeutic strategy would be effective over a range of inflammatory conditions that reflect DR onset and progression.

Our data demonstrate that exogenous administration of 11,12-EET and 19,20-EDP significantly decreased hMC cytokine expression induced by the two different inflammatory stimuli, PA and IL-1β. We also demonstrated that these epoxide-dependent activities manifest at the protein level in hMC when using experimental conditions that enhanced the levels and biological half-lives of the epoxides in culture (**Supp. Fig. 2, Appendix**). While both epoxygenated fatty acids decreased PA-induced *TNF* expression, they exacerbated IL-1β-induced *TNF* expression, suggesting a different mechanism of action in the two cases. The exact mechanism of action by which EET and EDP function has yet to be determined, though the results of our NF-κB-luciferase experiments indicated that both epoxygenated fatty acids decrease cytokine expression, at least in part, by modulating pathways that converge on NF-κB-dependent transcription. NF-κB is a pro-inflammatory transcription factor that controls the expression of inflammatory cytokines and plays an important and well-recognized role in early DR pathogenesis[1,33]. Similar findings were obtained in our previous studies using human retinal microvascular endothelial cells, and there is ample evidence of this mechanism in other cells and tissues[56,97,223,224]. Saturated fatty acids activate toll-like receptors expressed by MC that are upstream of NF-κB-dependent transcription[225,226]. Additionally, the canonical IL-1β signaling pathway includes NF-κB activation[1]. Therefore, we speculate that EET and EDP decrease *IL1B*, *IL6*, *CXCL8,* and PA-induced *TNF* mRNAs in part by an NF-κB-dependent mechanism, while another signaling mechanism becomes overriding in the case of IL-1β-induced effects on *TNF*

mRNA. We do not consider this observation a deterrent to this therapeutic approach because we have previously shown that EET and EDP decrease TNFα-induced leukocyte adhesion functions in hRMEC[56]. Therefore, any potentially detrimental effects of MC-derived TNFα on the retinal endothelium would be mitigated downstream by EET/EDP treatment.

We also tested the capacity of sEH inhibitor GSK2256294 to reduce inflammatory cytokines in PA- and IL-1β-treated hMC. GSK2256294 blocks the hydrolysis of endogenous EET/EDP, raising their endogenous cellular concentrations to therapeutic levels. The results of several studies indicate that sEH inhibition is a promising therapeutic modality in a wide variety of systems. In our studies, we observed a consistent reduction of cytokine mRNAs across all GSK2256294 concentrations tested (0.1nM, 1.0nM, and 10nM). Interestingly, while hMC responded to sEH inhibition alone, hRMEC did not, suggesting that hMC may be a sight of bioactive sEH that modulates the paracrine effects of EET/EDP. Similarly, others have shown that sEH is highly expressed in MC[164]. While GSK2256294 potently inhibits sEH activity in hMC, it is important to note that it can exert off-target effects related to the endpoints explored in this study. For instance, in studies of severe asthma, sEH inhibition has been correlated with increased concentrations of lipoxin A4, an anti-inflammatory compound that reduced inflammation and leukocyte-platelet aggregates in this context[227]. However, in the present study, this metabolite was not detected when queried in the conditioned medium of MC by mass spectrometric analysis. sEH is constitutively expressed in the retina and is elevated in the diabetic murine retina as well as in the diabetic human retina and vitreous[164]. sEH activity in diabetes is thought to be responsible for pericyte loss and endothelial barrier dysfunction by promoting the production of pro-inflammatory diol 19,20-DHDP, the hydrolysis product of 19,20-EDP. 19,20-DHDP alters the localization of cholesterol-binding proteins in the cell membrane, disrupting pericyte-endothelial cell junctions and inter-endothelial cell junctions. Like the expression of sEH, the accumulation of 19,20-DHDP is significantly increased in samples from patients with diabetic retinopathy[164]. To ensure that potential activity from vicinal diols did not

confound any of the cytokine measurements observed in our experiments, we treated hMC with 11,12-DHET and 19,20-DHDP. Neither lipid metabolite induced any of the inflammatory cytokines that were assayed in this study.

Mimicking a chronic, multifaceted disease like DR is a challenge *in vitro*, but *in vitro* experiments remain crucial tools to dissect the mechanisms of disease in a controlled, stepwise fashion. We used primary human Müller cells in order to maintain physiological relevance in our studies and to more easily translate our findings to future clinical trials in humans. Our proposed therapeutic strategy provides a unique advantage in translation to the clinic because it relies on manipulation of an endogenous system, allowing for protection throughout multiple stages of DR progression, while at the same time minimizing or eliminating toxicity. Current mainstream therapies focus on mediating late-stage DR morbidities directly associated with vision loss, while herein we propose a strategy that would focus on chronic retinal inflammation in early-stage DR, before irreversible damage has commenced. Our results confirm the anti-inflammatory effects of epoxide elevation in hMC, paving the way for directed *in vivo* studies. In future studies, we hope to confirm the therapeutic potential of systemically administered epoxides over longer time spans of pathogenesis in *in vivo* models of DR. These studies will be enabled by the recent development of water-soluble analogs of the epoxygenated fatty acids, as they will overcome issues of hydrophobicity and turnover of the parent EET/EDPs, enhancing their systemic circulation and bioavailability[96]. In conclusion, our data indicate that therapeutic manipulations to increase retinal levels of epoxygenated fatty acids offer the potential to be highly efficacious in the treatment of DR.

CHAPTER IV

CANNABINOID RECEPTOR 2 AGONISM DEMONSTRATES THERAPEUTIC POTENTIAL IN EXPERIMENTAL MODELS OF RELEVANCE TO DIABETIC RETINOPATHY

From: Ontko, CD, Smith TE, Stark AK, Olson JC, Jackson AL, Newkirk IM, McCollum GW, Penn JS. Cannabinoid Receptor 2 Agonism Demonstrates Therapeutic Potential in Experimental Models of Relevance to Diabetic Retinopathy. In preparation.

Introduction

Systemic diabetes-linked hyperglycemia and dyslipidemia cause damage to retinal cells that triggers the production of inflammatory cytokines in the eye[1,49]. Animal models[33–36] and patients with DR[33,34,37–40], display increased retinal and/or vitreal levels of pro-inflammatory cytokines, and sustained, chronic retinal inflammation is crucial to the pathogenesis of DR[1]. The chronic inflammatory environment in the diabetic retina causes endothelial cells to produce increased leukocyte adhesion molecules, such as ICAM-1 and VCAM-1, resulting in the increased immobilization of leukocytes to retinal capillary walls, termed *leukostasis.* Experimentally, the use of exogenous TNFα and IL-1β can recapitulate features of leukostasis *in vitro* and *in vivo.* For example, rats receiving intravitreal injections of TNFα or IL-1β developed retinal leukostasis[200,228], and the genetic deletion of TNFα in mice inhibited diabetes-induced leukostasis[229]. Additionally, TNFα and IL-1β both activate NF-κB-dependent transcription and induce ICAM-1 and VCAM-1 in hRMEC[1,56,230,231]. These adhesion molecules facilitate increased leukocyte-endothelial cell interactions, providing a mechanistic link between EC activation by inflammatory cytokines and leukostasis.

Cannabinoids (CB) are bioactive compounds found in cannabis, and endocannabinoids (eCB) are endogenous forms of CB that serve as activating ligands for the two cannabinoid receptors. Inflammation has been associated with the dysregulation of the body's

endocannabinoid system (ECS), and altered eCB levels have been reported in inflammatory conditions such as brain injury[232], cerebral ischemia[233], hepatic ischemia-reperfusion injuries[234], Huntington's disease[235], multiple sclerosis[236], rheumatoid arthritis[237], atherosclerosis[238], ulcerative colitis[239], and inflammatory pain[179,240]. Inflammatory disease conditions play an important role in the pathogenesis of diabetic complications and affect ECS homeostasis by decreasing eCB synthesizing enzymes and increasing eCB hydrolyzing enzymes, presumably lowering eCB levels[121,159,241,242].

Our previous studies investigating epoxides provided the motivation and rationale to expand our investigations to the ECS. **Chapter III** demonstrated the capacity of epoxides to mitigate retinal inflammation in DR models, but other closely related CYP-epoxygenated AA and DHA metabolites may prove even more beneficial. Some eCB are found to be CYP-epoxygenated products of AA and DHA[83,141]; notably the eCBs 11,12-EET-EA and 19,20-EDP-EA are closely related to the epoxides 11,12-EET and 19,20-EDP. However, eCB may be more stable[243,244] and potent (**Appendix B**), with the additional benefit of known receptors, and thus the transition to their study was logical and promising.

CB and eCB activate cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2), both of which are seven-transmembrane, G protein-coupled receptors[137]. CB1 is found in the central nervous system and is responsible for the psychotropic effects induced by cannabis, while CB2 is found more broadly in the body and is believed to be responsible for cannabis's immunomodulatory effects[147–149]. Growing evidence indicates that CB2 activation is immunomodulatory, anti-inflammatory, neuroprotective, and anti-nociceptive in numerous animal models and disease contexts[147,150]. In non-ocular inflammation models, CB2 activation has been shown to decrease leukocyte recruitment and adherence, inflammatory cytokine production, barrier function deficits, and apoptosis – all events that are pathogenic components of early DR progression[147,151,152]. Therefore, testing the effectiveness of CB2 activation in experimental models of DR is both rational and compelling, and could lead to insights into its effectiveness as

a potential therapeutic strategy.

Results

In this study, we tested the efficacy of two CB2-selective agonists, HU-308 and CB65 to attenuate TNFα- and IL-1β-induced leukocyte adhesion characteristics in hRMEC and murine models of DR. *In vitro,* leukostasis readouts included the query of ICAM-1, VCAM-1, and E-selectin gene expression and protein levels in hRMEC, as well as PBMC adhesion to hRMEC monolayers. *In vivo* experiments tested the effects of CB2 agonism on retinal leukostasis in an acute model of retinal inflammation and a systemic model of murine diabetes. In total, our studies aimed to advance the knowledge and development of CB2-directed therapies against the onset and early progression of DR.

HU-308 and CB65 reduce *ICAM1, VCAM1* and *SELE* expression in TNFα-stimulated hRMEC, and HU-308 reduces *ICAM1, VCAM1* and *SELE* expression in IL-1β-stimulated hRMEC

hRMEC cultures were stimulated with (a) TNFα or (b) IL-1β for 4 hours to simulate diabetic retinal inflammation. Cells were co-treated with either HU-308 or CB65 to test the effect of CB2 agonism on cytokine-induced gene expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin. As shown in **Figure 11**, both HU-308 and CB65 reduced adhesion molecule expression in hRMEC when stimulated with TNFα. However only HU-308, not CB65, reduced adhesion molecule expression in hRMEC when stimulated with IL-1β. In hRMEC stimulated with TNFα (**Fig. 11a**), HU-308 reduced *ICAM1* expression by 24% ($p<0.0001$), *VCAM1* expression by 41% ($p<0.0001$) and *SELE* expression by 55% ($p<0.0001$). CB65 reduced *ICAM1* expression by 40% ($p<0.0001$), *VCAM1* expression by 27% ($p<0.0001$) and *SELE* expression by 44% ($p<0.0001$). In hRMEC stimulated with IL-1β (**Fig. 11b**), HU-308 reduced *ICAM1* expression by 22% ($p=0.0002$), *VCAM1* expression by 24% ($p=0.0019$) and *SELE* expression by 23% ($p=0.0033$), while CB65 treatment did not change any adhesion

molecule expression (ns). HU-308 or CB65 treatment in the absence of an inflammatory stimulus did not cause any significant changes to adhesion molecule expression in comparison to vehicle (ns). The concentration of HU-308 and CB65 used was determined via the comparison of their CB2 and CB1 binding affinities, to ensure selective activation of CB2.

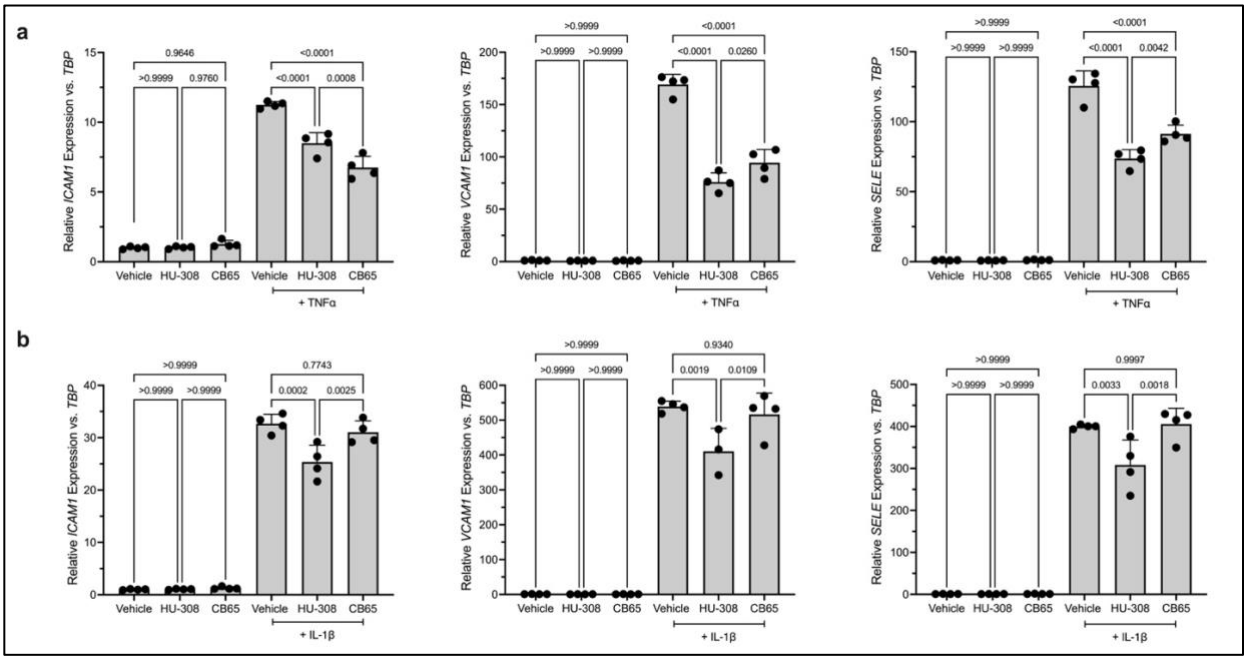


Figure 11: The effect of HU-308 and CB65 on TNFα- and IL-1β-induced adhesion molecule expression. hRMEC were stimulated with (a) TNFα or (b) IL-1β (0.1ng/mL), in the presence or absence of HU-308 or CB65 (1μM) for 4 hours. After treatment, total RNA was isolated and adhesion molecule expression was assayed via qRT-PCR. (a) *ICAM1, VCAM1* and *SELE* expression was significantly decreased by HU-308 and CB65 in hRMEC stimulated with TNFα. (b) *ICAM1, VCAM1* and *SELE* expression was significantly decreased by HU-308 in hRMEC stimulated with IL-1β, though CB65 was not effective in this context. HU-308 or CB65 treatment alone did not cause significant changes to adhesion molecule expression in comparison to vehicle. Each bar represents mean ± SD (n=3-4).

HU-308 and CB65 reduce ICAM-1 and VCAM-1 protein levels in TNFα- and IL-1β-stimulated hRMEC

Adhesion molecule protein levels (**Fig. 12**) were determined to compliment the above expression data (**Fig. 11**). In this experiment hRMEC cultures were stimulated with (a,b) TNFα or (c,d) IL-1β in the presence or absence of HU-308 or CB65 for 8 hours, and ICAM-1 and

VCAM-1 levels were determined via immunoblot analysis. As shown in **Figure 12**, both HU-308 and CB65 reduced adhesion molecule protein levels in hRMEC stimulated with TNFα or IL-1β. In the case of TNFα stimulation, HU-308 reduced ICAM-1 and VCAM-1 protein levels both by 30% (p=0.0027, p=0.0018) (**Fig. 12a**). Similarly, with TNFα stimulation, CB65 reduced ICAM-1 and VCAM-1 protein levels by 29% (p=0.0025) and 28% (p=0.0066) respectively (**Fig. 12b**). In the case of IL-1β stimulation, HU-308 reduced ICAM-1 and VCAM-1 protein levels by 17% (p=0.0148) and 36% (p=0.0002) respectively (**Fig. 12c**). Similarly, CB65 reduced ICAM-1 and VCAM-1 protein levels by 14% (p=0.0174) and 34% (p<0.0001) respectively (**Fig. 12d**). In this experiment as in **Figure 11**, HU-308 or CB65 treatment alone did not cause any significant changes to adhesion molecule levels in comparison to vehicle (ns).

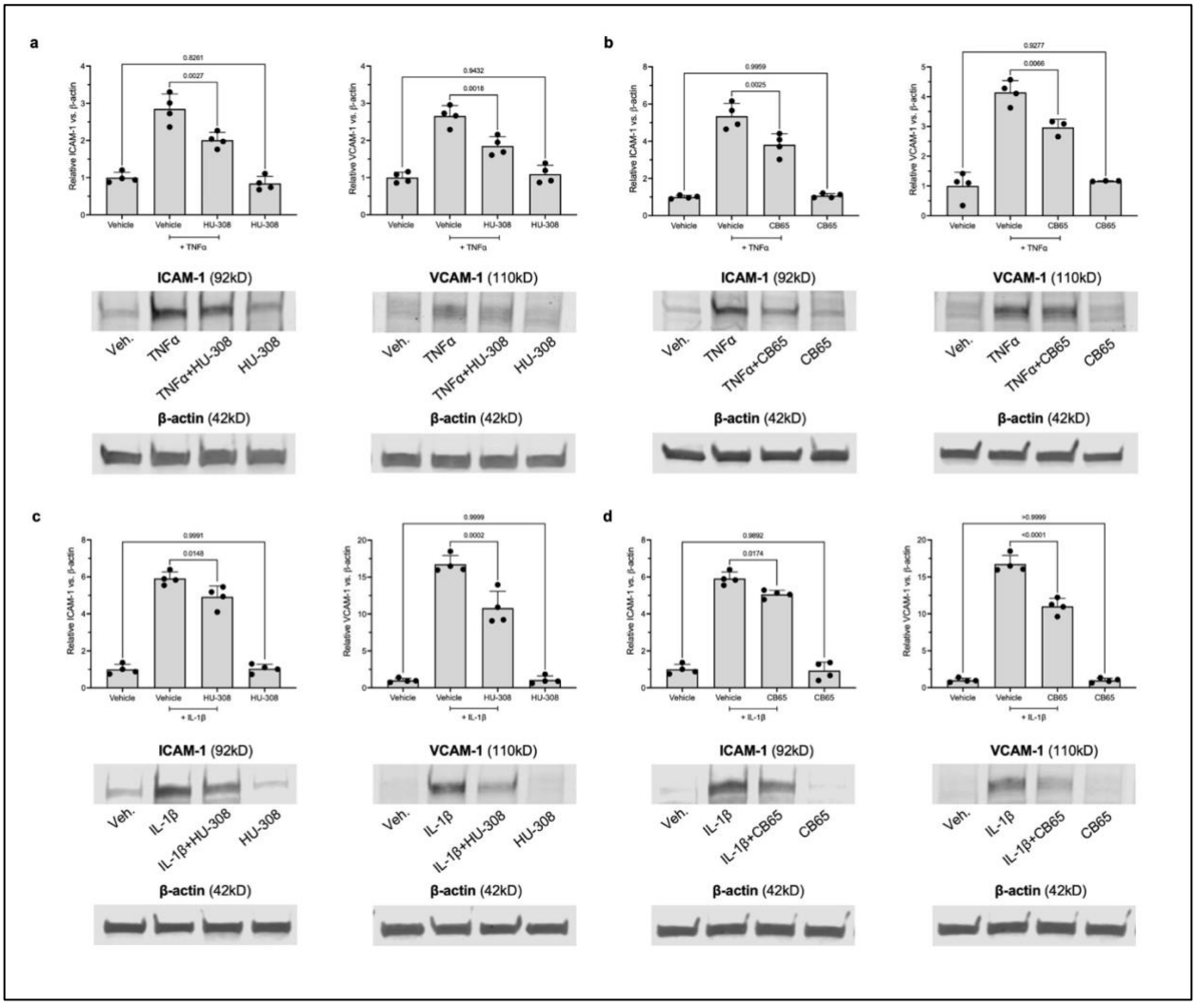


Figure 12: The effect of HU-308 and CB65 on TNFα- and IL-1β-induced adhesion molecule protein levels. hRMEC were treated with (a, b) TNFα or (c, d) IL-1β (0.1ng/mL), in the presence or absence of HU-308 or CB65 (1μM) for 8 hours, with IL-1β experiments including an additional 30-minute CB2 agonist pretreatment. After treatment, total protein was isolated and adhesion molecule protein levels were assayed via western blot. In hRMEC stimulated with TNFα (a, b), and in hRMEC stimulated with IL-1β (c, d), HU-308 and CB65 both significantly decreased ICAM-1 and VCAM-1. HU-308 or CB65 treatment alone did not cause any significant changes to adhesion molecule protein levels in comparison to vehicle. Representative blots are placed below each experiment's quantification. Each bar represents mean ± SD (n=3-4).

HU-308 and CB65 inhibit leukocyte adherence to TNFα- and IL-1β-treated hRMEC monolayers

Reduced adhesion molecule protein levels in hRMEC plasma membranes (**Fig. 12**) should result in a subsequent reduction in leukocyte adhesion to hRMEC. In a static adhesion assay, leukocyte adherence was measured after PBMCs were applied to cytokine-stimulated hRMEC monolayers. As shown in **Figure 13**, HU-308 and CB65 significantly reduced PBMC adherence to hRMEC monolayers, compared to hRMEC monolayers stimulated with cytokine alone. In hRMEC stimulated with TNFα, HU-308 reduced adherent PBMCs by 37% ($p=0.0061$) (**Fig. 13a**). In hRMEC stimulated with IL-1β, HU-308 reduced adherent PBMCs by 23% ($p<0.0001$), and CB65 reduced adherent PBMCs by 14% ($p=0.0304$) (**Fig. 13b**). Consistent with previous findings, hRMEC treated with HU-308 or CB65 alone did not significantly change the amount of adherent PBMCs per mm^2 in comparison to vehicle (ns). These results suggest that leukostasis might be inhibited by CB2 agonism in future *in vivo* studies.

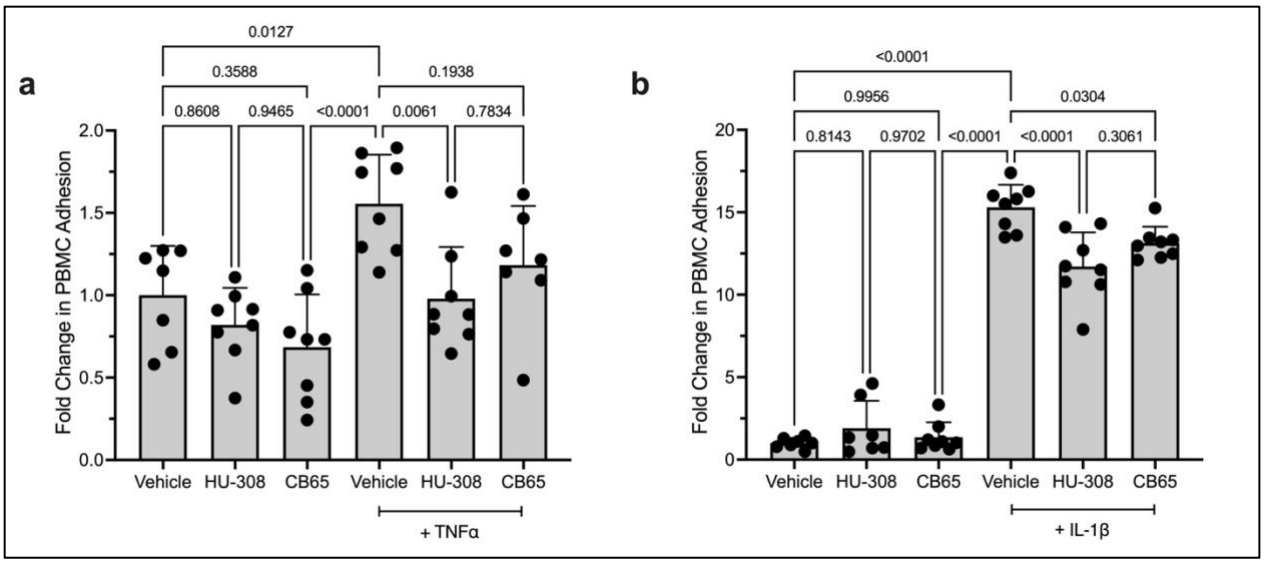


Figure 13: The effect of HU-308 and CB65 on TNFα- and IL-1β-induced leukocyte adhesion to hRMEC monolayers. hRMEC monolayers were treated with (a) TNFα (0.1ng/mL) for 4 hours or (b) IL-1β (0.1ng/mL) for 8 hours, in the presence or absence of HU-308 or CB65 (1μM). IL-1β experiments including an additional 30 minutes pretreatment when testing CB65. In a static adhesion assay, PBMCs were added to treated monolayers for 30 minutes, then monolayers were washed to remove nonadherent leukocytes. Monolayers were fixed and imaged, and the adhered PBMCs per mm^2 were quantified. (a) In hRMEC monolayers stimulated with TNFα, PBMC adhesion was decreased by the simultaneous treatment of HU-308 but not CB65. (b) In hRMEC monolayers stimulated with IL-1β, PBMC adhesion was significantly decreased by the simultaneous treatment of HU-308, and the pretreatment plus simultaneous treatment of CB65. In both experiments, HU-308 or CB65 treatment alone did not cause any significant changes to PBMC adhesion in comparison to vehicle. Each bar represents mean ± SD (n=6-8).

HU-308 and CB65 inhibit NF-κB nuclear translocation in TNFα- and IL-1β-stimulated hRMEC

Next, we sought to determine a potential mechanism for the above therapeutic effects demonstrated by HU-308 and CB65. NF-κB is a proinflammatory transcription factor that has been previously implicated in DR pathology and progression[1]. TNFα and IL-1β are important components of the NF-κB transcriptome, and these two cytokines also activate NF-κB[1]. In the absence of stimuli, NF-κB is cytoplasmic and bound to its inhibiting partner IκBα.

Phosphorylation via IKK causes IκBα degradation, allowing NF-κB nuclear translocation and transcriptional activation.

In **Figure 14**, immunofluorescent staining was used to visualize NF-κB translocation between the cytoplasm and the nucleus of hRMEC, suggesting its activation. In this experiment, hRMEC were treated with inflammatory cytokine (a) TNFα or (b) IL-1β in the presence or absence of HU-308 or CB65 for 30 minutes. After treatment, cultures where fixed with 4% PFA, stained for p65 NF-κB, and imaged using confocal microscopy. In this figure, it can be seen that NF-κB readily entered hRMEC nuclei when the cells were stimulated with TNFα or IL-1β. Co-treatment with HU-308 or CB65 mitigated this translocation significantly (**Fig. 14**). HU-308 and CB65 reduced TNFα-induced NF-κB translocation by 29% ($p<0.0001$) and 27% ($p<0.0001$) respectively (**Fig. 14a**). In the case of IL-1β-stimulation, both HU-308 and CB65 reduced NF-κB translocation by 44% ($p<0.0001$) (**Fig. 14b**). NF-κB translocation was not affected by treatment with HU-308 or CB65 alone (ns). These data suggest that CB2 agonism decreases inflammation in hRMEC, at least in part, by modulating NF-κB-dependent transcription.

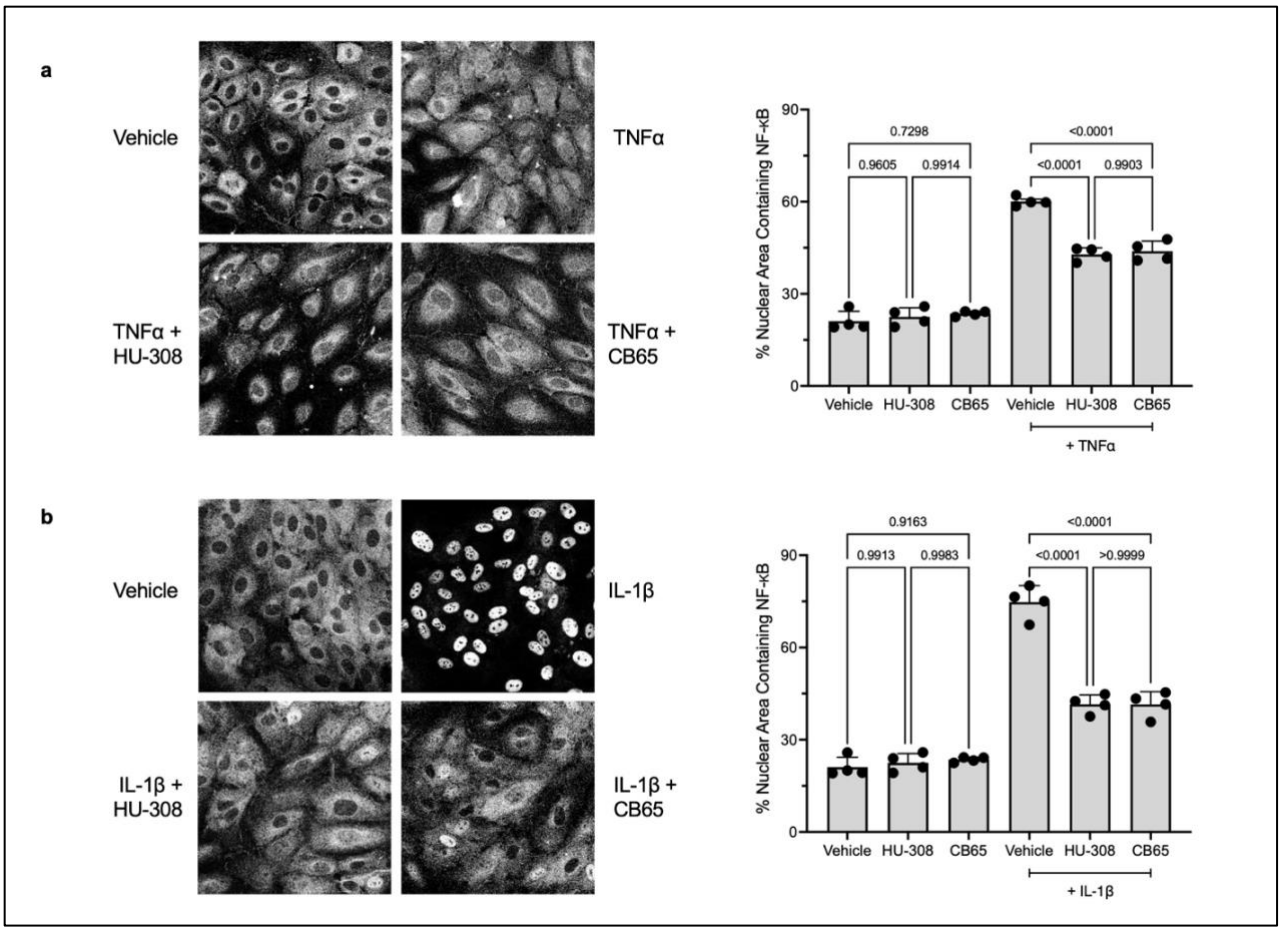


Figure 14: The effect of HU-308 and CB65 on TNFα- and IL-1β-induced NF-κB nuclear translocation. hRMEC were treated with (a) TNFα or (b) IL-1β (0.1ng/mL), in the presence or absence of HU-308 or CB65 (1μM) for 30 minutes. After treatment, cultures where fixed with 4% PFA, stained for NF-κB p65, and imaged using confocal microscopy. In hRMEC stimulated with (a) TNFα or (b) IL-1β, NF-κB enters into the nuclei of the cells. This translocation is significantly reduced when cells were co-treated with HU-308 or CB65. Representative images demonstrate NF-κB nuclear translocation when cells are stimulated with cytokine, and reduced translocation with the cotreatment of HU-308 or CB65. Quantification was performed by counting the percent of NF-κB positive pixels per nucleus of 2 fields per treatment group. The results of 2 independent experiments were averaged. HU-308 or CB65 treatment alone did not cause significantly different translocation from vehicle. Each bar represents mean ± SD (n=4).

HU-308 and CB65 inhibit NF-κB activation in TNFα- and IL-1β-stimulated hRMEC

While TNFα and IL-1β both induced NF-κB nuclear translocation in hRMEC (**Fig. 14**), we performed NF-κB luciferase activity assays to confirm and quantify NF-κB transcriptional activation (**Fig. 15**). In this experiment, hRMEC were transfected with the NF-κB-luciferase

promoter-reporter construct and stimulated with TNFα or IL-1β in the presence or absence of HU-308 or CB65. As seen in **Figure 15**, HU-308 and CB65 significantly decreased TNFα- or IL-1β-induced NF-κB-dependent luciferase activity compared to cytokine stimulation alone. HU-308 and CB65 reduced TNFα-induced NF-κB transcriptional activation by 49% (p=0.0016) and 37% (p=0.0279) respectively (**Fig. 15a**). HU-308 and CB65 reduced IL-1β-induced NF-κB transcriptional activation by 45% (p=0.0056) and 37% (p=0.0284) respectively (**Fig. 15b**). As consistent with other data, treatment with HU-308 or CB65 alone did not produce a significantly difference response from that of vehicle treatment (ns). In combination with the NF-κB translocation assay seen in **Figure 14**, these data further suggest that CB2 agonism decreases inflammatory phenotypes in hRMEC by modulating pathways that converge on NF-κB-dependent transcription.

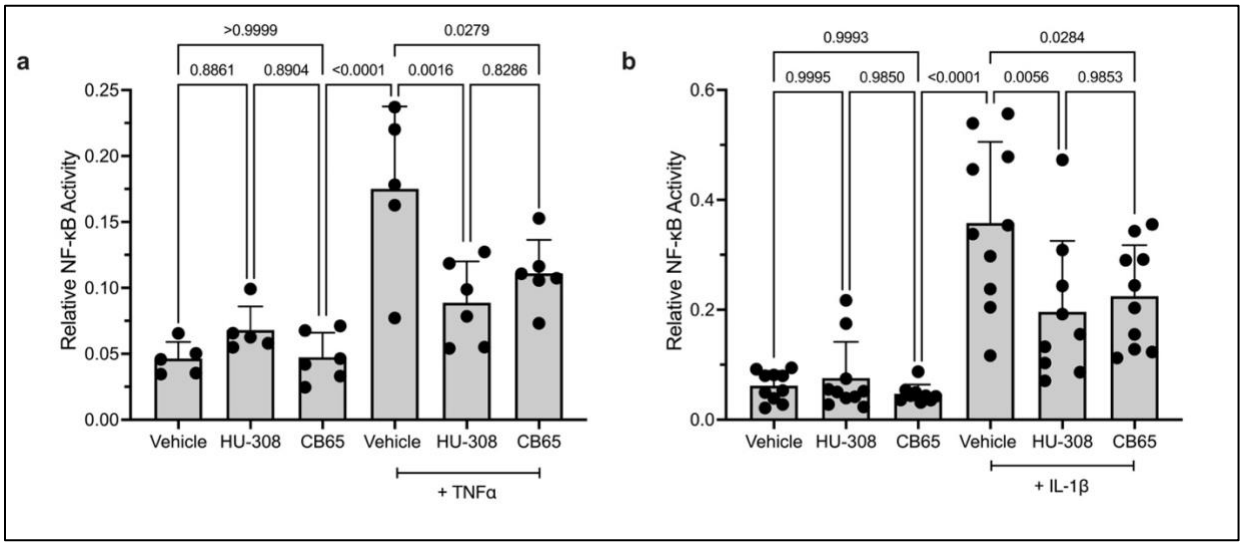


Figure 15: The effect of HU-308 and CB65 on TNFα- and IL-1β-induced NF-κB activation. hRMEC were transfected with luciferase construct and treated with (a) TNFα (1.5ng/mL) for 8 hours or (b) IL-1β (1ng/mL) for 6 hours, in the presence or absence of HU-308 or CB65 (1μM). After treatment, cells were lysed and NF-κB activity was determined by measuring the ratio of firefly-to-renilla luciferase. (a) In hRMEC stimulated with (a) TNFα, and (b) IL-1β, NF-κB activation was significantly decreased by HU-308 and CB65 treatment in comparison to stimulation alone. HU-308 or CB65 treatment alone did not cause significantly different activation from vehicle. Each bar represents mean ± SD (n=5-10).

HU-308 mitigates TNFα-induced retinal leukostasis in C57BL/6J mice

To complement our *in vitro* data, *in vivo* experiments were also performed. Previously, we demonstrated that CB2 agonism was efficacious against cytokine-induced inflammation in hRMEC (**Fig. 11-15**). Additionally, we sought to test the effects of CB2 agonism in a cytokine-induced mouse model of acute retinal inflammation[46,56,200,231,245] (**Fig. 16**). Mice received daily intraperitoneal injections of vehicle or HU-308 for one week prior to receiving an intravitreal injection of TNFα. 12 hours post-TNFα injection, the murine retinal vasculature is stained and adherent leukocytes are counted as a measure of leukostasis. As shown in **Figure 16**, systemic HU-308 administration significantly decreased TNFα-induced retinal leukostasis (p=0.0025) to vehicle levels. Representative images of the retinal vasculature in **Figure 16a** depict a visual presentation of leukocytes in the retinal vasculature. This experiment demonstrates that the beneficial effects of CB2 agonism against cytokine stimulation *in vitro* can be recapitulated *in vivo*.

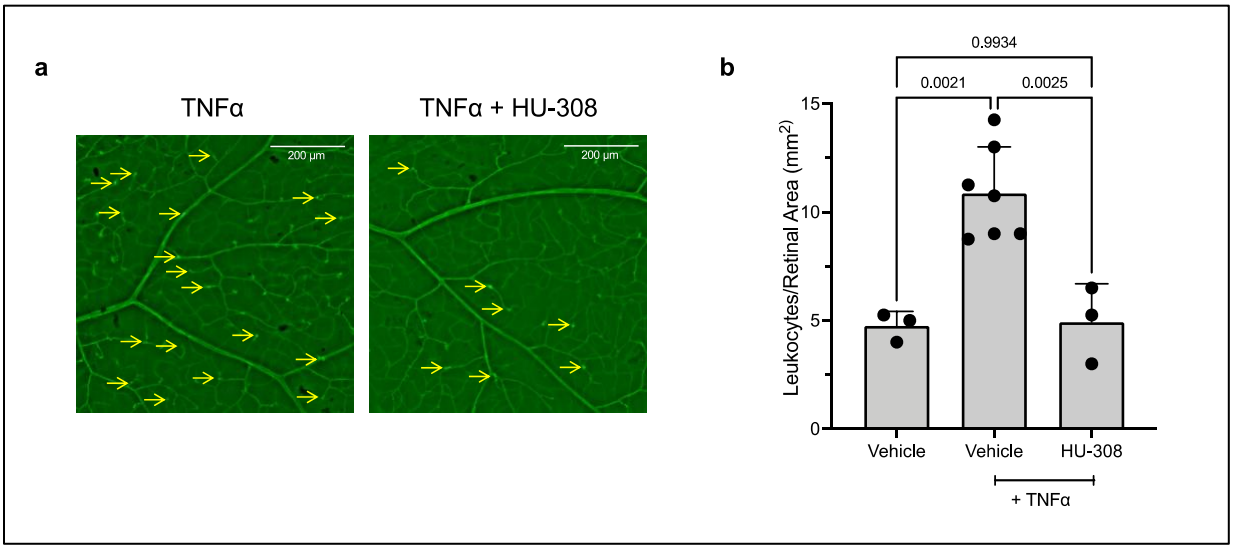


Figure 16: The effect of HU-308 on TNFα-induced retinal leukostasis *in vivo*. C57BL/6J mice received daily intraperitoneal injections of vehicle or HU-308 (5mg/kg) for one week prior to receiving an intravitreal injection of TNFα (25ng/mL). 12 hours post-TNFα injection, mice were anesthetized and infused with FITC-conjugated concanavalin-A to stain the leukocytes and vasculature in the retina. Retinas were dissected, flat-mounted, and imaged to count adherent leukocytes in the vasculature. (a) Representative images of retinal flatmounts; arrows showing adhered leukocytes. (b) Quantification of adherent leukocytes per mm^2 retinal area. *In vivo*,

systemic HU-308 significantly decreased TNFα-induced retinal leukostasis. Bars represent mean ± SD (n=3-7).

HU-308 inhibits STZ-induced retinal leukostasis in mice

The therapeutic potential of CB2 agonism against retinal leukostasis was also tested using the STZ model of systemic murine diabetes; both systemic and local HU308 administration were tested. In **Figure 17a**, mice received daily intraperitoneal injections of vehicle or HU-308 for 4 weeks, starting 6 weeks post-STZ injections. In **Figure 17b**, STZ-treated mice were given a single intravitreal injection of HU-308 the day before leukostasis readings. In both intraperitoneal and intravitreal models, sacrifice and leukostasis readings were performed 10 weeks post-STZ injections. In these experiments, both systemic and local HU-308 administration significantly decreased STZ/diabetes-induced retinal leukostasis ($p=0.0240$, $p<0.0001$), as analyzed by leukocyte adherence to the murine retinal vasculature.

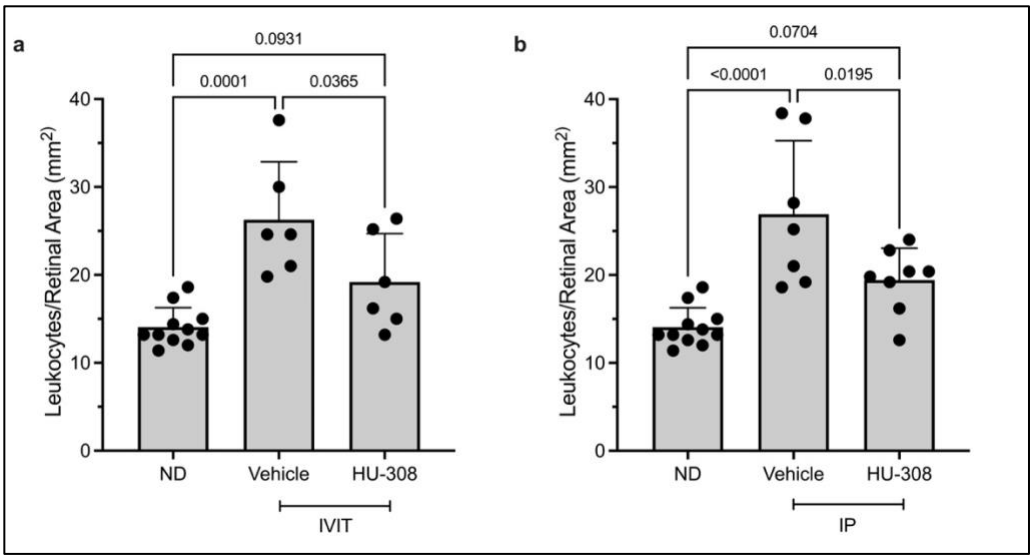


Figure 17: The effect of HU-308 on STZ-induced retinal leukostasis *in vivo*. For intraperitoneal (IP) administration, C57BL/6J mice were given daily intraperitoneal injections of vehicle or HU-308 (5mg/kg) for 4 weeks, starting 6 weeks post-STZ injection. For intravitreal (IVIT) administration, mice were given a single intravitreal injection of HU-308 (5uM) the day before leukostasis readings. 10 weeks post-STZ injections, mice were anesthetized and infused with FITC-conjugated concanavalin-A to stain the leukocytes and vasculature in the retina. Retinas were dissected, flat-mounted, and imaged to count adherent leukocytes in the vasculature. (a,b) Quantification of adherent leukocytes normalized to retinal area. *In vivo*, both

(a) systemic and (b) local HU-308 administration significantly decreased STZ/diabetes-induced retinal leukostasis. Each bar represents mean ± SD (n=5-7).

HU-308 and CB65 do not affect *Icam1, Vcam1 and Sele* expression in TNFα- and IL-1β-stimulated *Cnr2*$^{-/-}$ mRMEC

To verify the specificity of HU-308 and CB65 and to further confirm that CB2 activation is the mechanism of action of our agonists, both agonists were tested in mouse retinal microvascular endothelial cells (mRMEC) devoid of CB2. *Cnr2*$^{-/-}$ mRMEC were isolated from *Cnr2*$^{-/-}$ mice, and cultures were stimulated with (a) TNFα or (b) IL-1β in the presence or absence of HU-308 or CB65 for 4 hours. qRT-PCR was performed to assess gene expression changes to *Icam1, Vcam1 and Sele*. As shown in **Figure 18**, TNFα and IL-1β stimulation in mRMEC caused a significant increase in the expression of *Icam1, Vcam1 and Sele*, as previously observed in the corresponding genes in human cells (**Fig. 11**). Additionally, HU-308 and CB65 treatment did not significantly change adhesion molecule expression in *Cnr2*$^{-/-}$ mRMEC, and HU-308 or CB65 treatment alone did not cause any significant changes to adhesion molecule expression in comparison to vehicle (ns). These results indicated an absence of off-target effects when using HU-308 and CB65.

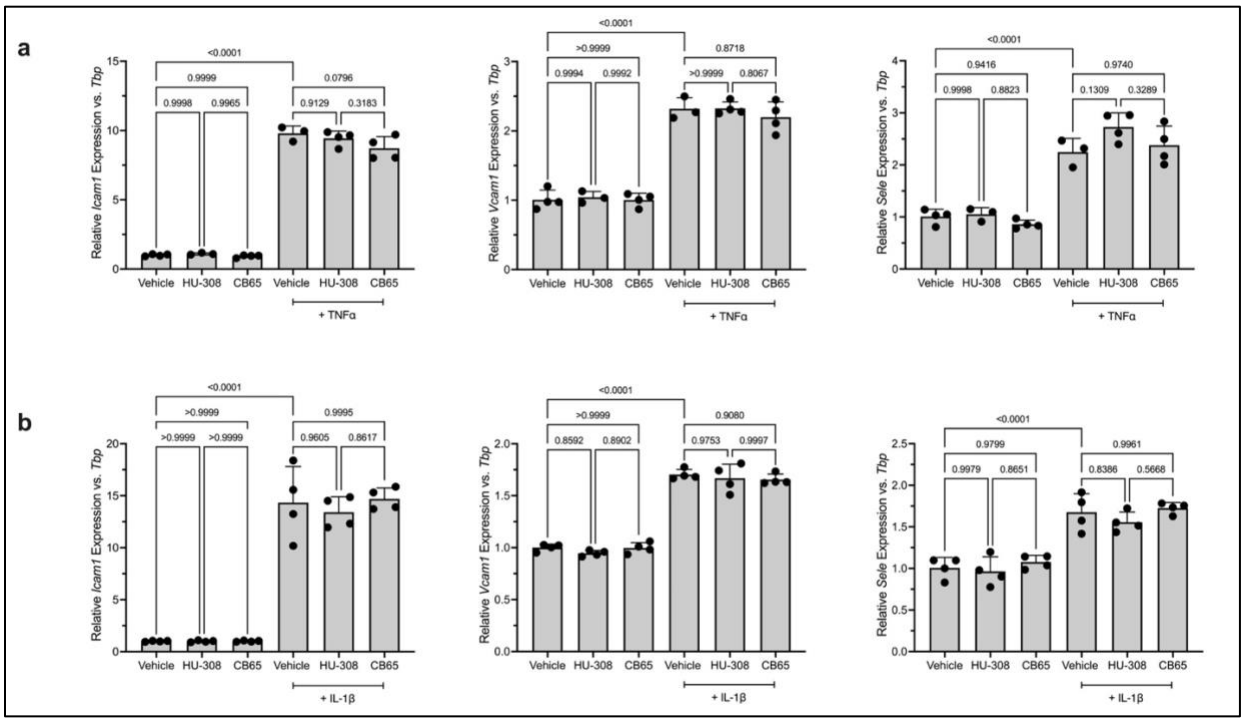


Figure 18: The effect of HU-308 and CB65 on TNFα- and IL-1β-induced adhesion molecule expression in *Cnr2*$^{-/-}$ mouse EC. *Cnr2*$^{-/-}$ mRMEC were stimulated with (a) TNFα or (b) IL-1β (1ng/mL), in the presence or absence of HU-308 or CB65 (1μM) for 4 hours. After treatment, total RNA was isolated and adhesion molecule expression was assayed via qRT-PCR. *Icam1, Vcam1 and Sele* expression was not significantly changed by HU-308 or CB65 treatment in hRMEC stimulated with TNFα or IL-1β. HU-308 or CB65 treatment alone did not cause significant changes to adhesion molecule expression in comparison to vehicle. Each bar represents mean ± SD (n=3-4).

HU-308 does not affect retinal leukostasis in STZ-induced diabetic *Cnr2*$^{-/-}$ mice

To confirm the absence of off-target effects *in vivo*, HU-308 was tested against leukostasis in STZ-induced diabetic *Cnr2*$^{-/-}$ mice. *Cnr2*$^{-/-}$ mice received daily intraperitoneal injections of vehicle or HU-308 for 4 weeks, starting 6 weeks post-STZ injections. 10 weeks post-STZ injections, mice were sacrificed and leukostasis readings were performed. As shown in **Figure 19**, HU-308 did not significantly affect retinal leukostasis in STZ-induced diabetic *Cnr2*$^{-/-}$ mice. Additionally, *Cnr2*$^{-/-}$ non-diabetic mice were seen to have more adherent leukocytes compared to WT non-diabetic mice (p=0074). This result is in line with previous literature, where

the lack CB2 receptor in mice is shown to coincide with inflammatory phenotypes such as increased leukocyte recruitment and pro-inflammatory mediator production[147].

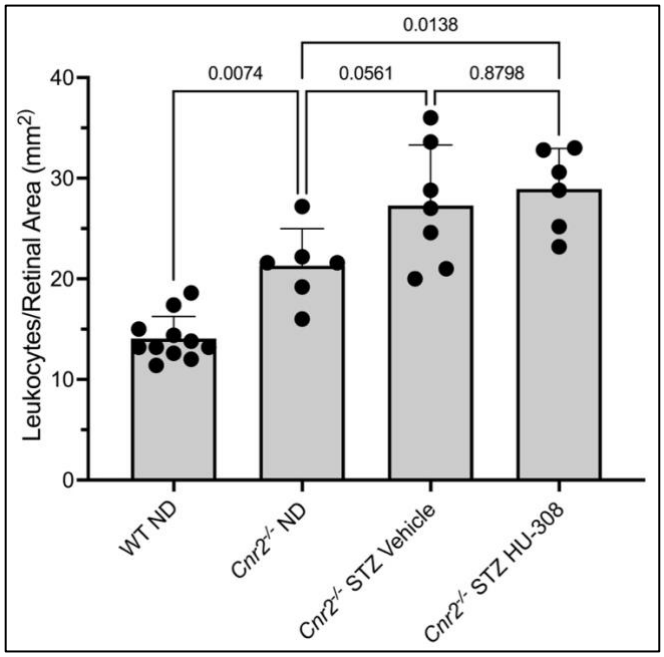


Figure 19: The effect of HU-308 on STZ-induced retinal leukostasis in *Cnr2*$^{-/-}$ mice. Via intraperitoneal administration, *Cnr2*$^{-/-}$ mice were given daily injections of vehicle or HU-308 (5mg/kg) for 4 weeks, starting 6 weeks post-STZ injection. 10 weeks post-STZ injections, mice were anesthetized and infused with FITC-conjugated concanavalin-A to stain the leukocytes and vasculature in the retina. Retinas were dissected, flat-mounted, and imaged to count adherent leukocytes in the vasculature. Adherent leukocytes were normalized to retinal area. *In vivo*, *Cnr2*$^{-/-}$ mice had an exacerbated inflammatory phenotype compared to WT mice, and systemic HU-308 administration had no effect on retinal leukostasis in STZ-induced diabetes. Each bar represents mean ± SD (n=6-11).

Conclusions

In this study, we sought to test the efficacy of CB2 agonists against experimental models of leukocyte adhesion or leukostasis in cellular and animal models of NPDR, respectively. The CB2 agonists used in this study, HU-308 and CB65, are both at least three orders of magnitude more selective for CB2 over CB1 and are structurally distinct, again indicating that our results are due to activation of CB2 signaling. To further verify the specificity of our agonists, mRMEC isolated from *Cnr2*$^{-/-}$ mice were stimulated with cytokine, and HU-308 and CB65 were found to

have no effect on mRMEC inflammatory response. These findings indicate that the efficacy we observed in hRMEC experiments was CB2-dependent (**Fig. 18**). In hRMEC, we tested the capacity of HU-308 and CB65 to reduce TNFα- and IL-1β-induced expression of leukocyte adhesion proteins. Both agonists caused significant reduction of the gene expression of *ICAM1, VCAM1,* and *SELE* (**Fig. 11**), and protein expression of ICAM-1 and VCAM-1 (**Fig. 12**) induced by either stimulus. Additionally, HU-308 and CB65 treatment reduced TNFα- and IL-1β-induced adhesion of PBMCs to hRMEC monolayers (**Fig. 13**), aligning with their capacity to reduce adhesion protein levels. In these experiments, we also measured *CNR2* expression to determine that our inflammatory stimuli were not negatively impacting receptor availability in hRMEC (**Supp. Fig. 3, Appendix**). Overall, these results agree with prior studies in non-ocular EC types demonstrating that CB2 agonists reduce the expression of inflammation-induced adhesion molecules and inhibit monocyte adhesion and extravasation[151,193,201].

Our *in vivo* experiments first tested CB2 agonism in a model of acute retinal inflammation. This model induces retinal leukostasis through the intravitreal injection of cytokine stimuli and thus complements the methodology used in our *in vitro* experiments. We chose to use HU-308 for our *in vivo* studies because HU-308 generally outperformed CB65 in our *in vitro* experiments, its characterization is more complete in the literature, and its formulation has proven to be consistent with systemic administration. Consistent with our PBMC adhesion assays *in vitro*, intraperitoneal injection of HU-308 mitigated cytokine-induced retinal leukostasis *in vivo* (**Fig. 16**). Notably, if systemically administered CB2 agonists were to be advanced to the clinic for DR complications, this would eliminate the risk of endophthalmitis often linked to anti-VEGF drugs currently administered by intravitreal injection[246].

Next, we tested HU-308 in a model of streptozotocin (STZ)-induced murine diabetes, a type 1 diabetes model more relevant to the links between systemic diabetes and retinopathy. STZ-treated mice consistently recapitulate progressive inflammatory features of NPDR, including increased retinal inflammatory cytokine levels, leukostasis, hyperpermeability, and

RMEC apoptosis[247,248]. In these experiments, it was found that diabetes induction caused an 86.7% increase in leukostasis, and *Cnr2* deletion in mice caused a 51.4% increase in leukostasis, though the difference between these two inductions was insignificant (**Fig. 17b** "Vehicle" and **Fig. 19** "*Cnr2*$^{-/-}$ ND"). This indicates that the deletion of CB2 alone is associated with a pro-inflammatory retinal phenotype[147]. HU-308 had no effect on retinal leukostasis in *Cnr2*$^{-/-}$ animals, indicating that off-target effects were not a complication. Conversely, in WT mice, systemically administered HU-308 significantly decreased diabetes-induced retinal leukostasis (**Fig. 17a**). Additionally, localized intravitreal injection also proved efficacious at reducing diabetes-induced retinal leukostasis (**Fig. 17b**). This demonstrates that HU-308 may *prevent* leukostasis through early, long-term administration (as demonstrated in our intraperitoneal methodology), as well as be able to *intervene* in leukostasis in later-stage disease progression (as seen in our intravitreal injection methods). Therefore, HU-308 may not only block the accumulation of leukocytes but also liberate those that already have become adhered. Additionally, in this model it was found that STZ did not impact retinal *Cnr2* expression, confirming CB2 to be an accessible and appropriate target (**Supp. Fig. 4, Appendix**).

Studies have confirmed the potential therapeutic value of CB2 in regulating systemic aspects of diabetes[155]. For example, CB2 agonists have been shown to increase insulin secretion and decrease plasma glucose levels in murine models of diabetes[155,178,249]. Similarly, CB2 agonists were seen to decrease lipid levels, glucose levels, inflammatory cytokine levels, and fat mass in obese mice[155,250]. In consideration of these findings, it is relevant to question whether the efficacy of HU-308 in retinal leukostasis arises from its direct effect on retinal cells, or rather the beneficial effects of CB2 agonism on diabetes-related systemic manifestations. *In vivo,* we saw no changes in fasting blood glucose or body weight across treatment groups (**Supp. Fig. 5, Appendix**) suggesting that HU-308 effects on systemic diabetes were absent or insignificant in our studies. In addition, and as previously stated, we tested the effects of HU-308 administration by local intravitreal injection and found a reduction in leukostasis comparable to

systemic administration (**Fig. 17b**). Given the levels of our intravitreal dosing, it is highly unlikely that this route of administration could have exerted an effect on systemic metabolic functions. This evidence implies that CB2 agonism had direct and beneficial effects on retinal cells in our *in vivo* experiments. Notably, HU-308 appeared to have a greater effect on retinal leukostasis *in vivo* than it did in hRMEC. This may be attributed to HU-308 benefitting other, non-endothelial cells in the retina. Retinal cells such as Müller glia, microglia, and photoreceptors also express CB2 at relatively high levels[251,252], and these cells also can serve as sources of inflammatory mediators. Therefore, it may be that HU-308 mitigates the inflammatory bioactivities of other retinal cell types, resulting in a more dramatic therapeutic effect *in vivo*. We have performed pilot studies in Müller cells that support this concept (**Appendix C**).

The results of our *in vivo* experiments with HU-308 are similar to those of other studies in experimental ocular inflammatory models. For example, topical HU-308 proved efficacious against endotoxin-induced uveitis in rats, reducing TNFα, IL-1β, and pro-inflammatory transcription factors such as NF-κB and AP-1[182]. Topical HU-308 also decreased leukostasis in the iridial vasculature[182]. Additionally, HU-308 was therapeutic in an experimental model of proliferative vitreoretinopathy, decreasing mean histopathological scores, number of microglia, and leukocyte adhesion[183]. Finally, HU-308 was shown to reduce corneal pain and inflammation in mouse models of corneal hyperalgesia[184]. In combination with our findings, these results demonstrate critical proof of concept that CB2 activation is therapeutic against important consequences of DR, and both local and systemic administrations can alleviate these consequences.

In order to explore one possible mechanism of action behind CB2's observed efficacy, we examined the CB2-dependent regulation of NF-κB in cytokine-activated hRMEC. The NF-κB pathway has long been recognized for its role in the development of inflammation, including the elaboration of inflammatory cytokines and chemokines (e.g., leukocyte recruitment molecules)[1,192,193]. Notably, each of the CB2-dependent ERK1/2, p38, and JNK pathways is

known to activate NF-κB and AP-1[194–197], which may then engage in synergistic pro-inflammatory activities. Thus, based solely on what is known currently about CB2 signal transduction, it can be concluded that modulation of these signaling intermediates by CB2 can both elevate tissue levels of anti-inflammatory cytokines (e.g., IL-4, IL-10) and suppress levels of pro-inflammatory cytokines (e.g., TNFα, IL-1β). Furthermore, there is also direct evidence that CB2 agonism suppresses NF-κB-dependent inflammatory events. For example, both HU-308 and JWH-133, another CB2-selective agonist, inhibited TNFα-induced *ICAM-1* and *VCAM-1* expression, NF-κB nuclear translocation, and RhoA activation in coronary artery EC[193]. In another study, 2-AG, a non-selective CB2 ligand, inhibited induction of IL-2 by PMA/ionomycin in mouse splenocytes and decreased NFATc and NF-κB promoter binding and activity[198]. Given the central role of NF-κB in CB2-mediated anti-inflammatory events, we chose to monitor NF-κB translocation and promoter activation in our *in vitro* experiments to establish a plausible mechanism for HU-308 and CB65 efficacy. In our experiments, HU-308 and CB65 decreased cytokine-induced NF-κB translocation and promoter activation in hRMEC (**Fig. 14, 15**). These results suggest that CB2 agonism exerts its anti-inflammatory activity at least partly through an NF-κB-dependent mechanism in hRMEC.

Mounting evidence increasingly supports the use of CB2 activation strategies to mitigate tissue inflammation, providing a promising approach to effectively address the chronic retinal inflammation associated with diabetes. CB2 agonists have already been tested in clinical trials for non-ocular inflammatory pathologies, lowering the bar for their clinical translation to DR treatments[173,253]. Currently, there are no FDA-approved therapeutics targeting NPDR, yet the development of an effective treatment strategy at this early disease stage could slow or prevent DR progression and limit or eliminate later-stage retinal damage. Indeed, inhibiting leukostasis occurring in NPDR may help prevent the disease transition to irreparable and vison-threatening PDR. Herein, we show that CB2 activation therapeutically addresses leukocyte binding in hRMEC and retinal leukostasis in diabetic mice, presumably by inhibiting of NF-κB-dependent

transcription of VCAM-1 and ICAM-1. Furthermore, our data illustrate the efficacies afforded by *systemically* administered CB2 agonists, with the potential to eliminate complications associated with intravitreal injection and improve patient compliance. We conclude that CB2 agonism constitutes a rational therapeutic approach for clinical application to patients with NPDR.

CHAPTER V

DISCUSSION

This dissertation work explores an innovative therapeutic approach to address early-stage DR, specifically focusing on a class of anti-inflammatory fatty acid metabolites and one of their corresponding receptors. AA and DHA are polyunsaturated fatty acids found at higher concentrations in the retina than any other tissue in the body; however, diabetes dysregulates their metabolism, modifying the levels of their metabolites in ocular tissues/fluids[19,37,85–87,155]. Thus, returning the tissue levels of these anti-inflammatory lipid metabolites to homeostatic levels may be beneficial in alleviating the diabetes-induced inflammatory burden[56,97,98]. The lipid metabolites under consideration here include the products of AA and DHA metabolism by CYP epoxygenases, termed *epoxides*, and the ethanolamide derivatives of those epoxides, termed *endocannabinoids*. Additionally, endocannabinoids have been associated with two *cannabinoid receptors*, one of which, *CB2*, demonstrates anti-inflammatory properties upon activation[147,150]. As our understanding of CYP-metabolites and their receptors grows, we gain insights into how their anti-inflammatory, and thus potentially beneficial, activities could be harnessed therapeutically in certain disease conditions that cause their dysregulation. **Chapter I** of this dissertation outlines the reasoning behind our proposed studies and provides information that supports the validity of our hypotheses. **Chapter II** lays out the methodology used in our studies in both *in vitro* and *in vivo* experimental settings. **Chapter III** demonstrates the efficacy of the CYP-epoxygenated products 11,12-EET and 19,20-EDP in mitigating cytokine- and FFA-induced inflammation in hMC. Finally, **Chapter IV** demonstrates the efficacy of activating the endocannabinoid receptor CB2 to target cytokine-induced leukostasis behaviors in cultured hRMEC and retinal tissue.

Our lab had previously determined the anti-inflammatory efficacy of 11,12-EET and 19,20-EDP in cytokine-stimulated retinal microvasculature *in vitro* and *in vivo*[56]. This work is attributed to former graduate student Megan Capozzi (now assistant professor at the University

of Washington), who pioneered the scientific exploration of epoxygenated fatty acids and their anti-inflammatory properties in models of DR. Her work was conducted in hRMEC and laid the groundwork for epoxide experiments in other retinal cell types, including hMC as seen in **Chapter III**. Her studies showed that hRMEC secreted less EETs when subject to inflammatory conditions[56], which was congruent with Schwartzman et al., who found that vitreous levels of EETs were significantly reduced in both NPDR and PDR patients relative to non-diabetic patients[37]. Additionally, co-treatment of exogenous 11,12-EET or 19,20-EDP in combination with sEH inhibition reduced cytokine-induced adhesion molecule expression in hRMEC and leukocyte adhesion to hRMEC monolayers. Finally, Dr. Capozzi demonstrated reduced leukostasis in a TNFα-induced acute retinal inflammation model *in vivo*. In these studies, 11,12-EET and 19,20-EDP were potently and equally anti-inflammatory[56], and thus, additional studies continued using both epoxides in other cellular contexts and DR models.

Dr. Capozzi's discoveries inspired the work in **Chapter III**, as I attempted to test the anti-inflammatory potential of 11,12-EET and 19,20-EDP in cytokine- and FFA-stimulated hMC. hMC are an obvious target cell to study due to their importance in retinal homeostasis and their important influence in DR pathogenesis. MC are particularly vulnerable and sensitive to disturbances in the retina, which can cause them to become destructive and promote DR pathogenesis[1,66]. MC are potent initiators and propagators of inflammation, and MC changes have been observed prior to the appearance of overt vascular pathology in DR[66–68], suggesting a crucial preliminary role in the onset of DR dysfunction. We selected PA as one of our inflammatory stimuli for this study because PA is a free fatty acid that is both diabetes-relevant and strongly pro-inflammatory[45,207]. Diabetic dyslipidemia is associated with DR progression, and circulating FFAs are proposed to be initiators of inflammatory cytokine release[22,23,204]. PA is elevated above other FFAs in the circulation and tissues of diabetic patients and experimental models of diabetes[45,205–208]. *In vivo*, STZ-induction of diabetes in mice caused PA to increase its relative percent of the total fatty acid content of the retina,[205] and *in vitro* MC are particularly

responsive to FFA[44,45]. While diabetic conditions are conventionally simulated using elevated glucose in the cell culture setting, we found that glucose elicited little to no response from several primary human retinal cells, including hMC[44,45]. Vitreous samples from diabetic patients and retina samples from experimental diabetes models exhibit elevated levels of TNFα, IL-1β, IL-6, and IL-8[33–38,40]. However, in our experiments using primary retinal cell types, we found that glucose did not elicit induction of these inflammatory cytokines, consequently yielding an inadequate cell culture model to study molecular mechanisms of DR. On the contrary, we have observed that PA reliably induced inflammatory cytokine production by hMC[45], and thus could serve as a reliable and robust *in vitro* model for diabetes-related dyslipidemia, complementing our ongoing studies of inflammation. Using RNA sequencing, we identified a variety of DR-relevant pathways, including NF-κB signaling and inflammation, intracellular lipid signaling, angiogenesis, and MAPK signaling, that were stimulated by PA, while elevated glucose alone did not significantly alter any diabetes-relevant pathways[45]. In **Chapter III** and the associated publication[57], we report that PA induces the expression of TNFα, IL-1β, IL-6, and IL-8 in hMC, all of which are known to be elevated in the diabetic retina. It is hypothesized that once inflammatory cytokines are produced in an early-DR response to metabolic stimuli, they amplify through autocrine and paracrine mechanisms, reaching levels that assume control of chronic and severe retinal inflammation. Accordingly, we chose to test IL-1β as a second stimulus to model the later-onset cytokine-dependent inflammation, as IL-1β is known to undergo MC-dependent auto-amplification and induce MC to express other inflammatory cytokines. Considering our choices of an early-DR metabolic stimulus (PA) and the later-DR cytokine stimulus (IL-1β), our main goal was to test whether increased cellular EET/EDP levels would provide continued efficacy along this hypothetical temporal axis of DR-pathogenesis.

Whether epoxides could mitigate FFA-induced retinal cell behaviors was uncertain; however, literary evidence suggested PA-induced inflammation might be susceptible to CYP-derived epoxide manipulation. Chen et al. demonstrated that 14,15-EET reduced PA-induced

inflammation, oxidative stress, NF-κB signaling, and ROS generation in hepatocytes[162]. Additionally, CYP2J2 overexpression in high-fat diet mice resulted in reduced serum and liver inflammatory cytokine levels and prohibited NF-κB/JNK activation[162]. Bettaieb et al. showed that sEH was upregulated in hepatocytes stimulated with palmitate, and that sEH inhibition attenuated markers of ER stress and JNK activation[213]. **Chapter III** confirmed the efficacy of the CYP-epoxygenated products to mitigate cytokine- and FFA-induced inflammation in hMC. Exogenous 11,12-EET and 19,20-EDP significantly decreased PA-induced gene expression of inflammatory cytokines TNFα, IL-1β, and IL-6 (**Fig. 6**), and IL-1β-induced expression of IL-1β, IL-6, and IL-8 (**Fig. 8**). sEH inhibition significantly reduced PA- and IL-1β-stimulated expression of all four cytokines queried: TNFα, IL-1β, IL-6, and IL-8 (**Fig. 7, 9**). 11,12-EET and 19,20-EDP were found to decrease PA- and IL-1β-induced NF-κB-dependent transcriptional activity (**Fig. 10**), which aligns with the findings from Chen et al.[162] mentioned above. These data suggest that experimental elevation of epoxides decreases inflammation in part by blocking NF-κB-dependent transcription, which is known to play an important role in DR pathogenesis.

Exogenous addition of epoxygenated fatty acids may have advantages over other anti-inflammatory therapeutic approaches, such as the use of corticosteroids, NSAIDs, or cytokine blockers. First, corticosteroids suppress pro-inflammatory transcription factors like NF-κB and are widely prescribed for inflammatory conditions such as IBS, arthritis, and asthma[254,255]. Corticosteroids have been tested in clinical trials for diabetic macular edema, and while they improved visual acuity after four months of treatment, they were less effective than laser coagulation after two years[255–257]. Moreover, corticosteroids exacerbate diabetes when administered orally[258], while intravitreally administered steroid therapy is known to increase the risk of glaucoma and cataract[255,256]. Second, NSAIDs that inhibit COX activity, such as aspirin and salicylate, have been shown to reduce DR sequelae in diabetic rats[259–261], and patients taking salicylates for rheumatoid arthritis were shown to have reduced incidence of DR[90].

Unfortunately, clinical trials of aspirin treatment for DR did not show benefits[91,92], and a study employing systemic COX-2-selective inhibitors increased the incidence of heart attacks and strokes and was terminated[262]. Finally, cytokine-directed therapies such as TNFα-blockers attempt to target specific inflammatory molecules pertinent to DR inflammation. Anti-TNFα therapy was found to block retinal inflammation, vessel leakage, and retinal cell injury in diabetic rats[54,263]. However, in human studies, patients with DME saw no statistically significant improvements in visual acuity, leakage, hard exudates, or hemorrhages[264], likely due to the influence of other inflammatory mediators. These three examples demonstrate the continued exploration of anti-inflammatory therapeutic approaches to address DR and underscore the ongoing quest for an ideal anti-inflammatory pharmacotherapy that is both safe and effective. We believe epoxides might address this need. Epoxide levels are decreased in hRMEC stimulated with cytokines[56] and in the vitreous of patients with NPDR and PDR[37], indicating that the exogenous addition of epoxides could be used to balance the endogenous system. This approach of homeostatic restoration would reduce the likelihood of toxicity or intolerance and impact a broader array of inflammatory influences, as opposed to a more targeted therapeutic with a narrower scope. The search for anti-inflammatory medications to treat early DR continues to be important, as it would prevent the development of irreversible changes that occur later in the pathologic course, including those that lead to total blindness.

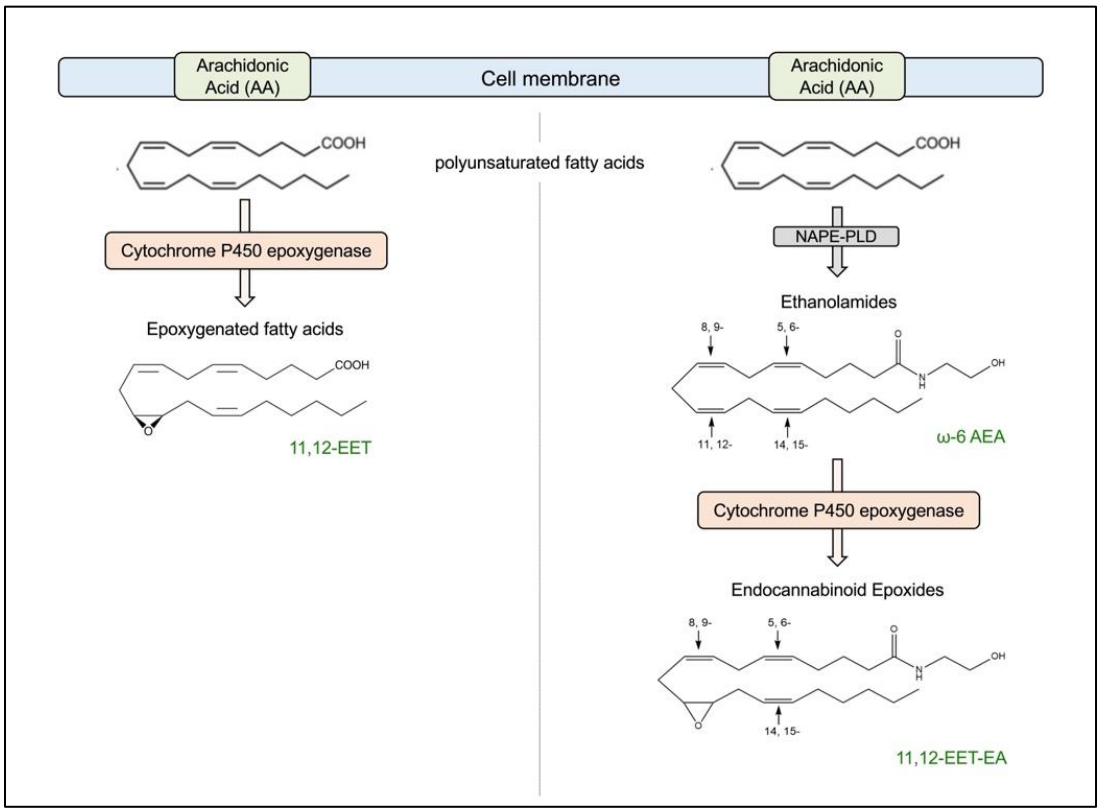


Figure 20. Overview of the synthesis of epoxygenated fatty acids vs. endocannabinoid epoxides. Arachidonic acid (AA) is stored in the plasma membrane and is the precursor for one group of epoxygenated fatty acids and endocannabinoid epoxides. Similar pathways exist for the production of epoxygenated fatty acids and endocannabinoid epoxides derived from docosahexaenoic acid (DHA). CYP-epoxygenation activity yields four regioisomers of epoxyeicosatrienoic acid (5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET), and this schematic shows the steps to synthesize 11,12-EET, and the corresponding endocannabinoid regioisomer, 11,12-epoxyeicosatrienoic acid ethanolamide (11,12-EET-EA). This schematic is one example that demonstrates the structural similarities of epoxygenated fatty acids and the respective regioisomer of endocannabinoid epoxides, and thus their potential similarities in bioactivity.

The success of epoxides at reducing retinal inflammation in DR models hints at the potential anti-inflammatory success of other CYP-epoxygenated products. As seen in **Figure 20**, the epoxygenated fatty acid 11,12-EET is closely related to the corresponding endocannabinoid epoxide, 11,12-EET-EA; the difference between the two being the addition of an ethanolamine group. While **Chapter III** demonstrated the anti-inflammatory properties of 11,12-EET and 19,20-EDP in DR models, the scope of that research remains limited without the knowledge of

the affiliated receptors. Therefore, another epoxygenated metabolite of AA (or DHA) with well characterized receptors may offer more opportunities for scientific exploration, potentially with similar beneficial characteristics in DR. Endocannabinoid epoxides are known to work through two receptors, CB1 and CB2. With this knowledge, the signaling mechanisms and bioactivities of these lipids can be more thoroughly analyzed, and further studies can incorporate techniques designed to manipulate the receptor, rather than just the ligand. We conducted preliminary work in hRMEC to examine the effects of exogenous eCB addition on retinal inflammation, similar to our approach in **Chapter III** using epoxides. In these experiments, we observed instances where eCBs exhibited greater anti-inflammatory effects than epoxides (**Appendix B**). Additionally, evidence in the literature suggested that eCB would be more stable *in situ*, extending their half-lives and potentially protective influences in retinal tissue[243,244]. Therefore, we pursued the study of the therapeutic potential of eCBs in DR.

There is a wide variety of eCBs that range in their affinities for CB1 and CB2. For example, as seen in **Figure 20**, anandamide (AEA) is a well characterized eCB known to bind with high affinity to CB1 and with low affinity to CB2[265]. In addition, another prominent eCB, 2-arachidonoylglycerol (2-AG), binds with similar affinity to both CB1 and CB2[266]. By contrast, the endocannabinoid epoxides EET-EA and EDP-EA are highly selective for CB2[83,141], which is why they were the initial focus of our studies. However, we ultimately chose to concentrate on synthetic CB2 receptor agonists, both as a means to mitigate receptor promiscuity and bypass the problem of ligand instability. CB2-specific agonists have extremely low affinity for other eCB receptors (e.g., CB1, transient receptor potential vanilloid 1 and 2 [TRPV1, 2]; other G protein-coupled receptors [GPR18, 55, 119]) that might otherwise confound mechanism of action. Additionally, CB2 agonists have already demonstrated acceptable safety profiles in clinical trials for non-ocular inflammatory pathologies, lowering the bar for their clinical translation to DR treatments[173,253].

Chapter IV describes our efforts to test the efficacy of CB2 agonists in cellular and

animal models of NPDR. Initially, we used pharmacologic tools in validated *in vitro* assays to evaluate the effects of CB2 activation on hRMEC and mRMEC stimulated with diabetes-relevant inflammatory cytokines. RMEC are the primary site of critical pathogenic events in early DR, including inflammation-induced leukostasis. Primary cells were used in all *in vitro* experiments, and leukostasis readouts included the query of ICAM-1, VCAM-1, and E-selectin mRNA and protein levels, as well as PBMC adhesion to hRMEC monolayers. Additionally, in our previous work we showed that among several DR-relevant cytokines, TNFα and IL-1β elicit the strongest responses from hRMEC [56,200,231,267,268]. Thus, these cytokines were used at pathophysiologic concentrations to stimulate hRMEC in an attempt to effectively model DR-related, cytokine-induced inflammation *in vitro*.

We believed this approach would prove successful because it is well-established that inflammation induces pathogenic behaviors in RMEC early in DR, and previous findings indicate that CB2 activation suppresses analogous responses in non-ocular tissues. For example, in TNFα-treated brain microvascular EC, two CB2 agonists inhibited the expression of 31 and 32 out of 33 upregulated pro-inflammatory genes that were monitored[199]. Additionally, inflammatory cytokines have been shown to induce leukostasis in multiple tissues, and CB2 agonism has been demonstrated to prevent inflammation-induced adhesion molecule expression, monocyte adhesion, and/or monocyte extravasation in a variety of non-ocular EC types[151,193,201]. Finally, akin to the elevated retinal permeability experienced in diabetic macular edema, CB2 agonism was shown to rescue cytokine- or endotoxin-induced hyperpermeability in brain microvascular endothelial cell monolayers as measured by Transendothelial Electrical Resistance[151,199]. The literature supports the hypothesis that CB2 activation will mitigate these RMEC pathogenic and pathologic behaviors in the context of DR, and our findings demonstrate, for the first time publicly, that CB2 agonism has therapeutic influence in the context of early DR intervention targeting leukostasis.

In **Chapter IV,** we demonstrate that CB2 agonism significantly reduced the gene expression of ICAM-1, VCAM-1, and E-selectin (**Fig. 11**) and decreased the protein levels of ICAM-1 and VCAM-1 (**Fig. 12**) in hRMEC. This reduction correlated to a decrease in leukocyte adherence to hRMEC, as shown in a static adhesion assay (**Fig. 13**). We acknowledge the challenge of modeling chronic disease processes in short-term cell culture experiments; however, our *in vitro* experiments faithfully recapitulate pathogenic events known to occur in DR (e.g., cytokine and leukocyte adhesion molecule elaboration, leukocyte-endothelial cell interactions). Our *in vitro* methods serve to elucidate key information in a controlled mono-cellular environment, isolated from the potentially confounding influences of other cells/tissues, allowing us to narrow our focus along a specific pathologic cascade. This approach enhances our mechanistic understanding of the effect of our therapeutic strategies on isolated processes. Importantly, our *in vitro* results successfully informed the translation of our experimental strategies to *in vivo* mouse models of retinal inflammation and diabetes, while also providing human relevance to those *in vivo* results. Subsequently, our *in vivo* models enable us to replicate inflammatory processes in complex biological systems, supporting our results from more isolated methods and allowing us to extrapolate our findings to a more relevant disease context.

Our *in vivo* experiments comprised two models: (1) a murine model of acute retinal inflammation and (2) a murine model of type 1 diabetes. The model of acute retinal inflammation is a well-established and characterized method[46,56,200,231,245] to induce retinal leukostasis *in vivo* through intravitreal cytokine-delivery. This model complements the methodology of our *in vitro* experiments, which also utilize direct cytokine-stimulation. In this model, mice received daily intraperitoneal injections of vehicle or HU-308 for one week prior to receiving a single intravitreal injection of TNFα. Leukostasis analyses were performed 12 hours later, revealing that TNFα caused a 2.3-fold increase in retinal leukostasis, which was reduced to vehicle levels in mice receiving systemic HU-308 (**Fig. 16**). These results demonstrate critical proof of concept that

CB2 activation is linked to important consequences of diabetes and underscore the high therapeutic potential of our treatment strategy. Most excitingly, these findings show the capacity of *systemic* CB2 agonism to alleviate effects of local retinal inflammation, suggesting strong potential for clinical translation to treat diabetic retinopathy. Notably, and by contrast, all currently approved drug treatments for DR require intraocular delivery.

The effects of CB2 agonism were also tested in a murine model of type 1 diabetes, in which streptozotocin (STZ) was used to ablate pancreatic beta cells. While many animal models, such as high fat diet (HFD) or db/db mouse models, are also conventionally used in diabetes studies, STZ-induced diabetes is a preferred model when studying DR. STZ-treated mice consistently recapitulate progressive chronic inflammatory features of NPDR, including increased retinal inflammatory cytokine levels, leukostasis, hyperpermeability, and endothelial cell and pericyte apoptosis[247]. Extensive evidence from human studies suggests that the last of these events leads directly to retinal vasoregression[27], followed by retinal hypoxia and eventually culminating in vision-threatening PDR onset. Models such as HFD or db/db have a slower onset and progression of DR pathologic features, if DR sequelae emerge at all. In STZ-treated mice, the emergence of DR pathogenic events follows a well-established timeline, allowing for justifiable planning of our leukostasis assessment in these animals. In this model, retinal inflammation develops soon after onset of diabetes (with detectable elevated cytokines at 4 weeks), followed by leukostasis and vascular hyperpermeability at 8 weeks, and pericyte death and capillary dropout at 12 weeks. In this experiment, mice received HU-308 either via systemic, intraperitoneal administration or local, intravitreal administration. Leukostasis readings were performed 10 weeks post-STZ injection, and results indicate that both systemic and local HU-308 administration significantly decreased retinal leukostasis in a STZ-induced model of murine diabetes (**Fig. 17**).

It was important in our studies to utilize CB2 knockout animals to confirm our experimental concept and validate the specificity of our agonists. We established a colony of

CB2 (*Cnr2*) null mice, previously demonstrated to exhibit worsened pathology in other, non-ocular models of tissue inflammation[147], and hypothesized that diabetic *Cnr2*$^{-/-}$ mice would demonstrate exacerbated retinopathy compared to diabetic wild-type mice. In these experiments, it was found that diabetes induction caused a near doubling of retinal leukostasis, and *Cnr2* deletion yielded a 50% increase in retinal leukostasis, though the difference between these two inductions was insignificant (**Fig. 17, 19**). This demonstrates that the deletion of CB2 alone can be associated with a pro-inflammatory retinal phenotype which is similar in scale to the influence of diabetes. Systemically administered HU-308 significantly decreased diabetes-induced retinal leukostasis in WT mice, while HU-308 had no effect on retinal leukostasis in *Cnr2*$^{-/-}$ animals, indicating the absence of off-target effects (**Fig. 17a**). This experiment supports the hypothesis that CB2 function is crucial to retinal homeostasis under diabetic conditions and strongly suggests that CB2 activation will prove beneficial in DR management. In addition to our *in vivo* studies, we also isolated mRMEC from the *Cnr2*$^{-/-}$ animals to test our agonists *in vitro*. *Cnr2*$^{-/-}$ mRMEC were stimulated with cytokine, and HU-308 and CB65 were found to have no effect on mRMEC inflammatory response, supporting our conclusion that the efficacy observed in hRMEC experiments was CB2-dependent (**Fig. 18**).

Our proposed therapeutic strategies provide advantages for translation to the clinic because they rely on manipulation of an endogenous system, allowing for protection throughout multiple stages of DR progression while minimizing toxicity. Additionally, some of the drugs we consider here, such as sEHi, FAAHi, and CB2 agonists, are already in clinical trials for other conditions, lowering the barrier to FDA approval, acceptance by the medical community, and the burden of protracted formulation efforts[165–167,170,171,173,253]. Most importantly, our work is significant because it focuses is on early events in the pathologic cascade of DR. We believe that the dysregulation of the ECS contributes to the chronic retinal inflammation experienced in NPDR, and the characterization of this relationship could be beneficial in elucidating a novel therapeutic strategy at this early stage. Currently, there are no approved therapeutics for NPDR,

and the development of an effective treatment that could delay DR onset, slow its progression, or limit or eliminate retinal damage done by the transition to PDR is, we believe, a worthwhile undertaking.

CB2 agonism can potentially influence systemic manifestations of diabetes. The CB2 receptor is broadly expressed in human tissue, including in critical immune organs or cell types such as the spleen, thymus, and tonsils, or T cells, B cells, and monocytes[147]. This attests to the overarching immunomodulatory role of CB2 in the body, consistent with numerous studies confirming the potential therapeutic value of CB2 in regulating systemic aspects of diabetes[155]. For instance, research demonstrates that CB2 agonists increase insulin secretion and decrease plasma glucose level in murine diabetes models[155,249]. Moreover, in obese mice, CB2 agonists lower lipid, glucose, and inflammatory cytokine levels in blood, and fat mass[155,250]. Thus, while CB2 activation may have exciting potential for general diabetes management, our findings attest to the specific benefits of both local and systemic CB2 agonism on eye tissues. Context is important here, because the eye, and particularly the retina, harbors a measure of immune privilege, which presents unique challenges for effective drug delivery and action[269]. Another concern for systemic distribution of CB2 agonists to the retina is the potential difficulty for some drug classes to cross the blood-retina barrier, which limits their bioavailability at the site of pathology. To overcome this issue, specialized drug delivery methods, such as intravitreal injections, intraocular delivery devices, or eye drops, may be required for ocular drug efficacy. Considering these issues, we tested and compared both systemic and local administrative routes when testing the efficacy of CB2 agonism *in vivo*. Our results show that HU-308 effectively mitigated ocular leukostasis, regardless of the administration route (**Fig. 17**). This suggests that, while CB2 agonism may provide systemic benefits from intraperitoneal administration, these benefits do not fully account for the beneficial effects observed, as mice receiving intravitreal administration experienced similar outcomes. Additionally, despite previous studies indicating that CB2 agonism improves diabetic complications at the systemic level, our

studies revealed no changes in fasting blood glucose or weight across treatment groups (**Supp. Fig. 5**).

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Owing to their important biological functions, the CYP-epoxygenated products of AA and DHA (i.e., epoxides and eCB), have been studied extensively for decades. With time, our understanding of the metabolism and regulation of these CYP products has improved, as we seek to better understand their production, hydrolysis, and bioactivities in both healthy and dysregulated disease systems and tissues. Herein, we attempt to address targeted questions in the expansive and complex landscape of prior work. CYP metabolites have been assigned hypothetical functional roles and signaling mechanisms as their physiologic significance in biologic systems has slowly come into focus. Now, studies of their pathogenic deviation and pathophysiological implications in various disease systems and tissue contexts can be used to inform the development of targeted therapies. Given our growing understanding of these CYP metabolites, findings from relevant disease models can now be used to design and implement effective clinical tools. The experiments described here represent preliminary efforts to that end.

While there are many different angles from which these lipids can be studied, the catalog of information for each remains incomplete. In this dissertation, we attempted to explore the therapeutic potential of epoxides and eCB in a novel context, guided by evidence from previous literature. The published studies referenced herein demonstrated CYP-epoxygenated fatty acid products to be anti-inflammatory and anti-apoptotic in various physiological contexts [94–96,162,270]. Moreover, the preponderance of evidence indicates that CYP levels decrease and sEH levels increase in many inflammatory[114–116] or diabetic states [117–120,122]. These findings encouraged and directed our efforts. Still, the literature lacked comprehensive studies in the eye. Vitreous sampling by Schwartzman et al. was instrumental to our hypotheses, as her group showed that levels of CYP-derived lipid metabolites in ocular fluids were inversely correlated with pathologic conditions in an ocular setting. Vitreous levels of EETs were found to be significantly reduced in both NPDR and PDR relative to non-diabetic patients[37], leading us to contemplate the effects of

returning these CYP-derived lipids to non-disease levels. **Chapter III** of this dissertation attempted to address the effect of increasing epoxides in DR-simulated retinal circumstances to guide our understanding of how pathogenic alteration of these metabolites could be targeted for clinical DR therapy. Still, our studies remain narrow and must continue to be expanded. Additional cell types, stimuli, epoxide regioisomers, animal models, agonists, etc., would all constitute compelling extrapolations of this project, and would further our understanding of the physiologic significance and therapeutic potential of CYP metabolites in DR.

We felt particularly compelled to test the effects of eCB, drawn by earlier studies in other ocular indications[271–273]. CYP metabolites EET-EA, and EDP-EA display anti-inflammatory activity via the CB2 receptor[83,141,144], and CB2 is widely expressed in the retina[251]. Notably, CB2 agonism had been tested previously in a variety of eye disease models, including uveitis[182], glaucoma[274], corneal injury[184], and proliferative vitreoretinopathy[183], leading us to believe this method could be applied to DR models as well. Moreover, in models of non-ocular inflammation, CB2 agonism was shown to decrease leukocyte recruitment and adherence, inflammatory cytokine production, barrier function deficits, and apoptosis[147,151,152]; however, minimal studies have determined if these beneficial effects would hold true for these pathogenic sequelae in DR[275]. Hence, in **Chapter IV,** we sought to close the gap in our knowledge by testing the effects of CB2 agonism in models of DR. While the introduction of this dissertation discusses many ways that CYP metabolites could be modulated for therapeutic benefit, and our early work focused on epoxides, we believe that CB2 agonism shows the most promise for DR treatment. In addition to the synthetic agonists discussed here, there are many naturally existing eCBs that also activate CB2, and these could be exogenously enhanced. EET-EA and EDP-EA are known to bind and activate CB2[83,141], though studies of these compounds and their effects in tissues and disease contexts like DR remain nascent, at best. Additionally, information about the synthesis, degradation, and receptor binding of these eCB isomers are largely the product of "test tube" studies[83], and have not been explored yet in biological systems. The gaps in

knowledge that exist for EET-EAs and EDP-EAs extend to other, especially lesser known, eCBs, as the ECS and its vast complexity currently remain sparsely explored. Still, CB2 activation with synthetic agonists demonstrated efficacy in our DR models, and thus, the study of exogenous EET-EAs and EDP-EAs would be a particularly interesting next step of this project.

This dissertation does not address strategies that attempt to influence epoxide and eCB synthesis or hydrolysis. Evidence suggests that increased CYP expression is beneficial in disease models like diabetes, presumably due to the increased synthesis of its metabolites[95,162]. While researchers have shown how interesting and beneficial CYP modulation can be in other disease contexts, CYP studies were not pursued in this project because we chose to focus on strategies that could be more easily translated to the clinic. Extending epoxide and eCB half-lives via hydrolysis inhibition may also constitute a rational method to therapeutically modulate CYP-metabolite levels. The effects of FAAHi and sEHi are widely studied, and their efficacy in non-ocular contexts has led to clinical trials testing their therapeutic potential in many pathological conditions[165–167,170,171]. Still, their efficacy in DR has not yet been studied, and virtually nothing is known regarding their potential to prolong epoxide or eCB levels in retina or their effects on retinal inflammation. Our preliminary experiments demonstrated that both FAAHi and sEHi could reduce inflammation in hRMEC (**Appendix A**), though we did not generate evidence that this anti-inflammatory activity was correlated with altered CYP metabolite levels in these cells. Still, the exogenous addition of naturally occurring EET/EDP-EAs in combination with the inhibitors of the hydrolytic enzymes that degrade them offers another avenue of investigation that may prove beneficial.

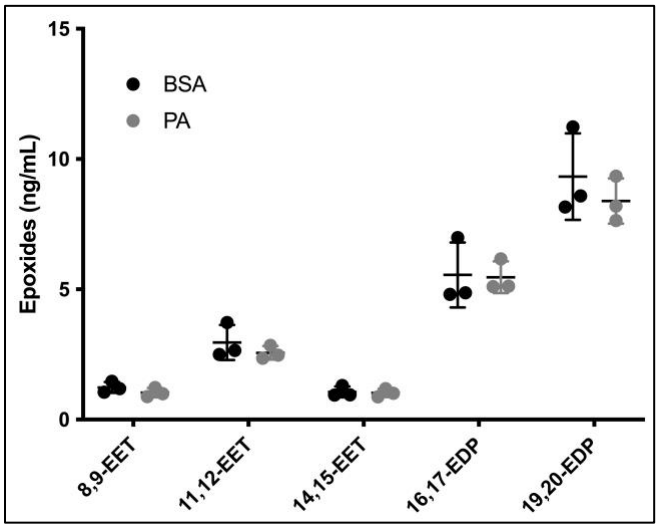
Logical next steps for this project would be to investigate the potential of CB2 activation in additional cell types and additional DR models. For example, preliminary experiments in hMC showed that both HU-308 and CB65 may be efficacious at reducing cytokine-induced cytokine expression (**Appendix C**). Additionally, preliminary experiments show that CB2 agonism may be efficacious in experimental models of retinal *vascular hyperpermeability* – a pervasive and

dominant feature of DR (**Appendix D**). In the eye, the blood-retinal barrier (BRB) plays a crucial role in protecting neurons, which require a delicate and unique environment for proper function and, importantly, cannot regenerate if damaged[276]. Therefore, maintaining tight vascular integrity through this tissue is crucial. Junctional complexes (tight and adherens junctions) between endothelial cells form a barrier that effectively separates circulating blood from the retinal interstitium, though these are selective to specific solutes necessary for retinal function[276]. These junctions include essential proteins such as zonula occludens 1, 2, and 3 (ZO-1, ZO-2, ZO-3), occludin, and claudin among others[276–280]. Vascular hyperpermeability both precedes and is associated with neovascularization. VEGF is a permeabilizing growth factor, as it causes disorganization of tight junction proteins, including phosphorylation of some proteins, increased extractability, morphology changes, and generally decreased protein levels[276,281,282]. VEGF therefore causes the breakdown of the existing BRB promoting hyperpermeability. As a potent endothelial cell mitogen, VEGF also promotes the proliferation new blood vessels which are highly permeable and prone to hemorrhage[1]. We have shown that CB2 agonism mitigates leukostasis in models of DR, and we have preliminary evidence suggesting that the same approach may also mitigate vascular hyperpermeability. MC, along with other cell types, release VEGF in response to the hypoxia caused by leukocyte capillary occlusion and vasoregression[1]. Therefore, these critical pathologic features of DR are functionally linked. Our preliminary *in vivo* experiments show the beneficial effects of CB2 agonism on vascular hyperpermeability; however, corresponding *in vitro* work studying junctional protein complexes and barrier integrity are necessary to complete this work.

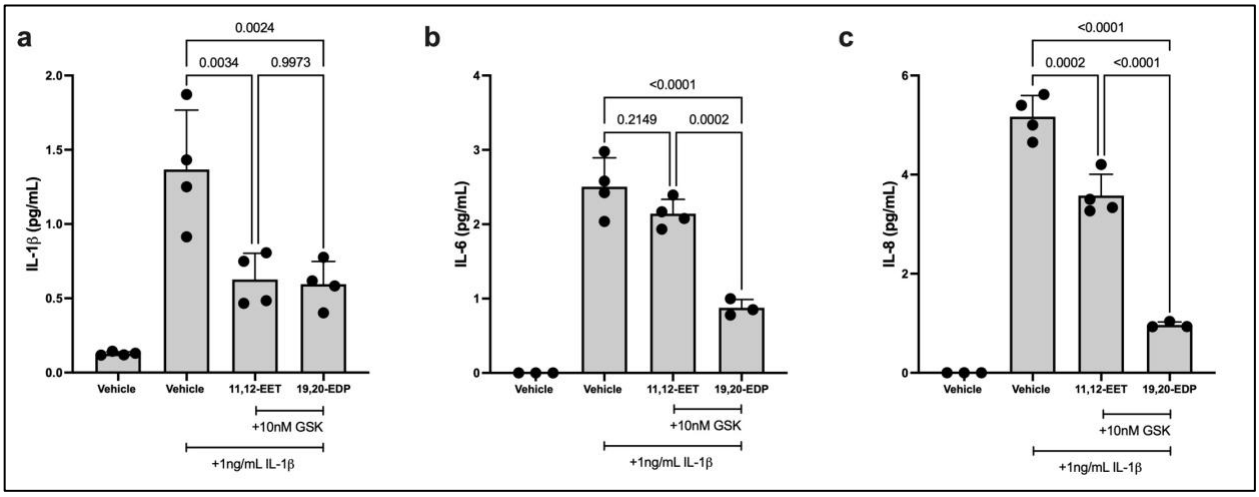
In summary, the experiments described herein have demonstrated the anti-inflammatory potential of epoxygenated fatty acids and endocannabinoids in two major cell types of the retina: Müller cells and endothelial cells. This work demonstrates the importance of CYP-metabolites to retinal homeostasis, their presumed dysregulation in DR, and the benefits of restoring CYP metabolite levels to homeostatic health. Our approach would mitigate DR pathogenesis at its

earliest stage, where no therapeutic strategies currently exist, potentially preserving vision quality globally for millions of people.

APPENDIX
Appendix, Supplementary Figures Related to Chapter 3

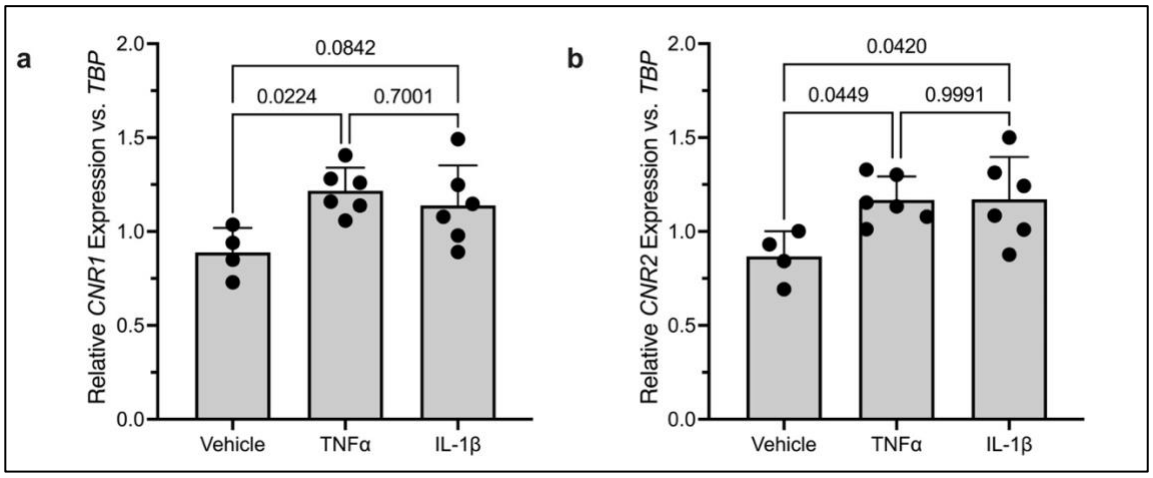


Supplementary Figure 1. The effect of PA on epoxide levels in hMC culture medium. hMC were treated with either BSA (vehicle; 100mg/mL) or BSA-conjugated PA (250μM) for 24 hours. AA (10μM) or DHA (10μM) was added for the last 3 hours of treatment. PA had no effect on epoxide levels (n=3).

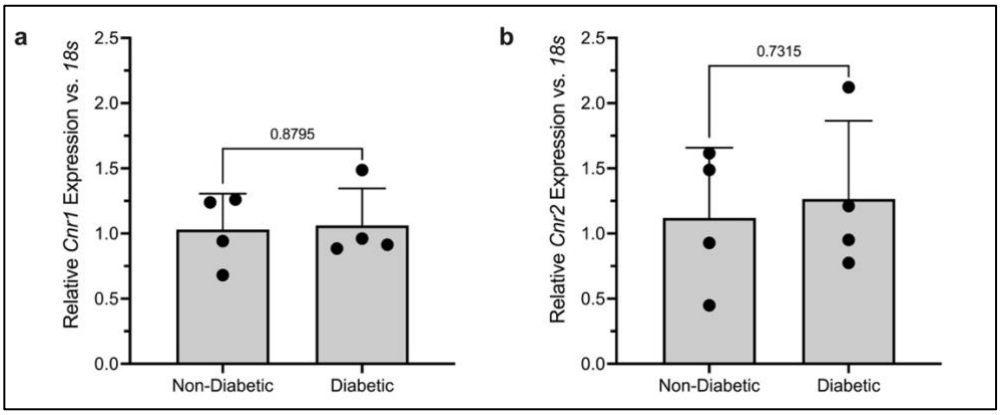


Supplementary Figure 2. The effect of 11,12-EET or 19,20-EDP and GSK2256294 on IL-1β-induced inflammatory mediator secretion in hMC. hMC were pretreated with 11,12-EET or 19,20-EDP (0.5μM) and GSK2256294 (10nM, sEH inhibitor) for 2 hours before IL-1β (1.0ng/mL) was added for 8 hours. The medium was changed to 11,12-EET or 19,20-EDP and GSK2256294 for another 8 hours. Cytokine secretion was assayed by (a) ELISA and (b,c) AlphaLISA. (a) IL-1β, (b) IL-6, and (c) IL-8 secretion was significantly decreased with the use of epoxygenated fatty acid and sEH inhibitor. Bars represent mean ± SD (n=3-4). Outliers removed with Grubbs Test.

Appendix, Supplementary Figures Related to Chapter 4

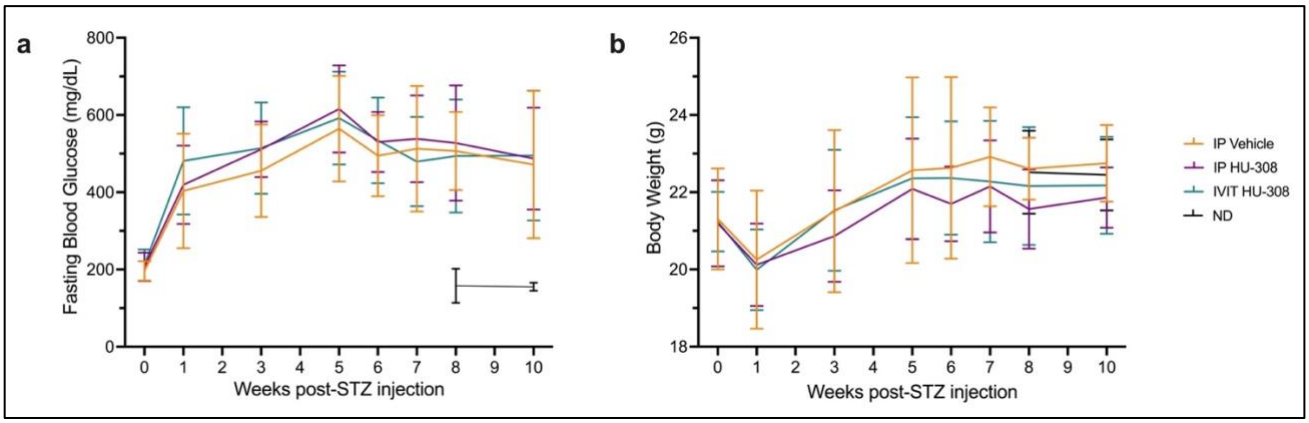


Supplementary Figure 3: The effect of TNFα- and IL-1β-stimulation on *CNR1* and *CNR2* expression in hRMEC. hRMEC were stimulated with TNFα or IL-1β (1ng/mL) for 4 hours. Total RNA was isolated and cannabinoid receptor expression was assayed via qRT-PCR. TNFα-stimulation increased *CNR1* and *CNR2* expression and IL-1β-stimulation increased *CNR2* expression in hRMEC. Each bar represents mean ± SD (n=4-6).



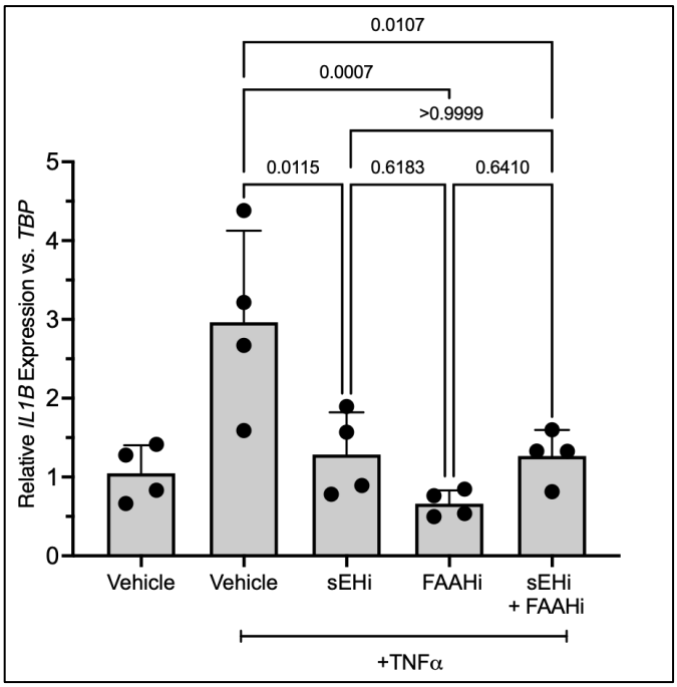
Supplementary Figure 4: The effect of STZ-induced diabetes on *Cnr1* and *Cnr2* expression in mouse retinal tissue. Mice were treated with STZ for 5 consecutive days. 6 weeks-post STZ administration, mice were anesthetized, sacrificed and their retinas were removed and vortexed. Total RNA was isolated and cannabinoid receptor expression was assayed via qRT-PCR. *Cnr1* and *Cnr2* expression in the retina was unaffected by the induction of diabetes in STZ-treated mice. Each bar represents mean ± SD (n=4).

Appendix, Supplementary Figures Related to Chapter 4, cont'd



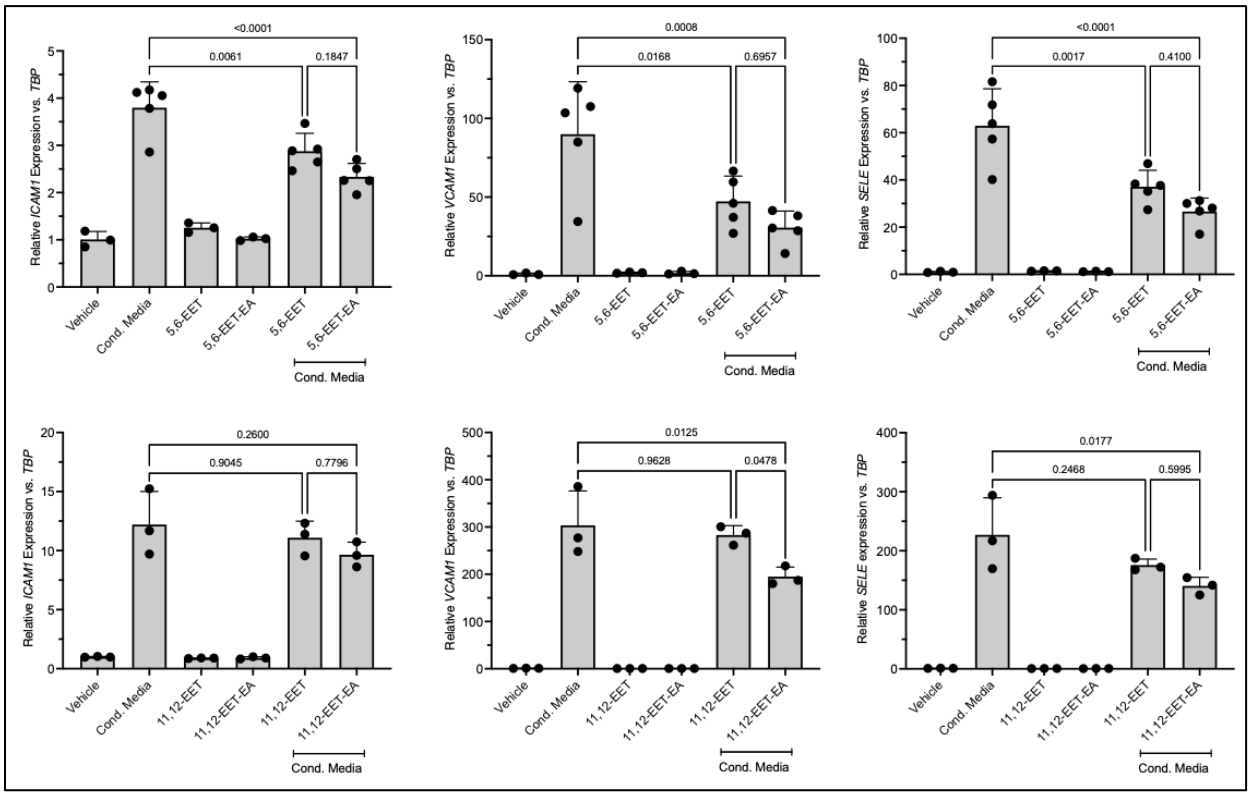
Supplementary Figure 5: The effect of HU-308 on fasting blood glucose and body weight in STZ-treated mice. For intraperitoneal (IP) administration, C57BL/6J mice were given daily IP injections of vehicle or HU-308 (5mg/kg) for 4 weeks starting 6 weeks post-STZ injection. For intravitreal (IVIT) administration, mice were given a single IVIT injection of HU-308 the day before sacrifice, 10 weeks post-STZ injection. *In vivo*, both systemic and local HU-308 administration had no significant effect on (a) fasting blood glucose nor (b) body weight in STZ-induced diabetic mice. Each bar represents mean ± SD (n=2-13).

Appendix A



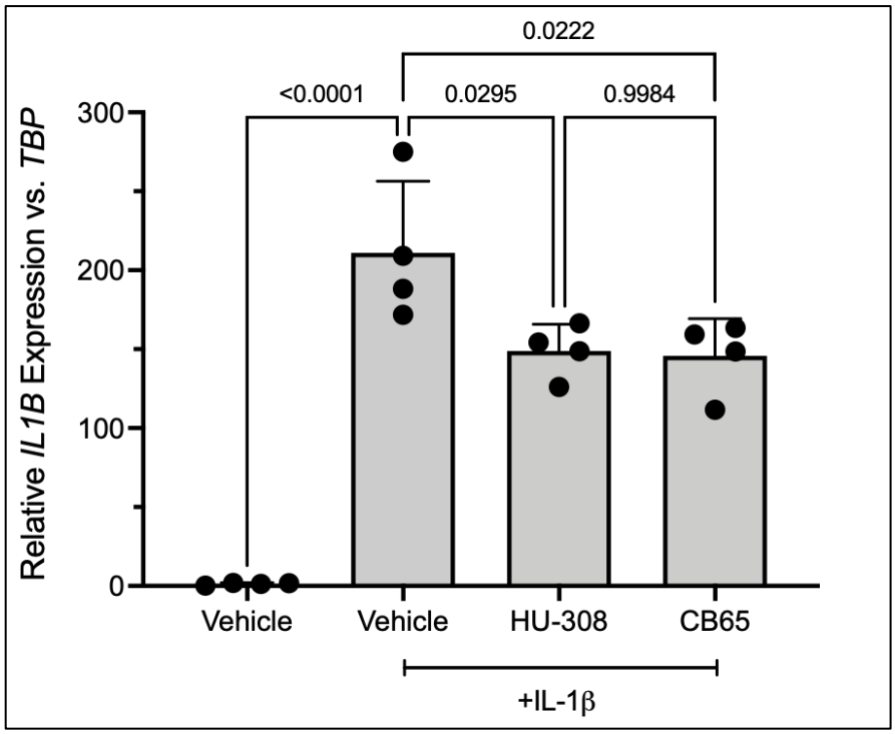
The effect of FAAH inhibition and sEH inhibition on TNFα-induced cytokine expression by hRMEC. hRMEC were pretreated with FAAHi (URB597, 1μM) and/or sEHi (GSK2256294, 0.1μM) for 2 hours, followed by the addition of TNFα (0.1ng/mL) for 4 hours. After treatment, total RNA was isolated and cytokine expression was assayed via qRT-PCR. *IL1B* expression was significantly decreased by the treatment of FAAHi alone, sEHi alone, and FAAHi and sEHi together. Bars represent mean ± SD (n=4).

Appendix B



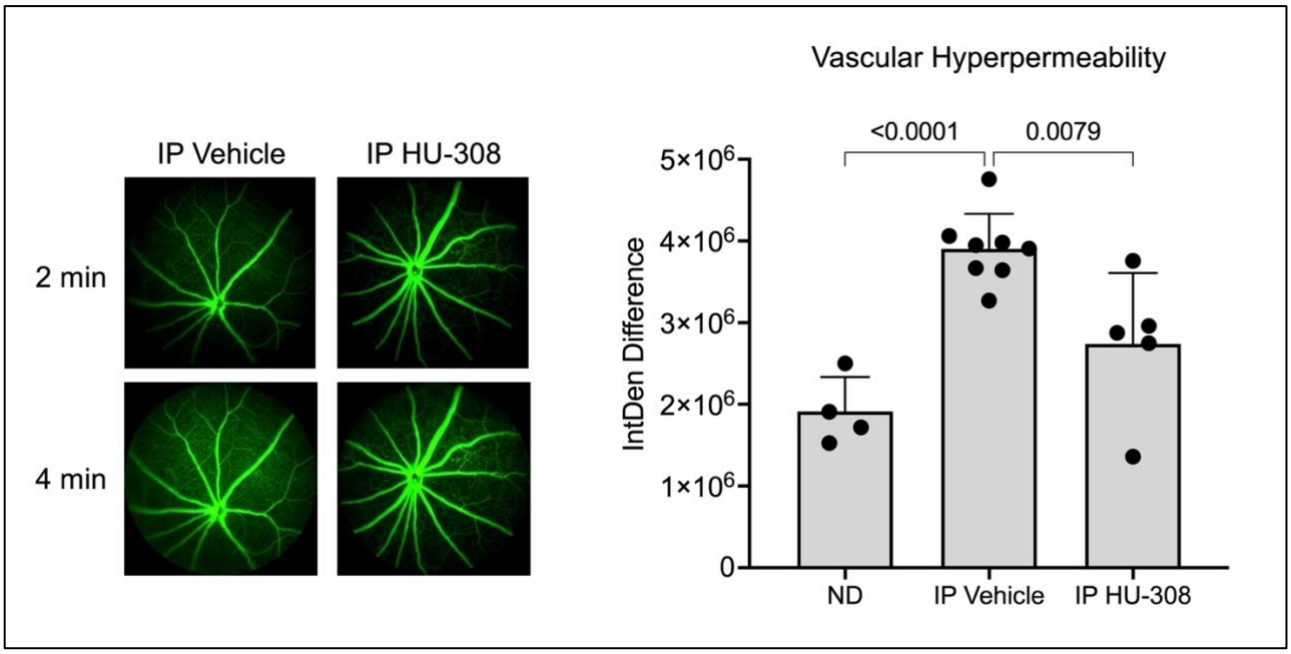
The effect of epoxides, 5-6-EET and 11,12-EET, and eCB, 5,6-EET-EA and 11,12-EET-EA, on conditioned media-induced adhesion molecule expression by hRMEC. hMC were treated with IL-1β (1ng/mL) +/- epoxide or eCB (0.5μM) for 2 hours. Media was replaced, and conditioned media was generated for 6 hours before being transferred to hRMEC for 2 hours. Total RNA was isolated and cytokine expression was assayed by qRT-PCR. The conditioned media produced by IL-1β-stimulated hMC caused significant increases in the expression of *ICAM1, VCAM1* and *SELE* by hRMEC. (a) For the 5,6-regioisomers, co-treatment of hMC with epoxide or eCB attenuated this induction, with 5,6-EET-EA showing a more potent effect than 5,6-EET (b) For the 11,12-regioisomers, statistically significant results were only seen using co-treatment with 11,12-EET-EA, not 11,12-EET, and results were not significant for *ICAM1*. Bars represent mean ± SD (n=3-5). Experiments conducted by Amy Stark.

Appendix C



The effect of HU-308 and CB65 on IL-1β-induced cytokine expression in hMC. hMC were introduced to serum-depleted (2% FBS) media for 12 hours, then stimulated with IL-1β (1ng/mL) in the presence or absence of HU-308 or CB65 (100nM) for 8 hours. After treatment, total RNA was isolated and cytokine expression was assayed via qRT-PCR. *IL1B* expression was significantly decreased by both HU-308 and CB65. Each bar represents mean ± SD (n=4).

Appendix D



The effect of systemic HU-308 administration on STZ-induced vascular hyperpermeability *in vivo.* C57BL/6J mice were given daily IP injections of vehicle or HU-308 (5mg/kg) for 2 weeks, 6 weeks post-STZ injection. Quantitative fluorescein angiography (qFA) readings were performed 8 weeks post-STZ administration to determine vascular permeability. Mice were anesthetized and a 10% sodium fluorescein solution was injected systemically to visualize the vessels of the eye. Vascular leakage was quantified by subtracting the integrated density of fluorescent fundus images taken 2 minutes and 4 minutes post-injection. (a) Representative fluorescent fundus images of vehicle vs. HU-308 treated mice, showing vascular leakage 2 minutes and 4 minutes after fluorescein injection. (b) Quantification of vascular leakage, shown as difference in integrated density between fundus images. Bars represent mean ± SD (n=4-8). Experiments conducted in collaboration with Taylor.

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