THE ROLE OF THE INTERLEUKIN-1 FAMILY IN THE DEVELOPMENT OF DIABETIC RETINOPATHY

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ABSTRACT

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Diabetic retinopathy is one of the most prominent complications of diabetes with approximately 67% of diabetic patients experiencing some form of retinopathy (1). Currently there is no cure for the disease, and in order to develop more reliable therapies a better understanding of mechanisms leading to disease onset and progression are crucial. The aim of this dissertation was to identify underlying mechanisms promoting diabetic retinopathy. Since there is a growing consent that chronic retinal inflammation might be causing and driving the progression of diabetic retinopathy the research was specifically focused on identifying potential mediators and cell types participating in the inflammation, and testing whether targeting specific inflammatory pathways will be a valid therapeutic strategy to treat diabetic retinopathy.

First, in order to understand the role of inflammation in diabetic retinopathy we examined the caspase-1/IL-1β pathway. We demonstrated that caspase-1 activity is increased in the retinas of streptozotocin (STZ)-induced diabetic mice at 10 and 20 weeks of diabetes. Interestingly, elevated caspase-1 activity was prevented in diabetic IL-1R1$^{-/-}$ mice at 20 weeks indicating that sustained caspase-1 activity is dependent on feedback through IL-1R1. The same phenomena was observed in Müller cells *in vitro*. Furthermore, we identified Receptor Interacting

Protein-2 (RIP2) as a central regulator of caspase-1 activity induced by either high glucose or IL-1β. It was further identified that this caspase-1 activity leads to Müller cell death both *in vitro* and *in vivo*.

To confirm that the activation of the caspase-1/IL-1β pathway is indeed responsible for retinal pathologies associated with retinopathy we used the galactosemic mouse model, another model of retinopathy leading to the same vascular pathologies seen in the STZ diabetic mouse model. Knockout of caspase-1 prevented the formation of acellular capillaries in galactosemic mice. When Müller cells were treated with elevated galactose levels, caspase-1 was activated and led to cell death. Interestingly, mediators associated with caspase-1 activation such as Thioredoxin Interacting Protein (TXNIP) and oxidative stress were not induced by galactosemia as they are in hyperglycemic conditions.

Finally, to identify potential roles of other IL-1 family members we began to examine the role of IL-1α in the activation of the caspase-1/IL-1β pathway. Our data suggest that IL-1α contributes to caspase-1 activity and Müller cell activation since treatment with an IL-1α neutralizing antibody inhibited high glucose induced caspase-1 activity. Furthermore, we observed that IL-1α appeared to translocate in to the nucleus under high glucose conditions in Müller cells *in vitro.*

Collectively, these findings indicate that caspase-1 activation and subsequent IL-1β production are crucial for the development and progression of diabetic retinopathy. Müller cells are a prominent site of active caspase-1 in the diabetic retina. It seems that targeting the caspase-1/IL-1β pathway might be a potential new strategy to develop therapies to treat diabetic retinopathy.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude for my mentor, Dr. Susanne Mohr, as without her guidance and support none of this work would have been possible. Throughout my training she was a constant source of encouragement and was always dedicated to ensuring that I achieved my goals. I will always appreciate the support she gave me, not only in lab so that I could learn the technical skills required to be a successful scientist, but also outside of lab so that I found myself in a position to put those technical skills into practice when I completed my training. Something I will always be thankful for is that Dr. Mohr always encouraged me to apply, participate and make the most of every opportunity. By doing so, I was able to become a more well-rounded scientist and also have opportunities that I would have never imagined and experiences I will never forget. Following her advice I was fortunate enough to attend and present at numerous international conferences, and it was at these conferences that she always made it a top priority to introduce me to some of the leading researchers in our field and include me at dinners and social events so that I could make contacts that would be crucial for success at the next level. She also encouraged me to apply to the Fundamental Issues in Vision Research course, a special topics course held at the Marine Biology Lab in Woods Hold, MA. This was an amazing experience where I was able to spend two weeks learning about vision science from leaders in the field and also meets other aspiring scientists with similar research interests. Additionally, Dr. Mohr always encouraged me to seek out an opportunity to research abroad, and it was through this

encouragement that I found a one-year internship at the pharmaceutical company Hoffmann La-Roche in Basel, Switzerland. This gave me my first taste of what it is like to work in industry rather than academia, and also gave me the opportunity to live in a new country for a year, an experience that provided many learning opportunities in itself.

Finally, in addition to her support and encouragement, Dr. Mohr always made sure that the lab had fun together. Although Dr. Mohr always pushed me in lab, it is the things we did outside of the lab that I will remember most, whether it be Segway tours or Giants games in San Francisco, white water rafting in Colorado, snorkeling in Hawaii, or seeing castles in Germany.

I would also like to thank the other members of the lab, most specifically Brandon Coughlin, Dr. Prathiba Jayaguru and Barbara Christian for their help and support in my experiments. Also, Dr. Lou Glazer has contributed greatly to help understand diabetic retinopathy at a clinical level. Finally, I would like to thank my entire family who has provided immense support outside of lab.

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KEY TO ABBREVIATIONS

AGE - Advanced Glycation End Product

AIF-Apoptosis-Inducing Factor

AIM2- Absent In Melanoma-2

AMD - Age Related Macular Degeneration

BID - BH3-Interacting Domain

CAPS - Cryopyrin-Associated Periodic Syndromes

CARD - Caspase Activation and Recruitment Domain

Casp-/- - Caspase-1 Knockout

CCL2 - Chemokine Ligand-2

CNV - Choroidal Neovascularization

COX2 - Cyclooxygenase-2

DAMP - Danger Associated Molecular Pattern

DCCT - Diabetes Control and Complications Trial

DED - Death Effector Domain

DIRA - Deficiency of IL-1ra

DISC - Death-Inducing Signaling Complex

DME - Diabetic Macular Edema

DMEM - Dulbecco's Modified Eagle's Medium

ETDRS - Early Treatment Diabetic Retinopathy Study

FBS - Fetal Bovine Serum

FMF - Familial Mediterranean Fever

GABA - γ-aminobutryic acid

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

GFAP - Glial Fibrillary Acidic Protein

GHb - Glycated Hemoglobin

GLAST - Glutamate Aspartate Transporter

H_2DCFDA - 2'-7'-dichlorofluroescin diacetate

HIF-1 - Hypoxia Inducible Factor-1

hMC - Human Müller Cell

ICE - Interleukin-1 Converting Enzyme

IFNγ - Interferon-γ

IKKβ - Inhibitor of NF-κB Kinase b

IL - Interleukin

ILM - Inner Limiting Membrane

INL - Inner Nuclear Layer

iNOS - Inducible Nitric Oxide Synthase

IRAK - IL-1R-Associated Kinase

MYD88 - Myeloid Differentiation Primary Response Protein 88

NLRP - Nod Like Receptor Family Pyrin Domain Containing

OCT - Optical Coherence Tomography

PAMP - Pathogen Associated Molecular Pattern

PEDF - Pigment Epithelium-Derived Factor

PGE2 - Prostaglandin E2

PI - Propidium Iodide

PRR - Pattern Recognition Receptor

PS - Penicillin/Streptomycin

PTP - Permeability Transition Pore

RIP2 - Receptor Interacting Protein-2

ROS - Reactive Oxygen Species

RPE - Retinal Pigment Epithelium

STZ - Streptozotocin

TH2 - T Helper Type-2

TIR - Toll/IL-1 receptor

TNFα - Tumor Necrosis Factor-α

TRAIL - TNF-Related Apoptosis Inducing Ligand

TUNEL - Terminal dUTP Nick End Labeling

TXNIP - Thioredoxin Interacting Protein

VEGF - Vascular Endothelial Growth Factor

WT - Wild Type

Chapter 1. Introduction

1.1 Diabetes Mellitus

Diabetes mellitus is a disease characterized by elevated fasting blood glucose (Fasting blood glucose ≥ 126 mg/dl) (2). Although there are numerous types of diabetes, the two most common types of diabetes are Type 1 and Type 2 diabetes. In patients with Type 1 diabetes, also known as insulin-dependent or juvenile diabetes, there is an autoimmune destruction of the pancreatic beta cells, the cell type that is responsible for producing insulin, which renders the body unable to produce the insulin required to stimulate glucose uptake from the blood stream. Patients with Type 2 diabetes have a similar increase in blood glucose but by an entirely different mechanism. Type 2 diabetes, also known as insulin-independent diabetes, is the most common form of diabetes and results from improper insulin signaling. Unlike patients with Type 1 diabetes, these patients are able to produce insulin, however the insulin cannot act on its receptor to trigger glucose uptake from the blood stream into target tissues such as muscle and adipose tissue, a process known as insulin resistance.

Diabetes is the first non-contagious disease labeled an epidemic by the World Health Organization. The number of people affected by the disease is staggering; approximately 29 million people in the United States have diabetes, a number that accounts for 9.3% of the total population. To make matters worse, 86 million people in the United States have pre-diabetes, meaning they have either elevated blood glucose levels or impaired glucose tolerance, which puts them at severe risk of developing diabetes in the future (2). According to the American Diabetes

Association the disease has cost the United States a total of $245 billion in 2012. These costs are a result of both the reduced productivity of the afflicted individuals in the work force, and also direct medical costs from the many complications that arise with the disease. Some of the common complications of diabetes include skin ulcers, impaired wound healing, nephropathy, neuropathy, and retinopathy.

1.2 Diabetic Retinopathy

1.2.1- Introduction to Diabetic Retinopathy

Diabetic retinopathy is one of leading causes of acquired blindness in working age adults. As the prevalence of diabetes increases so does the incidence of diabetic complications such as diabetic retinopathy. Diabetic retinopathy is a sight threatening disease that occurs in patients with both Type 1 and Type 2 diabetes. Duration of diabetes seems to be the strongest predictor of the incidence of retinopathy as approximately 8% of diabetic patients had retinopathy at 3 years, 25% by 5 years, 60% by 10 years and 80% by 15 years.

Another predictor of disease progression is glycemic control. In the Diabetes Control and Complications Trial (DCCT) a total of 1,441 patients who had either no retinopathy (primary prevention cohort) or minimal-to-moderate non-proliferative DR (secondary progression cohort) were treated by either conventional treatment using one or two daily injections of insulin or by intensive diabetes management with three or more daily insulin injections or a continuous subcutaneous insulin infusion. During the first 36 months, incidence of progression of retinopathy was similar between the two treatments in the primary prevention cohort. After 36

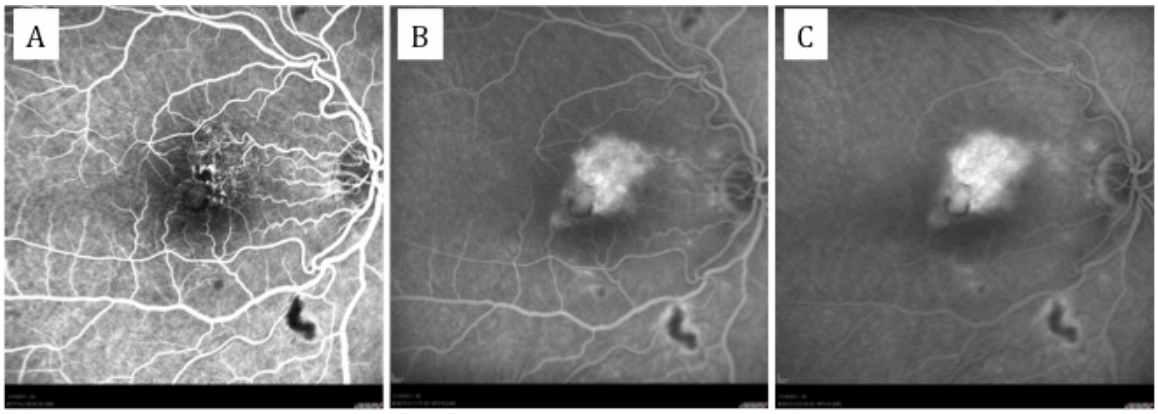
months however, there was a substantial decrease in incidence of retinopathy in the intensive treatment group compared to the conventional treatment group. In the secondary progression cohort, the intensive treatment group had higher initial incidence during the first year, however by 36 months the intensive group had lower risks of progression. Together, this indicated that glycemic control was important in both the prevention and intervention of diabetic retinopathy.

1.2.2 Non-proliferative Diabetic Retinopathy

The initial stage of diabetic retinopathy is referred to as non-proliferative, or background, diabetic retinopathy (Figure 1). The retinal changes associated with non-proliferative diabetic retinopathy include microaneurysms, lipid exudates, microhemorrhages, cotton-wool spots, and thickening of the basement membrane(3). These retinal changes are reversible and the defects in a patients vision are minimal(4). The associated changes in vision of patients with non-proliferative diabetic retinopathy include decreased contrast sensitivity unless the patient begins to show signs of edema, which can lead to decreased visual acuity(3).

Figure 1. Fluorescein angiography of patients with non-proliferative diabetic retinopathy.

A) Early frame fluorescein angiogram from a non-proliferative diabetic retinopathy patient showing microaneurysms. B) Mid and C) late frame non-proliferative angiogram from the same patient showing DME and leakage from microaneurysms.

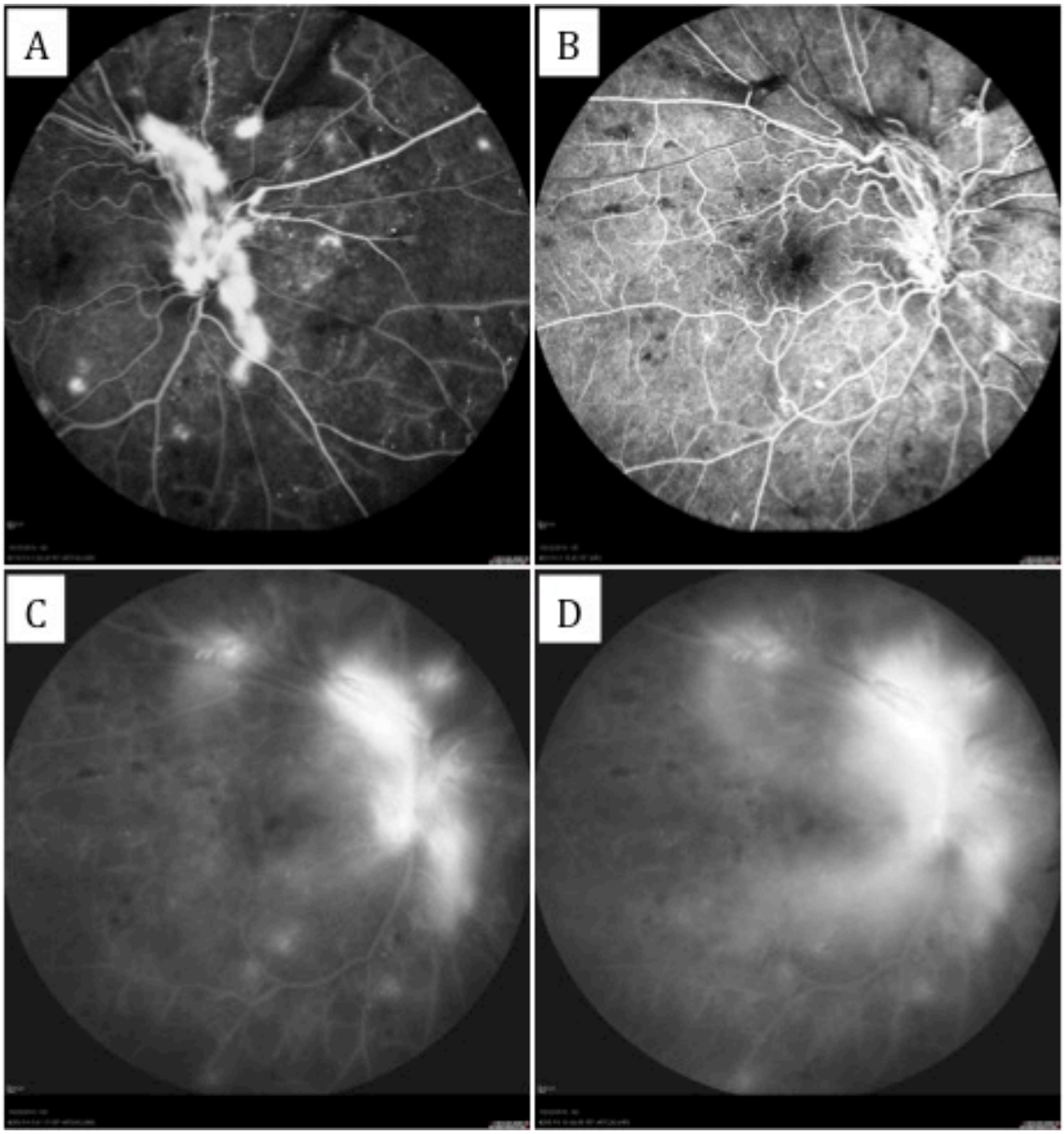


1.2.3 Proliferative Diabetic Retinopathy

As the disease progresses patients can develop more severe vision-threatening pathologies. These changes are commonly a result in the disease progressing from non-proliferative to proliferative diabetic retinopathy, where there is abnormal growth of new blood vessels. These new blood vessels can lead to leakage of blood into the retinal tissue or vitreous fluid which blocks light from passing through the fluid to activate photoreceptors in the back of the retina (Figure 2) Additionally, these blood vessels can lead to tractional-detachment of the retina which can result in permanent damage if not treated(3,4).

Figure 2. Fluorescein angiography of patients with proliferative diabetic retinopathy with neovascularization of the disc and elsewhere.

A) Early stage, imaged for disc. B) Early stage, imaged for macula. C) Mid stage D) Late stage.



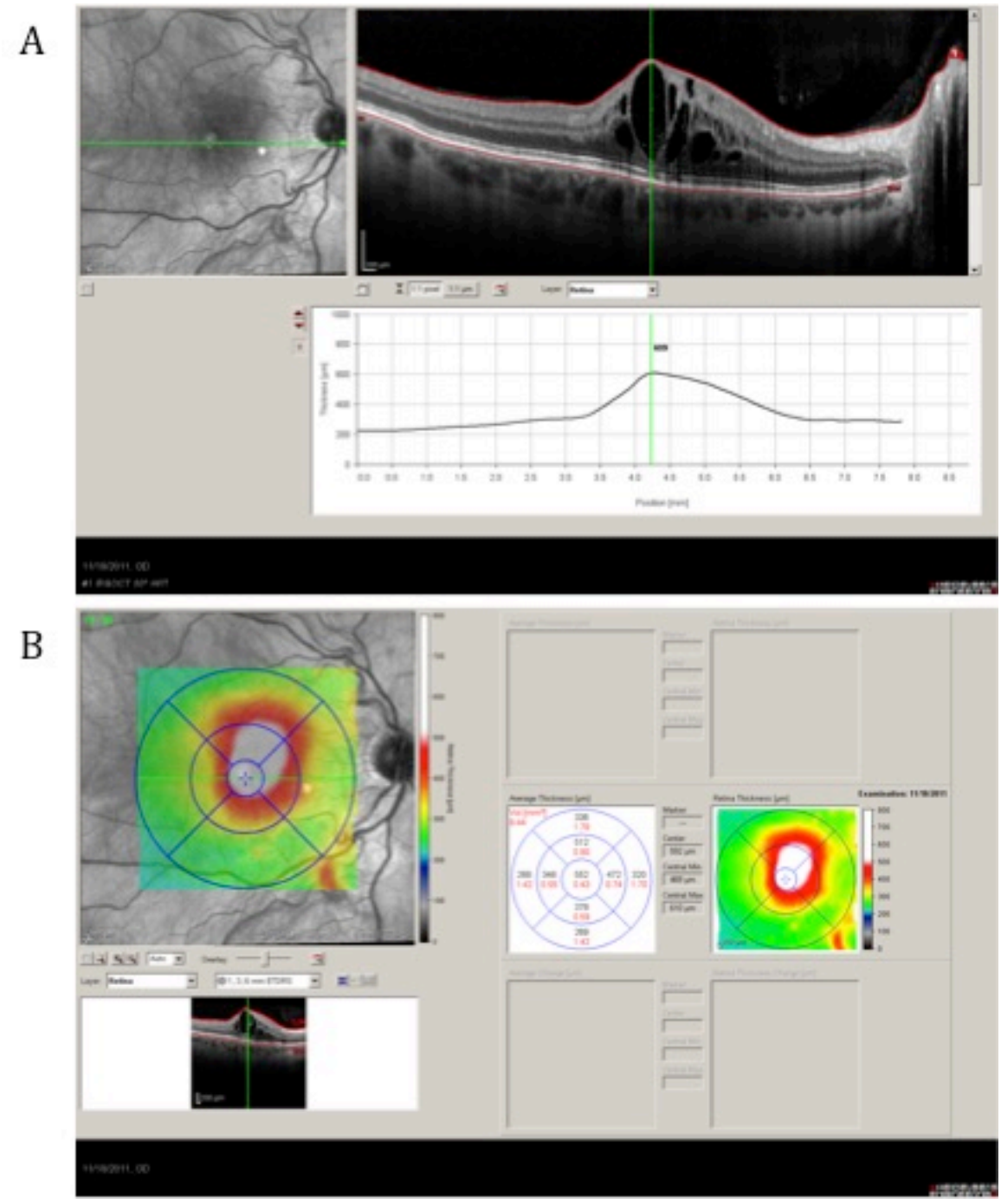
1.2.4 Diabetic Macular Edema

In addition to the non-proliferative and proliferative stages of diabetic retinopathy, diabetic patients are also at risk of developing diabetic macular edema (DME). DME is the most common cause of vision loss in patients with diabetic retinopathy and can occur in either the non-proliferative or proliferative stage of diabetic retinopathy. DME arises from the breakdown of the inner blood retinal barrier (primarily from endothelial cells) rather than the outer blood retinal barrier (primarily from retinal pigment epithelium(RPE)) and is caused by the redistribution of tight junction proteins such as claudin and occludin leading to leakage of fluid from the vasculature into the retina(5,6). In a healthy retina, excess fluid is removed primarily by the RPE pump, where RPE transport potassium and chloride ions out of the extracellular space allowing for passive removal of water via aquaporin-1 channels(7,8). In addition to the RPE pump, Müller cells also play a major role in the removal of fluid from the retina. Müller cells act as potassium shuttle by taking up potassium from the extracellular fluid through Kir2.1 potassium channels and depositing the potassium into the vasculature using Kir4.1 channels that are found on the Müller cell processes that encompass the blood vessels(9,10). This leads to osmotic fluid removal through aquaporin-4(8,10–12). In diabetes, Müller cells have been shown to downregulate the Kir4.1 channels, but not Kir2.1, leading to continued potassium uptake with no release into the microvasculature(13–15). This leads to subsequent swelling of Müller cells contributing to Müller cell dysfunction and decreased fluid removal contributing to DME. DME leads to thickening of the macula due to fluid accumulation and can be

observed by optical coherence tomography (OCT) (Figure 3). The thickening of the macula due to fluid accumulation typically leads to disruption of the retinal structure and changes in visual acuity.

Figure 3. OCT of patients with DME

(A) Line scan OCT from patient with DME with visible fluid accumulation in the macula. (B) Map scan from patient with DME showing increased macular thickening due to fluid accumulation.



1.2.5 Current Therapies for Diabetic Retinopathy

There are currently two main therapies for the treatment of diabetic retinopathy. The first are agents that target VEGF (vascular endothelial growth factor) and are typically used in the treatment of proliferative diabetic retinopathy and DME. Elevated levels of VEGF are found in the vitreous of patients with diabetic retinopathy and DME. VEGF is a growth factor that promotes neovascularization and also increases vascular permeability by increasing occludin phosphorylation and decreasing occludin levels in endothelial cells in the inner retinal vasculature. Therefore, neutralizing VEGF using specific antibodies has been successful in correcting vascular leakage and neovascularization. Unfortunately, not every patient responds to this treatment and patients that do require monthly injections of the drug to keep symptoms from reoccurring. Overall, this treatment strategy is a huge burden to the patient..

The second therapy is laser focal photocoagulation. The Early Treatment Diabetic Retinopathy Study (ETDRS) was the first large scale study examining the effects of using a argon blue-green or green laser to inflict 50-200μM moderate intensity burns in a grid pattern in the macula of the retina. In this study the laser burns were focused on microaneurysms, intraretinal microvascular abnormalities and other potential leakage or neovascular sites. It was observed that laser photocoagulation had a led to a significant improvement in visual acuity in treated eyes compared to untreated eyes 3 years later. Since this study, laser photocoagulation is still a common treatment for proliferative diabetic retinopathy, however, the methods have changed for focal laser treatment for DME. Currently,

burns are smaller (50 μM) and have a lighter intensity (using yellow and green, but no longer blue-green laser) to prevent scar expansion. Although, the exact mechanism by which focal and panretinal laser work is unclear, there have been numerous proposed mechanisms. The most common proposed mechanism is that the burns destroy the photoreceptors in the peripheral retina leaving more oxygen remaining healthy tissue. This eliminates areas of hypoxia, which is one of the main drivers of VEGF production. Additionally, photocoagulation destroys the leakage sources such as microaneurysms and leaky vessels. Other studies indicate that laser photocoagulation actually improves the RPE pumping of ions and water out of the retina and into the choroid.

In sum, the lack of available therapies points to the need for identifying new targets involved in the progression of diabetic retinopathy. In order to identify new targets there needs to be increased understanding of how the retina changes during diabetes.

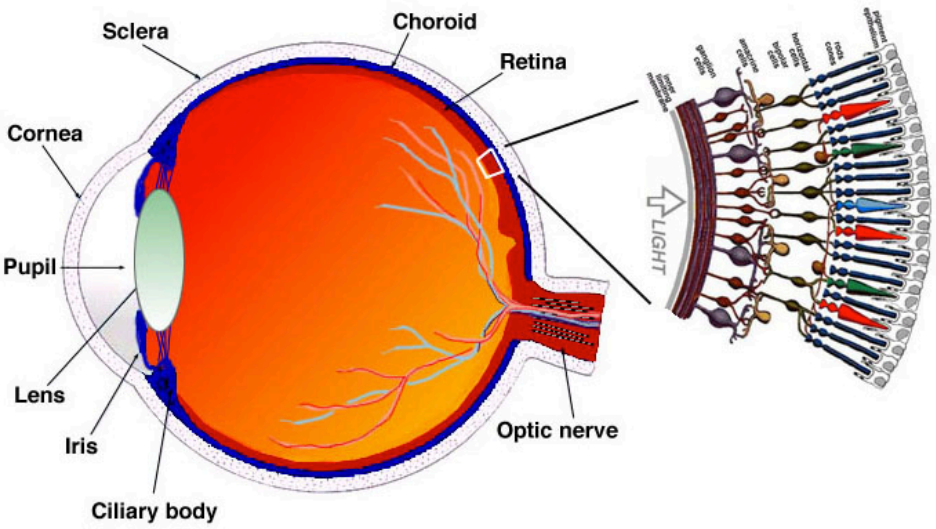
1.3 Retina Structure

The retina is a highly organized tissue consisting of various layers located in the back of the eye (Figure 4A). The cross-sectional layers of the retina from inner most layer to the outer most layer are ordered as follows (Figure 4B), the (1) Inner Limiting Membrane (ILM), which forms the boundary between the neural retina and the vitreous humor, a clear jelly-like fluid that fills the eye. The ILM is made up of the end feet of Müller cells, which are then coated in a mucopolysaccharide on the surface in order to form a true boundary between the

Figure 4. Anatomy of the Eye and Retina.

A) Anatomy of the eye showing location of the retina. B) Illustration of the cross section of the retina showing each retinal cell type. **This figure is used with permission from webvision.utah.med.edu*.

A



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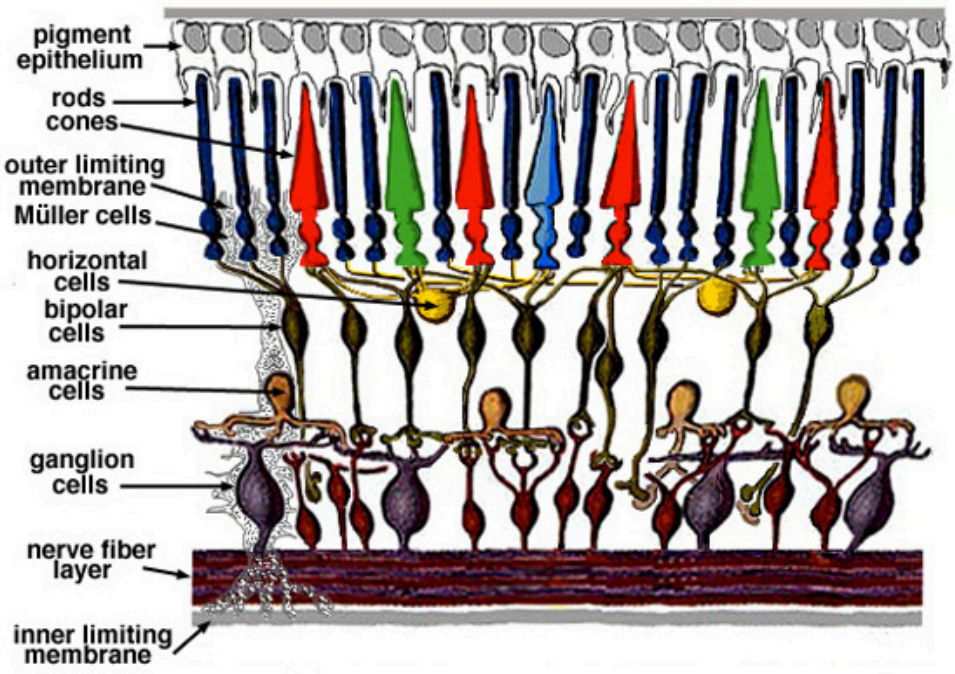


retina and vitreous humor(16,17). The (2) nerve fiber layer consists of the axons from the ganglion cells that run across the inner surface of the retina, just behind the ILM, and converge at the optic nerve to where they eventually return information to the brain for processing. They are supported by astrocytes, one of the three glia cell types in the retina. The (3) ganglion cell layer synapses in the (4) inner plexiform with the bipolar cells and amacrine cells. The (5) inner nuclear layer (INL) consists of the cell bodies of amacrine cells, bipolar cells, horizontal cells, microglia, and Müller cells, another glia cell type. The bipolar cells serve as a "bridge" to pass the signal from photoreceptor cells in the outer retina to the ganglion cells in the inner retina. The (6) outer plexiform layer is where the bipolar cells and horizontal cells synapse with the photoreceptor inner segments. Just behind this synapse is the (7) outer nuclear layer, which contains the highly organized nuclei of the photoreceptors, which are connected to the photoreceptor outer segments, which is the starting location of the visual cycle and signal transduction. The outer segment of the photoreceptors makes up the (8) photoreceptor layer. Just behind the photoreceptors is the final layer of the retina, which is the (9) retinal pigment epithelium cell layer.

The retina is unique in that it has two blood supplies. The first blood supply comes from the central retinal artery and branches after it enters through the optic nerve (Figure 5). These blood vessels are located within the retinal layers and can easily be seen in a fundus image. The blood supply from the central retinal artery is responsible for supplying nutrients and oxygen to the inner retina. The second blood supply is the choroid. The choroid lies just behind the retina and

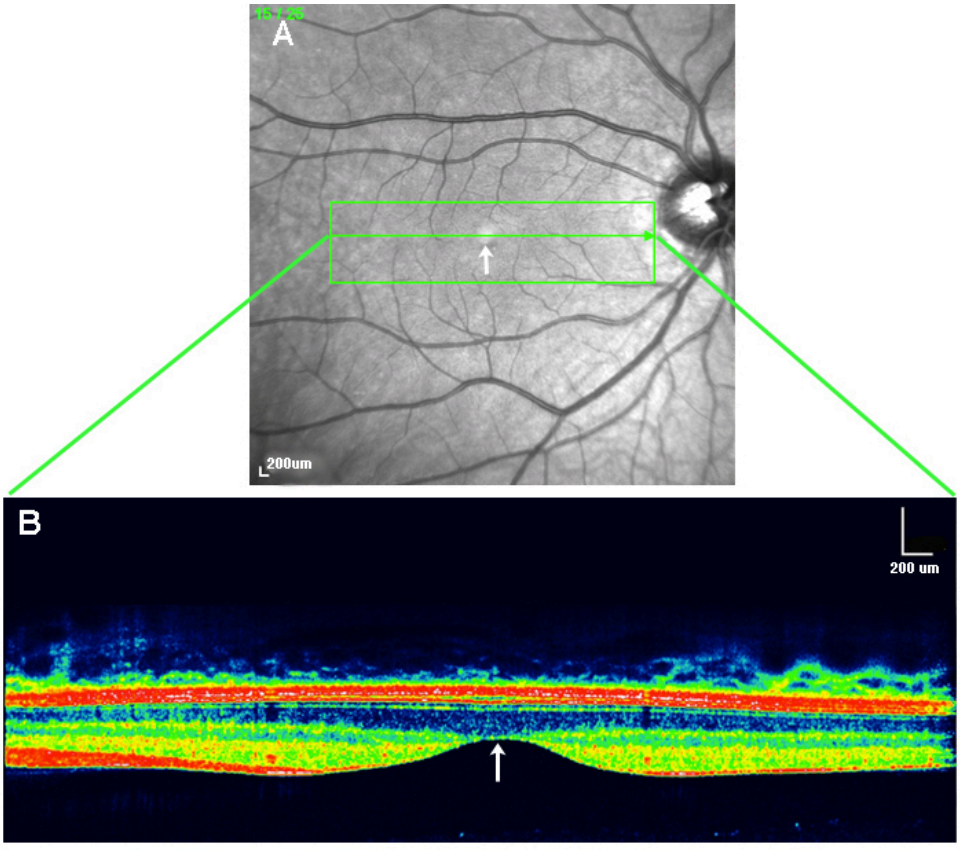
supplies oxygen and nutrients specifically to the RPE and photoreceptors that are located in the outer retina, one of the most metabolically active sites in the body. By having their own blood supply just posterior to the retina photoreceptors are very well suited to cope with the high metabolic demand. Interestingly, there are speculations that this unique design of the retina (having photoreceptors in the back instead on the front of the eye and retina) evolved exactly to guarantee the high metabolic demand could be supported. This is also the reason that the macula can survive as an avascular region; since it is a region that consists of only the photoreceptor cell layer that receives is nutrients solely from the choroid located behind the retina and doesn't depend on blood vessels within the retinal tissue that originate from the central retinal artery.

Looking at a fundus of the retina it becomes apparent that the retina is a tissue that is not only highly organized in the cross-sectional layers, but also organized spatially from the anterior view. The area in the center of the retina is known as the macula, which is then further divided into the fovea, parafovea and the perifovea (Figure 5B). This region of the retina is responsible for the highest visual acuity due to the high concentration of cone photoreceptors. Furthermore, this is an avascular region of the retina and also the thinnest layer of the retina as it contains only cone photoreceptors and end feet of Müller cells. Therefore, each photon of light can pass directly through the fovea to activate the photoreceptors with limited interference from the other retinal cell layers. The area outside of the macula is known as the peripheral retina. The peripheral retina is a highly vascular region

responsible for contrast sensitivity due to the increased concentration of rod photoreceptors.

Figure 5. Fundus and OCT of Healthy Retina

A) Fundus image of retina showing central retinal artery entering retina through the optic disc. The fovea (shown by arrow) can also be seen in the center of the retina. B) OCT showing the cross sectional layers of the same retina with arrow showing foveal pit that contains cone photoreceptors. **This figure is used with permission from webvision.med.utah.edu.*



1.4 Müller Cells

1.4.1 Müller Cells in the Healthy Retina

Müller cells are the principle glia of the retina. They are the only cells to span the entire width of the retina and have intimate contact with both the retinal blood vessels and retinal neurons (Figure 4B). Because of this arrangement, Müller cells have a variety of important functions in the healthy retina. Functions of Müller cells can be divided into 3 major categories: (1) Uptake and recycling of neurotransmitters, retinoic acid compounds, and ions (such as potassium K^+), (2) control of metabolism and supply of nutrients for the retina, and (3) regulation of blood flow and maintenance of the blood retinal barrier.

The extensive contact of Müller cells with retinal neurons allows Müller cells to actively participate in proper neurotransmission. They rapidly take up and clear glutamate and γ-aminobutryic acid (GABA) in the inner plexiform layer(18–21). Studies have shown that Müller cells take up extracellular glutamate through the Glutamate Aspartate Transporter (GLAST) and indicate that glutamate removal and prevention of neurotoxicity in the retina is achieved primarily by this mechanism(22,23). Once taken up, glutamate is converted to glutamine by glutamine synthetase and released back to neurons for re-synthesis of glutamate and GABA(24). This process provides substrate for neurotransmitter synthesis and also prevents glutamate toxicity. Müller cells further maintain proper retinal function by participating in a process known as "potassium spatial buffering", a process that redistributes and normalizes K^+ in the surrounding microenvironment to avoid prolonged accumulation of K^+(25). It has been shown that Müller cells can

take up K^+ from the inner and outer plexiform layers where neuronal synapses occur and release the K^+ into the vitreous humor in an effort to redistribute K^+ ions(26). This process is also involved in retinal fluid removal. Müller cells act as potassium shuttle by taking up potassium from the extracellular fluid through Kir2.1 potassium channels and depositing the potassium into the vasculature using Kir4.1 channels that are found on the Müller cell processes that encompass the blood vessels(9,10). This leads to osmotic fluid removal through aquaporin-4(8,10–12).

In addition to regulating neurotransmitters and ion levels within the retina, Müller cells also participate in the retinoid cycle with cone photoreceptors by taking up all-*trans* retinol from the subretinal space(27–30). During the visual cycle, photons of light lead to isomerization of 11-*cis* retinal to all-*trans* retinal in the rod and cone photoreceptors. Once isomerized, all-*trans* retinal is expelled from the opsin protein to be reduced by retinol dehydrogenases to all-*trans* retinol(31). The all-*trans* retinol from the cones is then released into the extracellular space where it is taken up by Müller cells, isomerized back to 11-*cis* retinol by all-*trans* retinol isomerase, and released back to the extracellular space to be taken up by the cone photoreceptors where it can finally be oxidized from 11-*cis* retinol back to original 11-*cis* retinal to restart the visual cycle(27–29,32).

Müller cells seem a primary site of nutrient storage for the retina. It has been shown that ATP production in Müller cells drastically declines when glycolysis is inhibited. However, ATP levels remained equal in aerobic versus anaerobic conditions as long as glucose was provided, indicating that Müller cells live primarily from glycolysis rather than oxidative phosphorylation(33). This is

important as it spares oxygen for retinal neurons and other cell types that use oxidative phosphorylation for ATP production. Furthermore, Müller cells are the primary site of glycogen storage in the retina(33,34). When nutrient supplies are low Müller cells can utilize this glycogen storage to provide metabolites for other cell types. Furthermore, the large amounts of lactate they produce via glycolysis and irreversible conversion of pyruvate to lactate due to a specific lactate dehydrogenase isoform can be transported to photoreceptors to be used as a potential alternative source of energy in case of need(33,35,36). Interestingly, studies suggest that the metabolism of glucose and glycogen by Müller cells is regulated by light being absorbed by the photoreceptors(24). This means that as photoreceptors absorb light, the Müller cells respond by metabolizing more glucose in order to provide more lactate for photoreceptors as needed, indicating that Müller cells and photoreceptors are tightly coupled in their respective functions by metabolism. In addition to providing lactate as a fuel source for photoreceptors, Müller cells can also regulate nutrient supplies to the retina via regulation of retinal blood flow. In a healthy retina, increased light stimulation results in increased retinal blood flow, which is required to supply the activated neurons with oxygen and other nutrients, a process termed neurovascular coupling. Müller cells play a crucial role in neurovascular coupling as they release metabolites controlling vasoconstriction and vasodilation of retinal blood vessels(37,38).

One of the most important functions of Müller cells is their regulation of retinal blood flow and contribution to the blood retinal barrier. The blood retinal barrier is essential for preventing leakage of blood and other potentially harmful

stimuli such as pathogens from entering the retinal tissue. It has been shown that Müller cells induce blood-barrier properties in retinal endothelial cells(39,40). Studies using conditional ablation of Müller cells showed severe blood retinal barrier breakdown(41). The exact mechanism of how Müller cells maintain the blood retinal barrier is debated but includes the secretion of factors such as pigment epithelium-derived factor (PEDF) and thrombospondin-1 which are anti-angiogenic and increase the tightness of the endothelial barrier(42,43).

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

1.4.2 Müller Cells in Diabetic Retinopathy

Functional changes that have been determined in Müller cells begin early in the disease, with significant decreases in glutamate transport via GLAST beginning after just 4 weeks of diabetes in rats(44). This is consistent with reports showing significantly increased glutamate accumulation in the retinas of diabetic rats(45,46). Furthermore, these studies have shown that there is decreased glutamine synthetase activity and a subsequent decrease in the conversion of glutamate to glutamine necessary for neurotransmitter regeneration(45,46). These results are in

line with reports demonstrating glutamate increases to a potentially neurotoxic level in the vitreous of diabetic patients(47). However, in neurological diseases such as stroke, therapies targeting glutamate increase have been ineffective indicating that increased glutamate levels might not play a pathophysiological role(48,49). Whether increased glutamate levels actually cause neurotoxicity over time in diabetic retinopathy has yet to be determined.

It seems that Müller cells not only contribute to glutamate toxicity directly by decreased glutamate uptake, but Müller cells also contribute indirectly via decreased K^+ uptake during the progression of diabetic retinopathy. There is decreased K^+ conductance on the plasma membrane of Müller cells isolated from rat retinas after 4 months of experimental diabetes(15). Redistribution of the Kir4.1 K^+ channel has been identified as the mechanism of decreased K^+ conductance(15). This decrease in K^+ conductance was also observed in Müller cells of patients with proliferative diabetic retinopathy(50). Alteration of the Kir4.1 K^+ channel localization in Müller cells in the diabetic retina has been attributed to the accumulation of advanced glycation end products (AGEs)(51). Together, this can lead to an imbalance in K^+ concentrations and altered K^+ homeostasis leading to neuronal excitation and subsequent glutamate toxicity. The contribution of K^+ spatial buffering in the context of DME has been discussed in section 1.2.4.

In addition to the defects in neurotransmitter clearance and K^+ homeostasis during diabetic retinopathy, Müller cells become activated as shown by increased expression of glial fibrillary acidic protein (GFAP), a common marker of reactive gliosis(45,52,53). Upon activation, Müller cells have been shown to secrete a

number of pro-inflammatory cytokines and pro-angiogeneic growth factors including vascular endothelial growth factor (VEGF), pigment epithelium-derived factor (PEDF), interleukin-1β (IL-β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), chemokine ligand-2 (CCL2), prostaglandin E2 (PGE2), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2)(54–60).

One specific pro-inflammatory pathway seems to stand out when talking about Müller cells and their role in inflammatory events. Once stimulated, Müller cells predominantly activate the caspase-1/interleukin-1beta (IL-1β) pathway(61–63) leading to speculations that IL-1β might not only be produced during inflammatory events but also for physiological events such as regulation of glucose consumption.

1.5 Caspase Family

1.5.1 Caspase Enzymes- Functions and Outcomes

Caspases are a family of enzymes responsible for regulating inflammation and cell death in both normal and disease states. These endoproteases share a common mechanism of action in which they recognize their specific substrate and use a catalytic cysteine residue in their active site to cleave the substrate after an aspartic acid residue. It is this "**C"**ysteine-mediated cleavage after an "**As"**partic acid residue from which the "**Cas"**pase name is derived. Caspases have been classified into two main categories, the pro-inflammatory caspases that regulate inflammation or pro-apoptotic caspases that initiate and carry out cell death. The pro-inflammatory caspases include caspase-1, -4, -5, -11, and -12(64). The pro-

apoptotic caspases can be further divided into subgroups including initiator caspases (caspase-8 and -9) or executioner caspases (caspase-3, -6, and -7)(64).

Caspases are typically produced as an inactive pro-caspase that requires dimerization and autocatalytic cleavage of their pro-domain before they can carry out their own enzymatic function. The mechanism of dimerization and activation is dependent on the specific caspase in question, however the binding of an adaptor protein to the pro-domain of the procaspase, allowing for autocatalytic cleavage between the pro- and active-domain on the caspase to produce the active caspase, typically facilitates activation. This mechanism is known as the proximity induced mechanism of caspase activation(65).

In the pro-apoptotic caspases the pro-domain contains a death effector domain (DED) to facilitate dimerization and activation. Activation of an initiator caspase via the DED typically leads to a cascade of caspase activation where one caspase cleaves and activates the next, leading to eventual cleavage and activation of executioner caspases resulting in cell death. With the pro-inflammatory caspases the adaptor protein typically binds the caspase activation and recruitment domain (CARD) rather than a DED, leading to dimerization and cleavage in order to activate the caspase. Furthermore, where as activation of pro-apoptotic caspases leads to activation of other caspases, activation of pro-inflammatory caspases typically results in activation of pro-inflammatory cytokines.

1.5.2 Caspase-1

Caspase-1 is arguably the most well studied member of the caspase family. Formerly known as interleukin-1-converting enzyme (ICE), the most prominent

action of caspase-1 is its ability cleave and activate interleukin-1β, a proinflammatory cytokine involved in innate immunity(66,67). Caspase-1 itself is produced as a 45 kDa inactive zymogen, and is cleaved at two locations resulting in three separate subunits- the CARD domain required for activation, with the remaining two subunits being 10 (p10) and 20 (p20) kDA polypeptides(68–71). Following cleavage of multiples caspases, two p10 subunits and two p20 subunits come together to form heterotetramer capable of cleaving various substrates(69,71,72). Caspase-1 is in the group of proinflammatory caspases rather than the pro-apoptotic group, and thus contains a CARD required for recruitment and activation rather that a DED. The activation of caspase-1 is typically facilitated by a molecular platform termed the inflammasome. The inflammasome platform usually contains a pattern recognition receptor (PRR), which is responsible for sensing various harmful stimuli, either pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). There are a number of known activators of caspase-1 including NLR family pyrin domain containing 1 (NLRP1), NLRP3, NLRP6, NLRP7, NLR family CARD domain containing protein 4 (NLRC4), absent in melanoma 2 (AIM2), RIG-1-like receptors, and receptor interacting protein-2 (RIP2)(73,74).

In addition to its primary function in IL-1β production, caspase-1 has been shown to be a relatively promiscuous enzyme that is able to cleave a variety of substrates. Caspase-1 can cleave and activate IL-18, a cytokine that can stimulate inflammation via induction of tumor necrosis factor-α(TNFα) and interferon-γ (IFNγ)(75–78). Furthermore, caspase-1 can be linked to adaptive immunity due to

it's role in activation of IL-33 which modulates T helper type 2 (TH2) immune response(74,79). Recent studies have shown that there may be at least 121 caspase-1 substrates, many of them related to cell death independent of IL-1β action(73).

1.5.3 Caspase-1 in Disease

Caspase-1 has been implicated in a number of diseases involving inflammation. These disease range from inflammatory disease such as septic shock, inflammatory bowel disease, rheumatoid and osteoarthritis, to neurodegenerative diseases such as Huntington's disease, amyotrophic lateral sclerosis and Alzheimer's disease, and also diseases of the eye including diabetic retinopathy and age related macular degeneration (AMD)(62,66,80–88). The harmful effects of caspase-1 can be mediated by cleavage of its vast number of substrates, although IL-1β and IL-18 are the most prominent effectors. The beneficial effects of caspase-1 inhibition have been implicated in a number of diseases ranging from rheumatoid arthritis, epilepsy, and diabetic retinopathy(88–90).

1.6 The Interleukin-1 Family and Signaling

1.6.1 The Interleukin-1 Family

Interleukin-1 (IL-1) was the first interleukin to be identified and the IL-1 family has since grown to a total of 11 members. These 11 family members include IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-18, IL-33, and IL-1F5-F10. The effects of these family members have increased in complexity with recent studies. Some of these family members seem to be inflammatory by acting as a Type 1 IL-1 receptor (IL-1R1) agonist (IL-1α, IL-1β, IL-18, IL-1F6, IL-1F8, IL-1F9, IL-1F11), some

can be suppressors of inflammation by acting as an IL-1R1 antagonist (IL-1Ra, IL-1F10), and some have even been shown to be anti-inflammatory in nature (IL-1F5, IL-1F7)(91–93). Furthermore, IL-1α, IL-33, and IL-1F7 have been found in the nucleus and exert their effects by acting as a nuclear factor by binding to DNA and influencing gene transcription(91,94–97). Interestingly, although the majority of these IL-1 family members act as extracellular cytokines, only IL-1ra has a traditional signal peptide allowing it to be secreted by the classical secretory pathway involving the endoplasmic reticulum and Golgi complex mechanism(93,97). The exact mechanism by which the other IL-1 members are secreted is currently unknown.

1.6.2 IL-1β, IL-1β Receptor Signaling, and Regulation

IL-1β is arguably the most widely researched member of the IL-1 family. This pro-inflammatory cytokine plays a crucial role in innate immunity, and is typically produced by monocytes, macrophages or microglia in response to DAMPs or PAMPs(92). IL-1β is produced as a biologically inactive 31 kDa precursor (pro-IL-1β) and requires cleavage by caspase-1 to produce the 17 kDa active form that is secreted and involved in inflammation and cell death in innate immunity(68,98–100). IL-1β signaling is facilitated by ligand binding to IL-1R1 on the cell membrane. Much like Toll Like receptors (TLRs), IL-1R1 contains an extracellular immunoglobulin domain to which IL-1β binds. Upon binding, IL-1R1 recruits the IL-1 receptor accessory protein (IL-1RAcP) that is necessary for signal transduction. Both IL-1R1 and IL-1RAcP have intracellular Toll/IL-1 receptor (TIR) domains that upon the formation of the heterodimer, come together and recruit myeloid

differentiation primary response protein 88 (MYD88) leading to phosphorylation of IL-1R-associated kinases (IRAKs) and inhibitor of NF-κB kinase β (IKKβ) resulting in activation of NF-κB and eventual gene transcription. Due to the severity of IL-1 signaling, this pathway is tightly controlled by a number of redundant negative regulators. The first level of regulation comes from within the IL-1 family itself, IL-1ra. IL-1ra can bind to IL-1R1 and prevent recruitment of the crucial IL-1RAcP, thus occupying IL-1R1 and preventing signaling(93,101). Another level of regulation comes from the Type 2 IL-1 receptor (IL-1R2) that serves as a decoy receptor. IL-1R2 is able to bind IL-1β but the receptor lacks the cytoplasmic TIR domain and is therefore unable to signal. In addition to serving as an IL-1β trap, IL-1R2 provides further sequestration of signaling since it recruits the IL-1RAcP after it binds IL-1β, leaving less IL-1RAcP to dimerize with IL-1R1.

1.6.3 Autoinflammatory Disease

IL-1β plays such a significant role in some inflammatory diseases that there is an entire class of syndromes that are characterized by aberrant IL-1β signaling. Termed "Autoinflammatory" diseases, these diseases involve increased IL-1β signaling, although it can come from either uncontrolled caspase-1 activation and IL-1β secretion as in the case of familial Mediterranean fever (FMF), cryopyrin-associated periodic syndroms (CAPSs), and Muckle-Wells syndrome, or decreased regulation of the IL-1β signaling as in the case of deficiency of IL-1ra (DIRA)(97,102–104). One common characteristic of these autoinflammatory diseases is their general responsiveness to IL-1β blockade, whether it be through an IL-1 receptor antagonist or an IL-1β neutralizing antibody(91,105–107).

1.7 Objectives of the Dissertation

In the last few years, it has become clear that diabetic retinopathy has multiple features of a chronic inflammatory disease. Interestingly, preliminary studies have indicated that the pro-inflammatory caspase-1/IL-1β pathway is activated in diabetic retinopathy. Since autoinflammatory diseases are defined by caspase-1 activation and IL-1β production it was an intriguing idea to investigate whether diabetic retinopathy has some features of an autoinflammatory disease. Supporting such an idea was the fact that Müller cells seem to preferentially activate this pathway once stimulated and could serve as a main source for active caspase-1 and IL-1β.

Therefore, the working hypothesis investigated in this dissertation is as follows:

Hypothesis: We hypothesize that hyperglycemia induces caspase-1 activity and IL-1β production in retinal Müller cells *in vitro* and retinal tissue *in vivo*. We postulate that caspase-1 is sustained by an IL-1β-induced feedback mechanism that requires signaling via a functional IL-1 receptor as it has been discussed for autoinflammatory diseases. We further hypothesize that intervening in the caspase-1/IL-1β pathway will prevent diabetic retinopathy.

The goal of this study was to identify mechanisms involved in the activation of the caspase-1/IL-1β pathway and potential feedback signaling involved in sustained inflammation. Furthermore, we aimed to identify the consequence of prolonged

caspase-1 activation in the fate of Müller cells and also in the development and progression of diabetic retinopathy in order to potentially identify new therapeutic targets in disease treatment.

Chapter 2. Interleukin-1 Receptor-Dependent and Independent Caspase-1 Activation in Diabetic Retinopathy

2.1 Introduction

Diabetes leads to many complications, one of them being diabetic retinopathy. Diabetic retinopathy is characterized by microaneurysms, vascular leakage, neovascularization and/or macular edema within the retina and ultimately leads to vision loss(108). Diabetic retinopathy was once thought of as a purely microvascular disease, however more recent studies indicate that the disease may actually originate within the retinal tissue. Sustained low-grade retinal tissue inflammation has been discussed as the cause for retinal cell dysfunction and cell death leading to subsequent microvascular changes(109,110). Despite increasing evidence that chronic inflammation and cell death contribute to development and progression of diabetic retinopathy, there have been few treatments aimed at preventing these events. In order to develop such treatments, a better understanding of mechanisms underlying initiation and maintenance of chronic inflammation is crucial.

Interleukin-1β (IL-1β) has emerged as one of the prominent pro-inflammatory cytokines associated with diabetic retinopathy(111–115). Levels of IL-1β are elevated in the vitreous and serum of patients with proliferative diabetic retinopathy compared to healthy individuals(113). Caspase-1, originally named Interleukin-1β Converting Enzyme (ICE), is the enzyme responsible for producing IL-1β by converting pro-IL-1β into its active form, which in turn exerts its effects via the Type 1 receptor (IL-1R1). Our previous work has indicated that caspase-1 is

activated throughout the progression of diabetic retinopathy in STZ and galactosemic mice(62,88,116). Caspase-1 activity was also elevated in retinal tissue samples from diabetic donors(62,117). Müller cells are one of the cell types identified as being a source of active caspase-1 and IL-1β production.

Typically, caspase-1 activation occurs in response to a bacterial infection. This mechanism is well established and usually involves sensing of the pathogen via the Nlrp3 inflammasome(118). In the diabetic retina, caspase-1 activation is unique in that it occurs in response to elevated glucose levels rather than a pathogen. Very little is known about caspase-1 activation in so called "sterile inflammation." Alternative pathways that are independent of the Nlrp3 inflammasome have been suggested to activate caspase-1 in pathogen free conditions. For example, Receptor Interacting Protein 2 (RIP2) can act as a potential activator of caspase-1 in sterile inflammation(119–123). In Huntington's disease, which is considered to be a sterile inflammatory disease, RIP2-mediated caspase-1 activation leads to chronic tissue inflammation and cell death(82). To date, the mechanism by which caspase-1 activation is initiated, and more importantly sustained, in the retina and retinal cells under diabetic conditions is not known.

Therefore, this study aimed to identify mechanisms of how caspase-1 activity is induced and maintained in hyperglycemic conditions *in vitro* and *in vivo* and how this process contributes to the sustained low-grade inflammation seen in diabetic retinopathy.

2.2 Research Design And Methods

Antibodies and Reagents. Caspase-1 antibody was from Invitrogen (Burlington, Ontario). Goat Anti-Rabbit IgG conjugated to horseradish peroxidase (HRP) and 4-20% Gradient Tris-SDS-PAGE gels were from BioRad (Hercules, CA). TRIzol, 7-amino-4-trifluoro-methylcoumarin (AFC) and glucose assay kit were from Sigma (St. Louis, MO). GHb kits were from Glyc-Affin (Rockford, IL). Caspase-1 substrate, YVAD-fmk and elastase Calbiochem (San Diego, CA). Human IL-1β, IL-1 receptor antagonist (IL-1ra), and high sensitivity IL-1β ELISA was from R&D Systems (Minneapolis, MN). Rabbit polyclonal anti-RIP2 antibody (ab8428) was from Abcam (Cambridge, MA). ON-TARGET*plus* SMARTpool Human RIPK2 and ON-TARGET*plus* Control siRNA Non-Targeting siRNA #1 were purchased from Thermo Scientific Dharmacon. Amaxa Cell Line Nucleofector Kit L was purchased from Lonza (Basel, Switzerland). Mouse anti-Glutamine synthetase (#610517, clone 6) was obtained from Transduction Laboratories.

Animal Models. Caspase-1 knockout mice (*Casp1*$^{-/-}$) (gift from Dr. T. McCormick, Case Western Reserve University), and IL-1 receptor knockout mice (IL-1R1$^{-/-}$) (Jackson Laboratories; strain name: *B6.129S7-Il1r1*^{tm1jm}) (both in a C57BL/6 background) mice were bred using homozygous breeding pairs. Recent studies have demonstrated that these caspase-1 null mice (derived from the original strain described by Li et al (66)) lack both caspase-1 and caspase-11 (*Casp1*$^{-/-}$ *Casp11*$^{-/-}$) (124,125). Male mice (Wild-type (WT) C57BL/6, *Casp1*$^{-/-}$, and IL-1R1$^{-/-}$) weighing 20 g were randomly assigned to be either diabetic or non-diabetic controls. Streptozotocin (STZ) injections (60mg/kg body wt i.p. on 5 consecutive days) were

utilized to induce diabetes as previously described (62). Diabetic animals were maintained with insulin injections (0.1-0.2 units of NPH insulin subcutaneously) as needed. Animals had free access to food and water and were maintained under a 12 h on/12 h off light cycle. Treatment of animals conforms to the Association for Research in Vision and Ophthalmology Resolution on Treatment of Animals in Research. GHb levels were measured at the end of the each study to determine severity of diabetes (Table 1).

Histologic assessment of retinal vascular pathology. Isolated retinas were washed in running water overnight and then digested in elastase solution (0.4U/ml in 100mmol/L sodium phosphate buffer, 150 mmol/L sodium chloride, 5 mmol/L EDTA) for 30-45 min at 37°C. The retinal tissue was transferred into 100mmol/L Tris-Hydrochloric Acid buffer (pH 8.5) and left overnight at RT. The cleaned vessel network was dried onto a mounting slide, stained with hematoxylin and PAS, dehydrated and covered with coverslip. Numbers of acellular capillaries were counted in 8 areas per retina (20X magnification), averaged, and reported per mm^2 retina as previously described(126).

Tissue Culture. Human retinal Müller cells (hMC): Handling of human tissue conformed to the tenets of Declaration of Helsinki for research involving human tissue. Human Müller cells were isolated from retinal tissue of healthy donors with no history of diabetes and chronic inflammatory diseases as previously described(127,128).

Treatment: hMC ($1x10^6$) were treated with either 5 mmol/L glucose DMEM or 25 mmol/L glucose DMEM supplemented with 2% FBS, 1% P/S for either 48 or 96

hours. Cells treated with 5 mmol/L glucose medium served as controls. For IL-1β studies, recombinant human IL-1β (1-5ng/ml) was used. For IL-1ra studies, following pretreatment with 100ng/ml IL-1ra for 1 hour, hMC were incubated in 25 mmol/L glucose DMEM for 48 or 96 hours, or in 5 mmol/L glucose DMEM plus recombinant human IL-1β (2ng/ml) for 24 hours.

siRNA Electroporation: hMCs were electroporated with either siRNA against RIP2 (50 nmol) or scramble RNA control (50 nmol) using a Nucleofector II device from Amaxa Biosystems (Cologne, Germany).

Preparation of cytosolic lysates. Following treatment, hMC were lysed in 100µl of lysate buffer [CHAPS Buffer (100mM HEPES, pH 7.5 containing 10% sucrose, 0.1% CHAPS), 1mmol/L EDTA, 1 mmol/L PMSF and leupeptin (10µg/ml)] as described previously(126).

Caspase-1 Activity Assay. Caspase-1 activities were measured as described previously (62,117,126–129). Briefly, equal amounts of sample protein (15 µg) were incubated in the presence of the specific caspase-1 substrate (YVAD-AFC; 2.5µmol/L) for 1 hour at 32°C. AFC fluorescence was detected by a Tecan Spectra FluorPlus fluorescence plate reader (excitation: 400 nm, emission: 510 nm). Release of AFC by active caspase-1 was calculated against an AFC standard curve and expressed as pmol AFC/mg protein/min.

Cytokine Assays. Medium (150µl) from hMC treated with 5 mmol/L or 25 mmol/L glucose containing medium was added to pre-coated 96 well plates. IL-1β ELISAs were performed according to the manufacturer's directions. Levels of cytokine were normalized to mg of total protein.

Glucose Consumption Assay. hMC ($1x10^6$) were cultured with either 5 mmol/L or 25 mmol/L glucose media for 96 hours. Media was changed at 48 hours. After collection of media at 48 and 96 hours, glucose concentration in media was measured using glucose assay kits according to manufacturer's directions.

Western Blot Analysis. Proteins (40μg) were separated in 4-20% SDS gradient gels and blotted on nitrocellulose membrane. Membranes were incubated with primary antibody against RIP2 (1:1000 dilution in PBS/0.05% Tween 20) overnight at 4°C followed by incubation with secondary antibody (1:5,000 dilution) for 1 hour at RT and developed using LICOR Biosciences Odyssey Imaging System (Lincoln, NE). Membranes were re-probed for β-actin and relative densities of RIP2/β-actin were calculated.

Müller Cell Death Detection. In vivo: Animals (see above) were sacrified after 6 months of diabetes. Eyes were fixed in formalin, embedded in paraffin, sectioned (10μM), and blocked with mouse on mouse (M.O.M.) Ig blocking solution for 1 hour at RT. Slides were incubated with primary anti-Glutamine synthetase antibody (1:1000) for 1 hour at RT. Following staining with secondary antibody, slides were treated with Flureseeine Avidin DCS for 10 min in dark and cover-slipped with DAPI containing mounting media. Blinded samples were visualized using scanning laser confocal microscopy (LSM 510; Carl Zeiss Meditec, Göttingen, Germany) and a water objective (63X Plan-Neofluor; Carl Zeiss Meditec). The number of Müller cells (GS positive cells) per standard retinal area (143μm x 143μm) was established from eight independent areas per section. Five individual sections per experimental group were counted and the average number of Müller cells was determined and

graphed. *In vitro:* Following treatment, cells were suspended and 100µL of cell suspension was mixed with 100µL of trypan blue solution. Cell death was quantified using hemocytometer.

Statistical Analysis. Analysis of data was performed using the Anova One-Way (correlated samples, $p<0.05$) test followed by Tukey's post analysis or Kruskal-Wallis test (ordinal data; $p<0.05$) followed by Dunn's post analysis to determine statistical significance among groups. For details in statistical analysis see VasserStats Statistical Computation Web Site. All data are presented as mean ± SDEV.

2.3 Results

2.3.1 Inhibition of Diabetic Retinopathy in STZ mice by Caspase-1 Knockout.

We have previously shown that diabetes leads to sustained caspase-1 activation throughout the development of diabetic retinopathy(126). To demonstrate that this activation of caspase-1 is necessary for the development of diabetic retinopathy, a long-term study using 4 experimental groups non-diabetic WT, diabetic WT, non-diabetic *Casp1*$^{-/-}$ and diabetic *Casp1*$^{-/-}$ mice was initiated. After 6 months of diabetes, there was a significant increase in the number of acellular capillaries/mm^2 retina in diabetic WT mice (12.2 ± 1.6) compared to non-diabetic WT mice (5.2 ± 0.8). Caspase-1 knockout prevented diabetes-induced formation of acellular capillaries indicating that activation of caspase-1 is indeed crucial for the development of diabetic retinopathy (Figure 6). Supplemental Table 1 shows that inhibition of diabetic retinopathy was not due to changes in severity of diabetes.

2.3.2 Sustained Caspase-1 Activity in STZ Mice Dependent on Feedback Through the IL-1 Receptor.

Caspase-1 activation leads to IL-1β production. Reports in the literature have suggested that IL-β itself can promote activation of caspase-1 via signaling through the IL-1R1 receptor(130,131). Since sustained caspase-1 activation is important for the development of diabetic retinopathy we tested whether intervention in the downstream signaling of the caspase-1/IL-1β/IL-1R1 signaling pathway using IL-1R1 knockout mice would impact caspase-1 activation throughout disease progression. After 10 weeks of diabetes, caspase-1 activity was significantly increased in both diabetic WT and diabetic IL-1R1$^{-/-}$ mice by 59.6 ± 15.3% and 33.2 ± 9.8% respectively compared to non-diabetic mice (Figure 7A). At 20 weeks of diabetes, caspase-1 activity was increased by 30.2 ± 3.8% in the retinas of diabetic WT mice compared to non-diabetic WT mice. However, there was no significant increase in caspase-1 activity in retinas of diabetic IL-1R1$^{-/-}$ mice compared to non-diabetic IL-1R1$^{-/-}$ or WT mice indicating that caspase-1 activation progresses from an IL-1R1 independent mechanism at 10 weeks of diabetes to an IL-1R1 dependent mechanism at 20 weeks of diabetes (Figure 7B). These data are consistent with a caspase-1/IL-1β/IL-1R1 feedback signaling mechanism.

2.3.3 Inhibition of Caspase-1 Activity in Human Müller cells by IL-1 Receptor Antagonist.

Previously we have shown that hyperglycemia induces caspase-1 activation and IL-1β production in human Müller cells(54,62,88). Therefore, these cells were used to explore mechanisms of caspase-1/IL-1β/IL-1R1 feedback signaling under

hyperglycemic conditions. To determine whether caspase-1 activation progresses from an IL-1R1 independent mechanism to an IL-1R1 dependent mechanism in this cellular model, human Müller cells (hMCs) were treated with 5 mmol/L or 25 mmol/L glucose media in the presence or absence of an IL-1 receptor antagonist for 48 or 96 hours. At 48 hours, caspase-1 activity was increased by 32.5 ± 3.6% in hyperglycemic conditions compared to normal. IL-1ra treatment had no significant effect on caspase-1 activity (Figure 8A). At 96 hours, treatment with IL-1ra led to a 97.7 ± 5.3% reduction in caspase-1 activity in cells incubated in hyperglycemic conditions indicative of a caspase-1/IL-1β/IL-1R1 feedback signaling (Figure 8B). To further confirm the concept of caspase-1/IL-1β/IL-1R1 feedback signaling, we tested whether IL-1β alone can induce caspase-1 activity in hMCs. hMCs were treated with increasing concentrations of IL-1β (0.5-2 ng/mL) in 5 mmol/L glucose conditions for 24 hours. IL-1β induced caspase-1 activation in a concentration dependent fashion demonstrating that IL-1β is capable of inducing caspase-1 activity (Figure 8C). Treatment with IL-1ra led to an 87.2 ± 3.4% inhibition of IL-1β-induced caspase-1 activity demonstrating that the effects seen by IL-1β were mediated by the IL-1 receptor (Figure 8D).

2.3.4 Glucose Consumption in Müller Cells Exposed to Hyperglycemic Conditions.

In order to understand how sustained caspase-1 activity becomes dependent on feedback signaling through the IL-1 receptor despite the constant presence of a high glucose stimulus, we determined how much glucose Müller cells consume when exposed to prolonged periods of hyperglycemia. Over the first 48 hours, hMCs cultured in 25 mmol/L glucose consumed nearly twice as much glucose (6.3 ± 0.4

mmol/L) as cells cultured 5 mmol/L glucose (3.3 ± 0.4 mmol/L). Interestingly, between 48 and 96 hours there was no significant difference in glucose consumption between Müller cells cultured in 25 mmol/L glucose (3.5 ± 1.0 mmol/L) compared to cells cultured in 5 mmol/L glucose (3.2 ±0.5 mmol/L) (Figure 9). These data indicate that glucose consumption is drastically increased at early time points at which treatment with IL-1ra is ineffective. At later time points however, glucose consumption decreases to normal levels and chronic caspase-1 activation becomes less dependent on hyperglycemia and mostly dependent on IL-1β, which can directly activate caspase-1 as we have shown above, explaining the effectiveness of IL-1ra treatment in hyperglycemic conditions.

2.3.5 RIP2 Mediated Caspase-1 Activation by Hyperglycemia.

Our results so far indicate that caspase-1 activation in Müller cells is seemingly maintained by two stimuli. Initially, activation of caspase-1 is predominantly driven by hyperglycemia. Once activated, caspase-1 activity is sustained by feedback signaling of IL-1β through the IL-1 receptor. Therefore, we were interested whether both stimuli activate caspase-1 by the same mechanism. Several known activators of caspase-1 were tested; only RIP2 played a significant role in hyperglycemia-induced caspase-1 activation. As shown in Figure 10A, RIP2 protein is significantly upregulated in hMCs cultured in 25 mmol/L glucose (77.6 ± 25.5%) compared to control. To confirm that increased RIP2 levels are responsible for increased hyperglycemia-induced caspase-1 activity, siRNA against RIP2 was used. Caspase-1 activity was significantly increased in hMCs transfected with scramble siRNA or left non-transfected in hyperglycemic conditions compared to

control. Knock down of RIP2 attenuated high glucose-induced caspase-1 activity by 85.8 ± 1.8% demonstrating that RIP2 is necessary for hyperglycemia-induced caspase-1 activity (Figure 10B). Consistently, knock down of RIP2 also prevented IL-1β production under these conditions (Figure 10C).

2.3.6 Inhibition of IL-β-Induced Caspase-1 Activity by RIP2 Knock Down.

Since RIP2 upregulation was crucial for hyperglycemia-driven caspase-1 activation, we further investigated the role of RIP2 in IL-1β-induced caspase-1 activation. Treatment of hMCs with IL-1β in 5 mmol/L glucose media led to a 2.38±0.6 fold increase in RIP2 protein levels (Figure 11A). siRNA against RIP2 prevented IL-1β-induced caspase-1 activation as seen in Figure 11B. RIP2 is not only involved in hyperglycemia-induced caspase-1 activation but also promotes caspase-1 activation by IL-1β. Together, this indicates that RIP2 is a central regulator of the caspase-1/IL-1β/IL-1R1 feedback signaling that controls Müller cell function and viability.

2.3.7 Hyperglycemia-Induced Müller Cell Death Mediated by Caspase-1/IL-1β/IL-1R1 Feedback Signaling.

We have previously shown that prolonged exposure to hyperglycemia causes Müller cell death. To determine the role of caspase-1/IL-1β/IL-1R1 pathway and feedback signaling in Müller cell death, hMCs were treated with 5 mmol/L or 25 mmol/L glucose media in the presence or absence of RIP2 siRNA or an IL-1ra for 96 hours. Upstream inhibition of the caspase-1/IL-1β signaling by RIP2 knock down prevented hyperglycemia mediated cell death of Müller cells (Figure 12A). Additionally, IL-1ra treatment prevented hyperglycemia-induced cell death

demonstrating that feedback signaling affects proper Müller cell function and viability (Figure 12B). These data indicate that interference in caspase-1/IL-1β/IL-1R1 feedback signaling at any point of the pathway is beneficial for Müller cell survival.

2.3.8 Diabetic Caspase-1$^{-/-}$ Mice Are Protected from Müller Cell Loss.

We have demonstrated that hyperglycemia-induced sustained caspase-1 activation is detrimental for Müller survival *in vitro*. To confirm the importance of caspase-1 activation on Müller cell viability *in vivo*, numbers of Müller cell were counted in retinal sections of non-diabetic or STZ-diabetic WT and non-diabetic or STZ-diabetic caspase-1$^{-/-}$ mice. Diabetes caused a 12.2 ± 2.0% loss of Müller cells after 6 months of diabetes. Knock out of caspase-1 prevented diabetes-induced Müller cell loss indicating that activation of caspase-1 is detrimental for Müller cell survival not only *in vitro* but more importantly *in vivo* (Figure 13).

Figure 6. Retinal capillary degeneration in non-diabetic and diabetic wild type and caspase-1 knockout mice.

Retinas of non-diabetic (n=6) and diabetic (n=8) wild type mice, and non-diabetic (n=6) and diabetic (n=5) *casp1*$^{-/-}$ mice were isolated at 7 months of diabetes. Number of acellular capillaries was determined and expressed as mean ± SDEV.

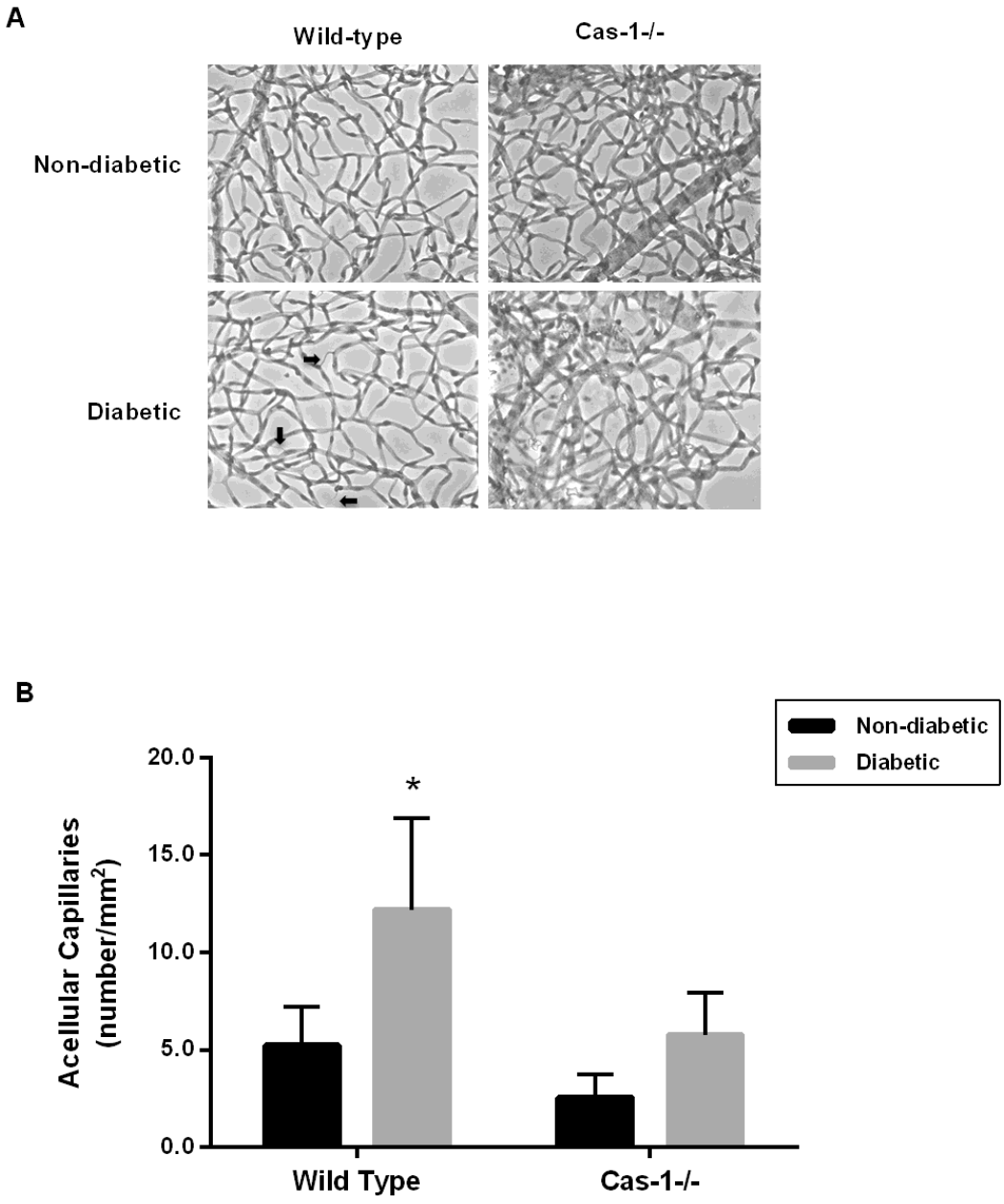


Figure 7. Caspase-1 activity pattern in the retinas of non-diabetic and diabetic wild type and IL-1R1$^{-/-}$mice.

Retinas of non-diabetic (n=10) and diabetic (n=10) wild type and non-diabetic (n=12) and diabetic (n=10) IL-1R1$^{-/-}$ mice were isolated at 10 weeks **(A)** and 20 weeks **(B)**. Caspase-1 activity was measured and expressed as mean ± SDEV with (*) = $p < 0.05$ compared to non-diabetic animals.

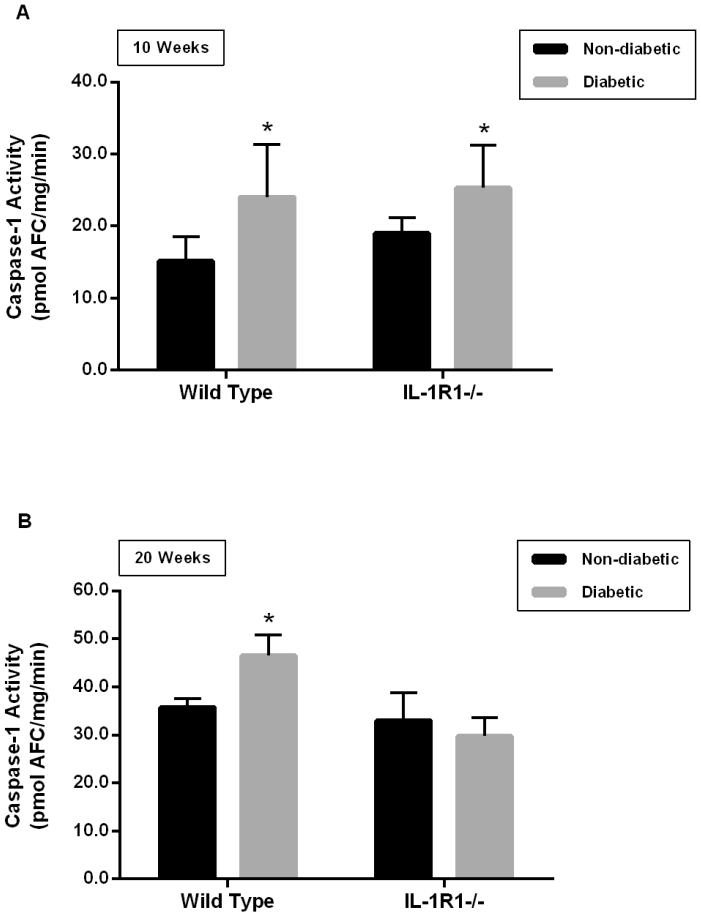


Figure 8. Inhibition of hyperglycemia-and IL-β-induced caspase-1 activity in human Müller cells by IL-1 receptor antagonist.

hMCs were cultured in either 5 mmol/L or 25 mmol/L glucose media in the presence or absence of 100ng/ml IL-1ra for **(A)** 48 or **(B)** 96 hours. **(C)** hMCs were treated in 5 mmol/L glucose or 5 mmol/L glucose + IL-1β (0.5, 1, or 2ng/ml) containing media for 24hrs. **(D)** hMCs were treated with IL-1ra (100ng/ml) in either 5 mmol/L glucose or 5 mmol/L glucose + IL-1β (2ng/ml) media for 24hrs. Caspase-1 activity was measured and expressed as mean ± SDEV with (*) = p< 0.05 compared to control and (#) = P<0.01 compared to 25 mmol/L glucose.

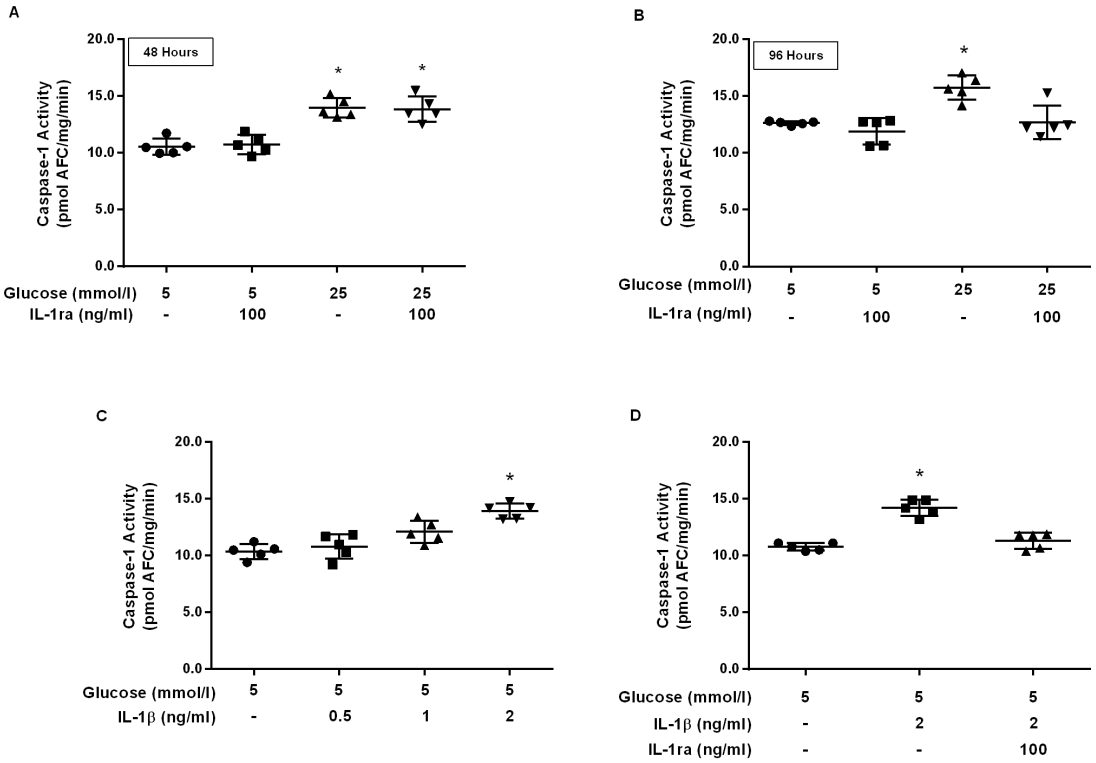


Figure 9. Glucose consumption normalized over time in Müller cells.

hMCs were incubated in either 5 mmol/l glucose or 25 mmol/l glucose for eitehr 48 or 96 hours. Glucose consumption was measured and expressed as mean ± SDEV, n=5, $p<0.05$.

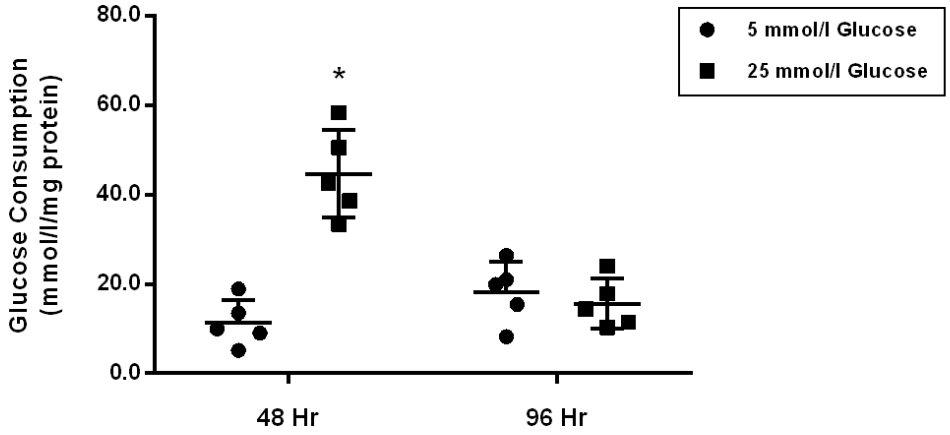


Figure 10. RIP2 mediated caspase-1 activity and IL-β production in Müller cells.

(A) hMCs were incubated in either 5 mmol/L glucose or 25 mmol/L glucose media for 48 hours. RIP2 protein levels were determined by Western Blot analysis, normalized to β-actin, and expressed as mean ± SDEV (n=5) with (*) = $p< 0.05$ compared to 5 mmol/L glucose. **(B)** hMCs transfected with either scramble RNA or siRNA were incubated in 5 mmol/L glucose or 25 mmol/L glucose media for 48 hours. Caspase-1 activity was assessed and expressed as mean ± SDEV (n=5) with (*) = $p< 0.01$ compared to 5 mmol/L glucose and (#) = $p<0.01$ compared to 25 mmol/L glucose. hMCs without transfection served as controls. **(C)** IL-1β release into media was measured using ELISA assays. IL-1β levels are expressed as mean ± SDEV (n=3) with (*) = $p< 0.05$ compared to 5 mmol/L glucose and (#) = $p<0.05$ compared to 25 mmol/L glucose.

Figure 10 cont.

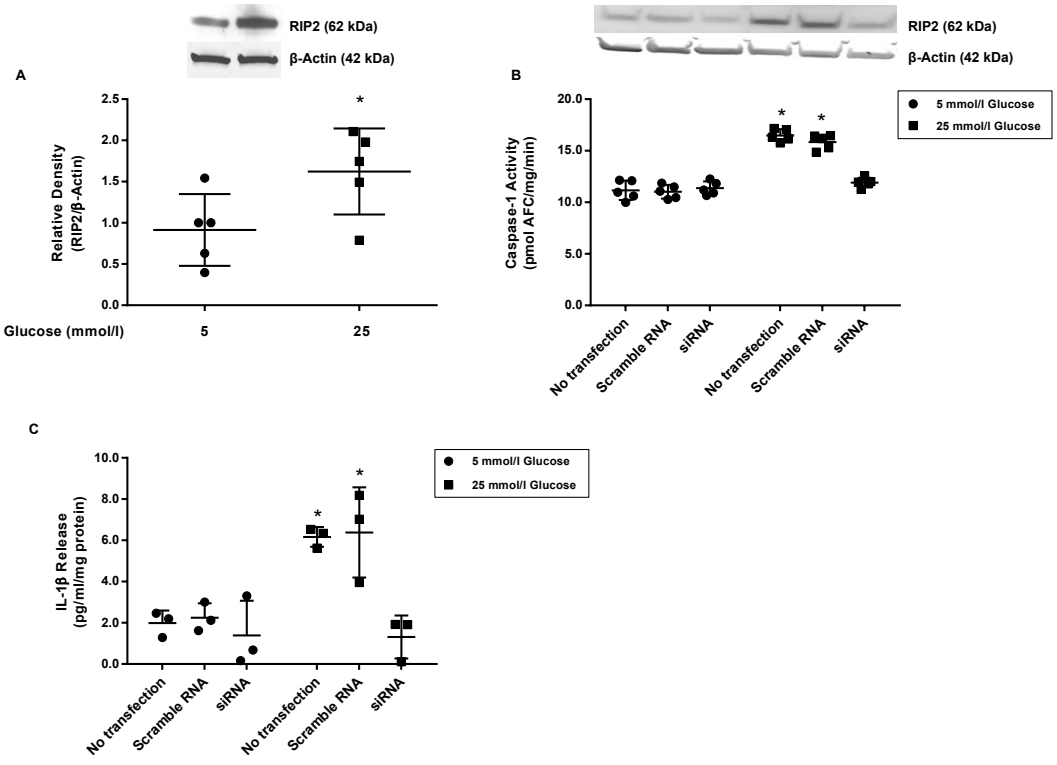


Figure 11: IL-1β-induced caspase-1 activity mediated by RIP2.

(A) hMCs were incubated in either 5 mmol/L glucose or 5 mmol/L glucose + IL-1β (2ng/ml) for 24 hours. RIP2 protein levels were determined by Western Blot analysis, normalized to β-actin, and expressed as mean ± SDEV (n=5) with (*) = p< 0.05 compared to 5 mmol/L glucose**. (B)** hMCs transfected with either scramble RNA or siRNA were incubated in 5 mmol/L glucose or 5 mmol/L glucose + IL-1β (2ng/ml) for 24 hours. Caspase-1 activity was assessed and expressed as mean ± SDEV (n=5) with (*) = p< 0.01 compared to 5 mmol/L glucose and (#) = p<0.01 compared to 25 mmol/L glucose.

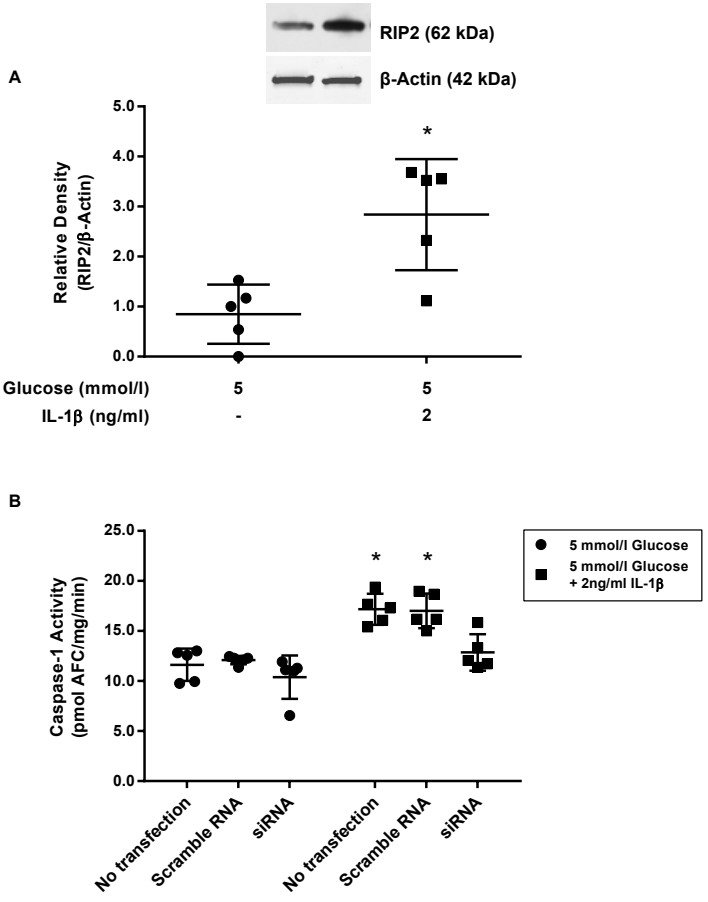


Figure 12: Inhibition of hyperglycemia-induced Müller cell death by IL-1ra, YVAD-fmk, and RIP2siRNA.

hMCs were cultured in either 5 mmol/L or 25 mmol/L glucose media in the presence or absence of **(A)** RIP2 siRNA or **(B)** 100ng/ml IL-1ra for 96 hours. At 96 hours, cell death was determined using the Trypan Blue Exclusion assay and expressed as mean ± SDEV (n=5) with (*) = p<0.01 compared to 5 mmol/L glucose, (#) = P<0.01 compared to 25 mmol/L glucose.

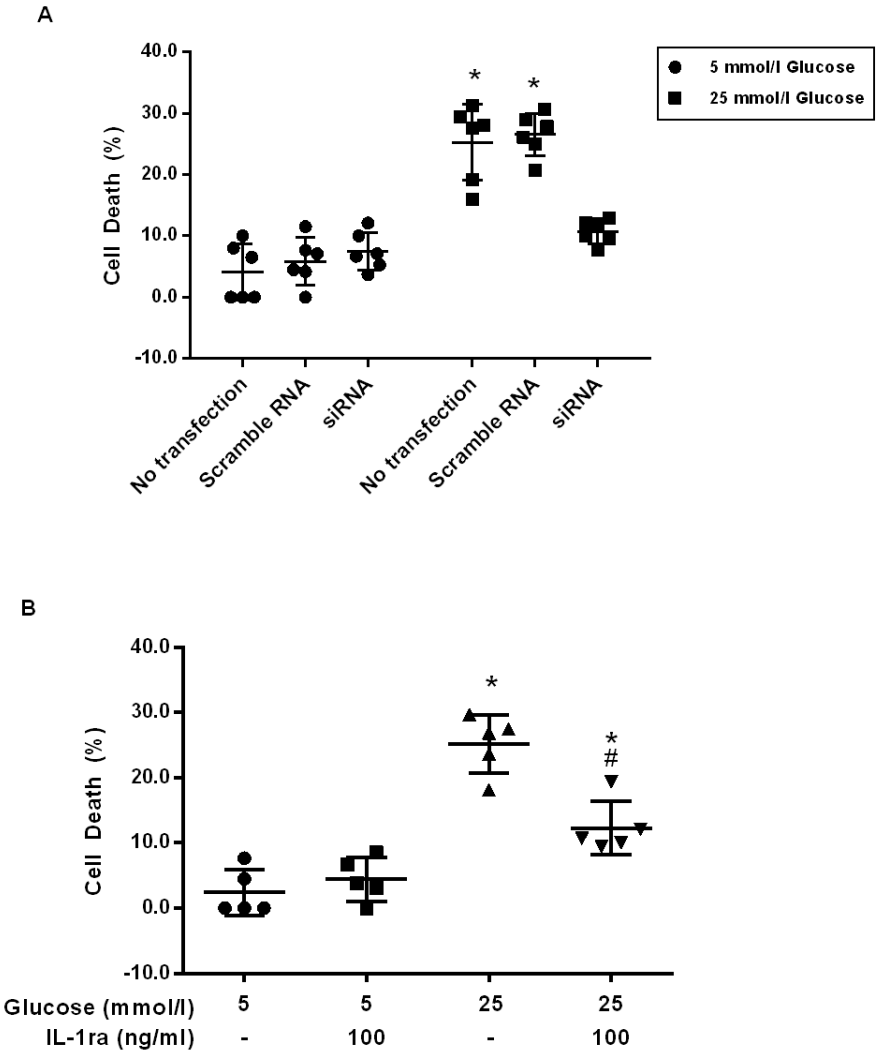


Figure 13: Caspase-1 dependent Müller cell death in retinas of diabetic mice.

Eyes of STZ-diabetic wt (n=10) and non-diabetic wt (n=10), STZ-diabetic cas-1-/- (n=5) and non-diabetic cas-1-/- (n=5) mice were isolated after 7 months duration of diabetes. Number of Müller cells is presented as mean ± SDEV with (#) = $p<0.02$.

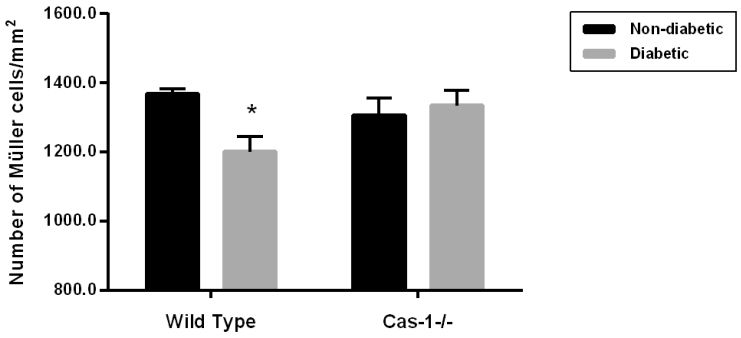


Figure 14: Proposed mechanism of sustained caspase-1 activation via feedback signaling in Müller cells.

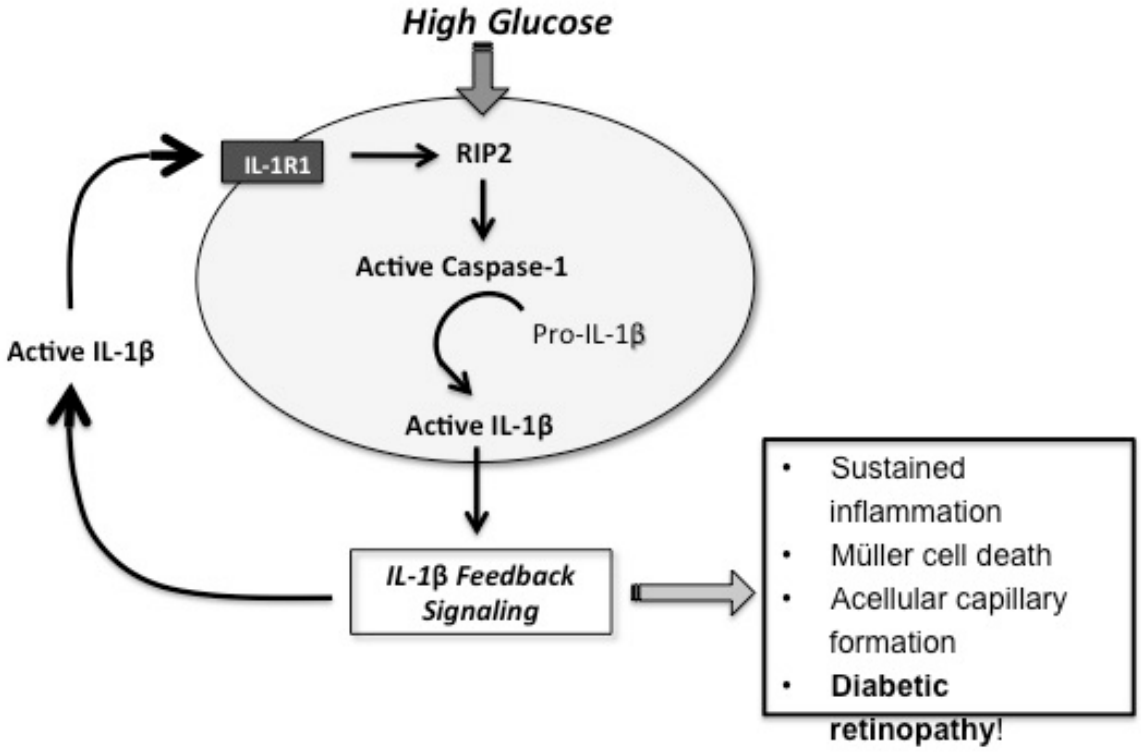


Table 1: Summary of Animal Data.

Severity of diabetes was estimated by measuring non-enzymatically glycated hemoglobin (GHb) levels using affinity chromatography (Glyc-Affin; Pierce, Rockford, IL). (*) = $p < 0.05$ significantly different from normal wild type animals compared to diabetic wild type animals and normal Cas-1 -/- (A) or IL-1R1 -/- (B) animals compared to diabetic Cas-1 (A) or IL-1R1 -/- (B) animals .

Experimental Group	Duration of diabetes (weeks)	GHb (%)	GHb (mmol/mol)
A) WT normal	24	4.8±0.10	29±1
A) WT diabetic	24	13.8±0.50 (*)	127±5
A) Cas-1-/- normal	24	3.65±0.32	16±3
A) Cas-1-/- diabetic	24	13.7±2.11 (*)	126±21
B) WT normal	10	4.34±0.63	24±6
B) WT diabetic	10	10.47±3.95 (*)	91±39
B) IL-1R 1 -/- normal	10	3.36±0.42	13±4
B) IL-1R1 -/- diabetic	10	10.39±0.87 (*)	90±9
B) WT normal	20	4.36±0.76	24±8
B) WT diabetic	20	13.22±0.76 (*)	121±8
B) IL-1R 1 -/- normal	20	3.69±0.17	17±2
B) IL-1R1 -/- diabetic	20	9.42±1.12 (*)	79±11

2.4 Discussion

The importance of inflammation in the progression of diabetic retinopathy has become increasingly apparent. A variety of pro-inflammatory cytokines have been identified in the vitreous of patients compared to healthy individuals, among them IL-1β(111–115,132). Our previous studies have shown that caspase-1 activity is consistently increased in the retinas of diabetic animals and patients(62,133). This new study provides novel mechanistic insights into the process of prolonged caspase-1 activation and IL-β production in diabetic retinopathy. First, using caspase-$1^{-/-}$ mice we demonstrated that caspase-1 activation is indeed crucial for the development of diabetic retinopathy. Furthermore, using IL-$1R1^{-/-}$ mice we identified that diabetes-induced caspase-1 activity progresses from an IL-1R1 independent mechanism to an IL-1R1 dependent mechanism throughout disease progression. This is consistent with our previous observation that knock down of the IL-1R1 receptor prevented the development of diabetic retinopathy(133). These data provide for the first time an indication of a caspase-1/IL-1β/IL-1R1 feedback signaling mechanism that keeps caspase-1 active in the diabetic retina. Using Müller cells known to produce active caspase-1 and IL-1β under hyperglycemic conditions we confirmed that prolonged exposure to hyperglycemia leads to caspase-1/IL-1β/IL-1R1 feedback signaling causing sustained caspase-1 activity. We were able to show that hyperglycemia initiates caspase-1 activity and IL-1β continues to promote caspase-1 activation despite the fact that initial increased glucose consumption has been normalized. In addition, activation of caspase-1 by both stimuli, hyperglycemia and IL-1β, was mediated by RIP2 as determined by RIP2 knockdown experiments.

Finally, we determined that activation of the caspase-1/IL-1β/IL-1R1 feedback is detrimental leading to Müller cells death. Hyperglycemia is not only detrimental for Müller cells *in vitro* but most importantly *in vivo*. We confirmed that Müller cell loss *in vivo* is dependent on activation of caspase-1. Taken together, this indicates that hyperglycemia induces caspase-1 activation and IL-1β production initiating a seemingly IL-1β driven caspase-1/IL-1β/IL-1R1 feedback cycle that is detrimental to the viability of retinal cells such as Müller cells and promotes the development and progression of diabetic retinopathy (Figure 14).

The idea of a caspase-1/IL-1β/IL-1R1 feedback signaling is intriguing for several reasons. Although our study used Müller cells to demonstrate hyperglycemia-induced caspase-1/IL-1β/IL-1R1 feedback signaling, other retinal cells might be capable of producing similar feedback cycles leading to sustained caspase-1 activation and prolonged IL-1β production. Several retinal cell types such as astrocytes and microglia have been identified to produce IL-1β when exposed to elevated glucose levels(134–136). This leaves the strong possibility that IL-1β produced by one retinal cell type feeds into IL-1β feedback signaling of another retinal cells type even further augmenting IL-1β production. Despite our *in vitro* studies showing that autocrine activation is sufficient to induce caspase-1/IL-1β/IL-1R1 feedback signaling the combination of several IL-1β-producing cells amplifying feedback signaling might actually be necessary to reach high enough IL-1β levels for IL-1β to drive caspase-1 activation via the IL-1R1 in the diabetic retina. This could explain the long duration of diabetes required for caspase-1 activity to become

dependent on IL-1β feedback *in vivo*. However, more studies need to be done to positively confirm this concept of caspase-1/IL-1β/IL-1R1 feedback signaling in other retinal cell types. Future studies will also have to identify how other cytokines such as TNFα that have been associated with the development of diabetic retinopathy influence the regulation of the caspase-1/IL-β/IL-1R1 feedback signaling.

Looking deeper into the switch of caspase-1 activation from IL-1R1 independent to IL-1R1 dependent mechanisms despite the presence of an obvious hyperglycemic environment we examined glucose consumption in Müller cells. Müller cells are unique in their metabolism since they derive their energy primarily from glycolysis and are known to take up large amounts of glucose(33). Although hyperglycemia initially drives caspase-1 activation, over time glucose consumption surprisingly decreased and eliminated glucose as a mediator of prolonged caspase-1 activity. This indicates that Müller cells may be capable of regulating glucose consumption overtime to avoid excessive caspase-1 activation by hyperglycemia. However, cells are unable to compensate for the increased levels of IL-1β, which itself is capable of driving caspase-1 activation. This leads to caspase-1 activation being driven solely by IL-1β feedback signaling, which explains the effectiveness of IL-1ra treatment since glucose is playing a minor role in caspase-1 activation once the feedback signaling is set in motion. How much glucose is actually needed to trigger caspase-1 activation and to support ongoing caspase-1/IL-1β/IL-1R1 feedback signaling has to be determined in more detail in future studies. Interestingly, although caspase-1 activation during this feedback signaling is mediated by two distinct stimuli,

hyperglycemia and IL-1β, both pathways are controlled by one regulator, RIP2. RIP2, a 62 kDa CARD domain containing protein, can act as a scaffold protein capable of binding and activating pro-caspase-1 via CARD-CARD interaction(82,119,120). Aberrant RIP2 activity has been implicated as a driver of inflammation in diseases such as Huntington's Disease, where it causes increased caspase-1 activation and IL-1β production, ultimately leading to neuronal cell death(82). For the first time this study shows that RIP2 plays a major role in hyperglycemia-mediated caspase-1 activation and cell death in retinal Müller cells. This is interesting since the more prominent mechanisms of caspase-1 activation seem to be inflammasome-mediated mechanisms involving NLRP3(118). NLRP3 upregulation has been shown in some retinal cells(137) but was not observed in our studies in Müller cells (data not shown).

Prolonged activation of caspase-1 and IL-1β production causes Müller cell death *in vitro* and *in vivo*. Müller cell death in diabetic retinopathy was first identified in 1980, however, few studies have looked at actual mechanisms underlying Müller cell death(54,133,138–140). Intervention in the caspase-1/IL-1β/IL-1R1 feedback signaling by inhibition of caspase-1 as shown in this study or by the IL-1 receptor as we previously reported prevents Müller cell death in diabetic retinopathy demonstrating the importance of this signaling pathway to Müller cell viability(140). The protective effect of caspase-1 inhibition on Müller cell viability is not surprising. Increasing evidence shows that there are a number of modes of programmed cell death in addition to the classical apoptosis(141,142). One of the more recently identified types of cell death, termed pyroptosis, is an inherently

inflammatory based cell death defined as being caspase-1 dependent and preventable by inhibition of caspase-1(142). This current study demonstrates that hyperglycemia-induced Müller cell death fulfills all criteria for pyroptosis and links the pro-inflammatory function of caspase-1 and IL-1β production to cell death. Excessive IL-1β production by Müller cells might not only affect viability of Müller cells but also affect viability of surrounding retinal cells as we have previously shown for endothelial cells(143). Furthermore, Müller cell death preceded acellular capillary formation suggesting a potential role for Müller cells in the progression of diabetic retinopathy.

In this study we have outlined a mechanism of prolonged caspase-1 activation that contributes to chronic inflammatory events in the diabetic retina and while set in motion by hyperglycemia seemingly becomes independent of its original hyperglycemic insult. If confirmed in diabetic patients it could potentially explain why diabetic retinopathy still progresses despite good control of blood glucose levels. It also might open new venues for treatment of diabetic retinopathy. Currently the most common therapy seeks to inhibit VEGF, a growth factor that promotes increased vascular permeability and neovascularization(144–148). While these drugs have been successful in some patients, they do not provide reliable benefits for all patients. Our data suggest that treatment of inflammatory events are potentially more pressing than inhibition of growth factors such as VEGF. Several studies have shown that IL-1β can lead to increased production of VEGF(149–151). Inhibition of caspase-1/IL-1β signaling may provide a therapeutic target upstream of VEGF, without inhibiting VEGF actions directly.

Chapter 3. Modes of Retinal Cell Death in Diabetic Retinopathy

This chapter is a modified version of an article published in Journal of Clinical and Experimental Ophthalmology

Authors: Derrick J. Feenstra, E. Chepchumba Yego, and Susanne Mohr

3.1 Abstract

Cell death seems to be a prominent feature in the progression of diabetic retinopathy. Several retinal cell types have been identified to undergo cell death in a diabetic environment. Most emphasis has been directed towards identifying apoptosis in the diabetic retina. However, new research has established that there are multiple forms of cell death. This review discusses the different modes of cell death and attempts to classify cell death of retinal cells known to die in diabetic retinopathy. Special emphasis is given to apoptosis, necrosis, autophagic cell death, and pyroptosis. It seems that different retinal cell types are dying by diverse types of cell death. Whereas endothelial cells predominantly undergo apoptosis, pericytes might die by apoptosis as well as necrosis. On the other hand, Müller cells are suggested to die by a pyroptotic mechanism. Diabetes leads to significant Müller cell loss at 7 months duration of diabetes in retinas of diabetic mice compared to non-diabetic, which is prevented by the inhibition of the caspase-1/IL-1β (interleukin-1beta) pathway using the IL-1 receptor knockout mouse. Since pyroptosis is characterized by the activation of the caspase-1/IL-1β pathway subsequently leading to cell death, Müller cells seem to be a prime candidate for this form of

inflammation-driven cell death. Considering that diabetic retinopathy is now discussed to potentially be a chronic inflammatory disease, pyroptotic cell death might play an important role in disease progression. Understanding mechanisms of cell death will lead to a more targeted approach in the development of new therapies to treat diabetic retinopathy.

3.2 Introduction

The most common features of diabetic retinopathy are alterations to the retinal microvasculature leading to microaneurysms, macular edema, leakage of blood into the retinal tissue and vitreous, and eventual blindness(3,108). Endothelial cells, which line the microvasculature and provide the blood-retinal barrier, have long been regarded as a scapegoat for explaining changes in the increased vascular permeability in the course of diabetic retinopathy. However, the blood-retinal barrier function of the endothelial cells is supported by surrounding cells, such as Müller cells, pericytes, and astrocytes(39). Since the blood-retinal barrier depends so heavily on this interdependent microenvironment where the function of one cell type depends on support from other cell types, any cellular injury and cell loss will have vast effects on proper retinal barrier function and for that matter any retinal function(143,152,153).

Indeed, loss of retinal cells seems to be a prominent feature of diabetic retinopathy. Diabetes-induced cell death has been observed in numerous retinal cell types such as endothelial cells(143,154–156), pericytes(156–158), neural retinal cells such as ganglion cells(159–161), and retinal glial cells such as Müller cells,

astrocytes, and microglia(5,52,88,135,162–166). Endothelial cell death and pericyte loss have long been assumed to play an important role in the loss of proper blood-retinal barrier function(152,156,167,168). Despite increasing efforts to demonstrate retinal cell death in diabetic retinopathy, mechanisms leading to cell death by diabetes are only poorly understood to date. Identifying potential modes of cell death is complicated by the fact that for some forms of cell death, the pathways and markers are poorly understood and are still being discovered. In recent years, existing types of cell death (apoptosis and necrosis) have been re-classified and new subtypes of cell death have been added.

According to the most recent cell death nomenclature paper published by the Nomenclature Committee on Cell Death (NCCD) there are now 13 subroutines of regulated cell death identified(142). These include anoikis, autophagic cell death, caspase-dependent intrinsic apoptosis, caspase-independent intrinsic apoptosis, cornification, entosis, extrinisic apoptosis by death receptors, extrinsic apoptosis by dependence receptors, mitotic catastrophy, regulated necrosis, netosis, parthanatosis, and pyroptosis. Each type of cell death has different, and often not fully defined, characteristics and markers leading to increased complexity in correct identification of cell death mechanisms both *in vitro* and more importantly *in vivo*. Apoptosis is the most studied type of cell death in diabetic retinopathy. It has well-defined features and is easily detectable with established techniques, such as TUNEL (Terminal dUTP Nick End Labeling) assay. However, some of the cell death types are far more difficult to detect due to the lack of established markers and techniques available.

In order to establish mechanisms underlying the development of diabetic retinopathy and to determine whether cell death is crucial for the progression of the disease, a better understanding of potential types of cell death in the diabetic retina must be achieved. This will then allow for more targeted therapies to combat cell death in diabetic retinopathy. This review will provide an overview of the various retinal cell types undergoing cell death in diabetic retinopathy and attempt to assign cell death classification to these dying cells.

3.3. Apoptosis: Extrinsic Versus Intrinsic

The most well defined form of cell death is apoptosis. Apoptosis, originally introduced by Kerr et al. in 1972, is a term that describes a form of programmed cell death resulting in cytoplasmic and nuclear condensation, a specific pattern of DNA fragmentation, and eventual demise of the cell into apoptotic bodies to be phagocytosed by surrounding cells with very little inflammation involved in the process(169). The common theme in identification of apoptotic cell death is the use of TUNEL staining or other methods that are aimed to specifically detect the apoptotic DNA laddering pattern(170–172). However, due to recent advancements in cell death studies and changes in cell death nomenclature, classification of apoptosis is not as simple as detection of specific DNA fragmentation.

Apoptosis can be divided into various subcategories according to both the stimuli and the pathways leading to execution of cell death, and should therefore be a term used with caution. 'Extrinisic Apoptosis' for example is used to define cell death induced by binding of lethal ligands including FAS/CD95 ligand, tumor

necrosis factor α (TNFα), or TNF-related apoptosis inducing ligand (TRAIL) to their respective death receptor(142,173). Upon binding of these ligands to the death receptor, the "death domain" of the receptor recruits the assembly of the "death-inducing signaling complex" (DISC), a platform of various proteins. The DISC can differ depending on the death receptor involved but typically results in activation of caspase-8(142,174–176). Depending on cell type, active caspase-8 initiates one of two distinct pathways. First, active caspase-8 can directly cleave caspase-3, known as an executioner caspase in the apoptotic process(177). Alternatively, active caspase-8 can cleave BH3-interacting domain death agonist (BID) creating truncated BID (tBID). tBID then binds Bcl-2 allowing BAX to form a pore in the outer membrane of the mitochondria enabling the release of cytochrome c into the cytosol. This triggers formation of the canonical 'apoptosome' via assembly of APAF1 with pro-caspase-9, cytochrome c, and dATP leading to caspase-9 activation, which in turn activates caspase-3(142,178).

Another type of apoptosis, 'intrinsic apoptosis' is similar to extrinsic apoptosis in that there is eventual activation of caspase-3 as the executioner caspase. However, rather than an extrinsic ligand binding to a death receptor, apoptosis is triggered by intracellular stress such as DNA damage, oxidative stress, or excitotoxicity(142). Regardless of the intracellular stress that initiates intrinsic apoptosis, the intrinsic and extrinsic pathways converge at the mitochondria. Increased pore formation by either bak or bax, or pore formation by a multi-protein complex termed the permeability transition pore (PTP) promotes the release of proteins such as cytochrome c, apoptosis-inducing factor (AIF), and endonuclease G

(ENDOG) from the mitochondria into the cytosol(142,179,180). In addition, alterations of the respiratory chain lead to increased reactive oxygen species (ROS) production(142). As described above, apoptosome formation induces caspase-9 and subsequent caspase-3 activation. Activation of caspse-3 initiates events that are responsible for the specific DNA cleavage pattern seen in apoptotic cell death. In contrast, AIF and ENDOG can translocate to the nucleus leading to DNA fragmentation that is independent of caspase activation(178,181–186). In this case, apoptotic cell death occurs even in the absence of active caspases or when caspases are pharmacologically inhibited. This allows for even further classification of intrinsic apoptosis into caspase-dependent and caspase-independent intrinsic apoptosis(142). Therefore, observation of DNA fragmentation alone by TUNEL staining is not sufficient to distinguish between the different types of apoptosis.

Much of the research in diabetes-induced retinal cell death has been focused on identifying apoptosis using the TUNEL assay as the method of choice. Some TUNEL based studies were supported by additional data identifying active caspase-3. TUNEL staining has identified increased endothelial cell apoptosis in retinas of diabetic and galactose fed rats, compared to control animals(156,187,188). A similar increase in TUNEL staining was seen in retinal endothelial cells of diabetic mice(189). A recent study confirmed these results in the retinas of human subjects with diabetic retinopathy compared to those without(190). TUNEL staining has also been used in studies showing that neutrophils from diabetic rats, when co-cultured with human endothelial cells, led to increased endothelial cell apoptosis indicating that other cells types when exposed to hyperglycemia induce endothelial cell death

via an apoptotic pathway(191). In other studies, propidium iodide (PI), which when injected intravenously will fluoresce after leakage through injured cell membrane and bind to DNA or RNA, has been used to detect endothelial cell apoptosis in diabetic rats(154). However, PI staining does not allow for a discrimination between cells undergoing apoptosis or necrosis(154). While much of the apoptosis research has used DNA fragmentation alone, some more detailed studies have shown that high glucose leads to cytochrome c release and changes in mitochondrial morphology in endothelial cells indicating a mitochondria-mediated apoptotic mechanism(192). This is further supported by a study that demonstrated that overexpression of bcl2, an anti-apoptotic member of the bcl2 family, prevented capillary degeneration in diabetic mice(193). Other studies have demonstrated that hyperglycemia can initiate pro-apoptotic pathways in endothelial cells by measuring caspase-8 and caspase-3 activity indicating the caspase dependency of the apoptotic process(143,189,194,195). Although these studies provide good evidence that caspase-dependent apoptosis is the predominant type of cell death for endothelial cells when exposed to a hyperglycemic environment, more studies are needed to determine whether apoptotic cell death in endothelial cells is mediated by an intrinsic or extrinsic mechanism in diabetic retinopathy.

Apoptosis has also been suggested as the type of cell death in pericytes during the progression of diabetic retinopathy. TUNEL staining demonstrated increased pericyte apoptosis in retinas of diabetic and galactose fed rodents compared to control animals(156,187,196). Increased pericyte apoptosis has also been shown in retinal tissue of diabetic patients compared to non-diabetic patients,

again using TUNEL staining(157,158). In addition, increased caspase-8 and caspase-3 activity is seen in rat retinal pericytes in high glucose conditions(197). Similarly to endothelial cells, mitochondria of retinal pericytes display significant fragmentation and metabolic dysregulation and this has been directly implicated in accelerated apoptosis in retinal pericytes in diabetic retinopathy(198). These studies all indicate a similar caspase-dependent apoptotic mechanism for pericytes as seen for endothelial cells.

Additionally, TUNEL staining has been used to identify apoptosis in a variety of retinal cell types, although numbers of these studies are limited. Increased apoptosis in neural retinal cells such as ganglion cells of diabetic rats compared to non-diabetic rats has been detected(135,159,160,188,196). Amacrine cells have also been shown to undergo apoptosis, as characterized by TUNEL staining and staining for active caspase-3, using the Ins2Akita mouse model(199). It has been suggested that there is selective S-cone loss as identified by TUNEL staining in diabetic retinopathy(200). However, more detailed studies are needed to further confirm the aforementioned mechanisms and to allow for a distinct classification of apoptosis in these cells. Although numerous studies have established that apoptosis of several cell types occurs in the diabetic retina, the next important step will be to identify the link between increased glucose levels and the initiation of caspase-dependent apoptosis.

3.4 Necrosis: A Regulated Pathway to Cell Death?

Historically, necrosis was considered the type of cell death "on the other end" of the cell death spectrum. Necrosis has been a term used for 'accidental cell death' rather than 'programmed cell death,' which was reserved for apoptosis. It was defined in the classification of cell death article published by the NCCD in 2005, as cell death with no apparent signs of apoptosis or autophagy(201). The morphological appearance of cells undergoing necrosis were described as having features such as cytoplasmic swelling, mechanical rupture of the plasma membrane, dilation of cytoplasmic organelles, and chromatin condensation(201). The understanding of pathways leading to necrosis in vivo was vague at best. Due to the lack of a clear mechanism for necrosis, new terms describing "necrosis-like" cell death were introduced. One of these new terms was "apoptonecrosis" where apoptosis evolves into necrosis, although use of this term was discouraged to avoid further confusion until pathways involved in this process were fully identified(201). However, out of this research, the picture of "regulated necrosis" and it's importance in various physiological and pathological settings evolved(142,202). Triggers for regulated necrosis include excitotoxicity, DNA damage resulting in DNA alkylation, and ligands such as TNF and FasL binding to their respective death receptors(142,203–207). These triggers initiate ubiquitination of receptor interacting kinase-1 (RIPK1) and subsequent activation of RIP3. Whereas RIP3 would activate pro-caspase-8 in apoptotic conditions, in experimental or pathological settings where caspase-8 is absent RIP3 can lead directly to execution of regulated necrosis(142,203–205). Crucial characteristics of regulated necrosis include death receptor signaling,

absence of caspase activity, and RIP1 and /or RIP3 activation. Activation of pathways in regulated necrosis still lead to the classical morphological features associated with necrosis(142). All these new studies indicate that the process of necrotic cell death can be regulated depending on microenvironment rather than being a random event as previously assumed.

Necrosis has been implicated in the process of diabetic retinopathy. Increased necrotic cell death of pericytes has been observed in the retinas of diabetic rats and humans using light and electron microscopy(208–210). This particular pericyte cell death was later described as "selective necrosis"(138). Reasoning for this designation was most likely due to the assumption that this cell death caused by diabetic conditions was accidental. Although the newer studies claim apoptosis as the major type of cell death for pericytes in diabetic retinopathy, one cannot exclude that some pericytes might undergo cell death via regulated necrosis depending on microenvironment and the progression of the disease. Further clarification of the definition for necrosis and the pathways involved may be necessary to better understand and identify this process in the diabetic retina.

3.5 Autophagic Cell Death

Autophagic cell death may be the most puzzling type of cell death identified to date. It is currently defined by the NCCD as "a type of cell death that occurs in the absence of chromatin condensation but accompanied by massive autophagic vacuolization of the cytoplasm"(141). The first study demonstrating that autophagic cell death exists *in vivo* showed that knockdown of key genes required for

autophagy reduced cell death in *Drosophila melanogaster*(211). Autophagic cell death has also been identified in cancer cells exposed to chemotherapeutic agents *in vitro*(212,213). Cells dying by autophagic cell death have very little association with phagocytes, contrary to cells dying by apoptosis which are eventually removed via phagocytosis(141). In order to determine autophagic cell death, cell death must be prevented by inhibition of the autophagic pathway either by chemicals or knockdown of essential autophagic proteins(141,142). Detection of common markers used to observe increases or decreases in autophagy, such as LC3 (microtubule-associated protein 1 light chain 3) or ATG (autophagy) family members, are not sufficient to indicate autophagic cell death.

Autophagy is the process of removing unwanted or damaged cellular material or organelles by packaging these materials into autophagosomes, which are then targeted for degradation. In most physiological settings, autophagy is considered a beneficial and a pro-survival mechanism used by the cell and inhibition of autophagy can actually lead to increased apoptosis(214–218). Therefore, an increase in autophagic flux does not always imply autophagic cell death. For example, if cell death occurs with increased markers of autophagy but cannot be blocked by autophagy inhibition, it is not indicative of autophagic cell death. To classify cells as dying by autophagic cell death, inhibition of proteins within the autophagic pathway must promote cell survival.

Whether autophagic cell death is occurring in the progression of diabetic retinopathy has not been determined to date. A recent study indicated increased autophagy by measuring levels of ATG5, but whether this ultimately leads to

autophagic cell death in retinal cells during diabetic retinopathy has not been addressed(219).

3.6 Pyroptosis: Inflammation Driven Cell Death

An emerging type of cell death that is attracting increasing attention is 'pyroptosis.' Pyroptosis is an inherently inflammatory-mediated form of cell death, defined as being caspase-1-dependent(73,142,220). During pyroptosis, there is assembly of a multiprotein platform allowing for induced proximity-mediated activation of caspase-1. Active caspase-1 then cleaves the pro-inflammatory cytokines IL-1β and IL-18 from their inactive precursors to their biologically active forms(73,75,76,220). The multiprotein platform allowing for caspase-1 activation is termed either the inflammasome or pyroptosome. Inflammasomes are comprised of the ASC (Apoptosis-associated Speck-like protein containing a CARD) adaptor protein and a cytosolic sensor of either DAMPS (Danger Associated Molecular Patterns) or PAMPS (Pathogen Associated Molecular Patterns) such as a NLRs (NOD-like receptors) or AIM2 (absent in melanoma 2)(221–225). The pyroptosome is an assembly of ASC dimers that can directly activate caspase-1(226). Prevention of pyroptosis is accomplished by inhibition of caspase-1 either by pharmacological intervention or caspase-1 knockout in animal models. Although it is now very well established that initiation of pyroptosis is caspase-1 and IL-1β driven, the execution phase of pyroptosis is not yet completely understood. It has been shown that pyroptosis shares traits with both apoptosis and necrosis in the execution phase(141,227). Execution of pyroptotic cell death might depend on cell type,

microenvironment, and stimulus. Different pathways might be involved in the execution of pyroptosis bringing into question whether TUNEL staining is actually able to identify all pyroptotic cells.

This inflammatory-mediated process of cell death is particularly intriguing in the context of diabetic retinopathy. A disease which was originally thought of as a purely microvascular disease, diabetic retinopathy is now being viewed as a potential chronic inflammatory disease leading to changes in the retinal microvasculature(3,110,228). Studies have demonstrated that diabetes leads to activation of caspase-1 and IL-1β production in the retinas of diabetic and galactosemic mice as well as diabetic rats(135,229,230). Active caspase-1 and IL-1β was also detected in retinal tissue of diabetic patients(88,162) and the vitreous of patients with proliferative diabetic retinopathy(113,231). Inhibition of the caspaspe-1/IL-β signaling pathway prevented the development of diabetic retinopathy in diabetic and galactosemic animals indicating that this inflammatory pathway is important for disease development, potentially via pyroptotic cell death of retinal cells that are crucial for proper retinal function(88,135).

When looking at specific cell types undergoing pyroptotic cell death in the course of diabetic retinopathy, retinal glial cells stand out. It has been shown that caspase-1 activity and IL-1β production is increased *in vitro* in Müller cells following exposure to hyperglycemic conditions and cells die as a consequence(116,232). Inhibition of the caspase-1 pathway prevented Müller cell death under these conditions. In Müller cells and microglia, it has been shown that use of minocycline, a drug that decreases caspase-1/IL-1β signaling, is able to prevent cell

death(88,135). These *in vitro* studies are an indication that glial cells might respond to chronically elevated glucose levels by undergoing pyroptotic cell death.

Since execution of pyropototic cell death lacks specific markers, identifying retinal cells dying by pyroptosis *in vivo* is a difficult task. Studies using EM show that there is Müller cell death occurring in diabetic retinopathy(138). Dying Müller cells are described as being hypertrophic consistent with the notion that during pyroptosis, cells swell rather than shrink as observed in apoptotic cell death(53). Other studies indicate that there is simply hypertrophy and glial dysfunction associated with the disease(135). A previous study by us has shown that GAPDH (glyceraldehyde-3-phosphate dehydrogenase) accumulates in the nucleus of Müller cells in the retinas of diabetic rats(163). Nuclear accumulation of GAPDH has been closely associated with cell death induction(233–235). Interestingly, hyperglycemia-induced nuclear accumulation of GAPDH was mediated by activation of the caspase-1/IL-1β pathway(54,139). Whether caspase-1/IL-1β-mediated GAPDH nuclear accumulation is part of the pyroptotic pathway in general has yet to be determined. As our results indicate (Figure 15), diabetes leads to Müller cell loss in the retinas of diabetic mice. Due to the lack of specific markers for pyroptotic cell death, Müller cells were stained against glutamine synthetase and CRALPB and counted. To confirm that Müller cell loss was dependent on the activation of the caspase-1/IL-1β pathway, IL-1 receptor knockout mice were made diabetic and Müller cells were counted in retinas of non-diabetic and diabetic IL-1 receptor knockout mice. Inhibition of the caspase-1/IL-1β pathway prevented diabetes-induced Müller cell loss. These studies are the first to clearly demonstrate Müller cell loss in diabetes

and to suggest that cell death might occur via a pyroptotic mechanism. Based on our studies, we suggest that hyperglycemia leads to activation of caspase-1 and subsequent production of IL-1β leading to Müller cells death via pyroptosis (Figure 16). Since glial cells in general respond to hyperglycemia by producing pro-inflammatory cytokines, future studies need to determine whether all glial cell types are able to undergo pyroptotic cell death.

Figure 15: IL-1R1 Dependent Müller cell death in diabetes.

Diabetic wild type (gHb= 11.0 ± 1.8) and IL-1R1-/- (gHb= 12.1 ± 0.4) were sacrificed after 7 months of diabetes along with age matched normal controls (wild type gHb= 3.8 ± 0.55, IL-1R1-/- gHb= 3.2 ± 0.2). Animals were sacrified and eyes were isolated and fixed in formalin. Retinal sections were processed for both glutamine synthase, CRALBP, and DAPI staining and blinded samples were visualized using confocal microscopy Z-sections. The number of Müller cells was determined by counting three independent areas per retinal section and three retinal sections per animal. The number of Müller cells per mm^2 retina is expressed as mean ± STDV (n=10 per group).

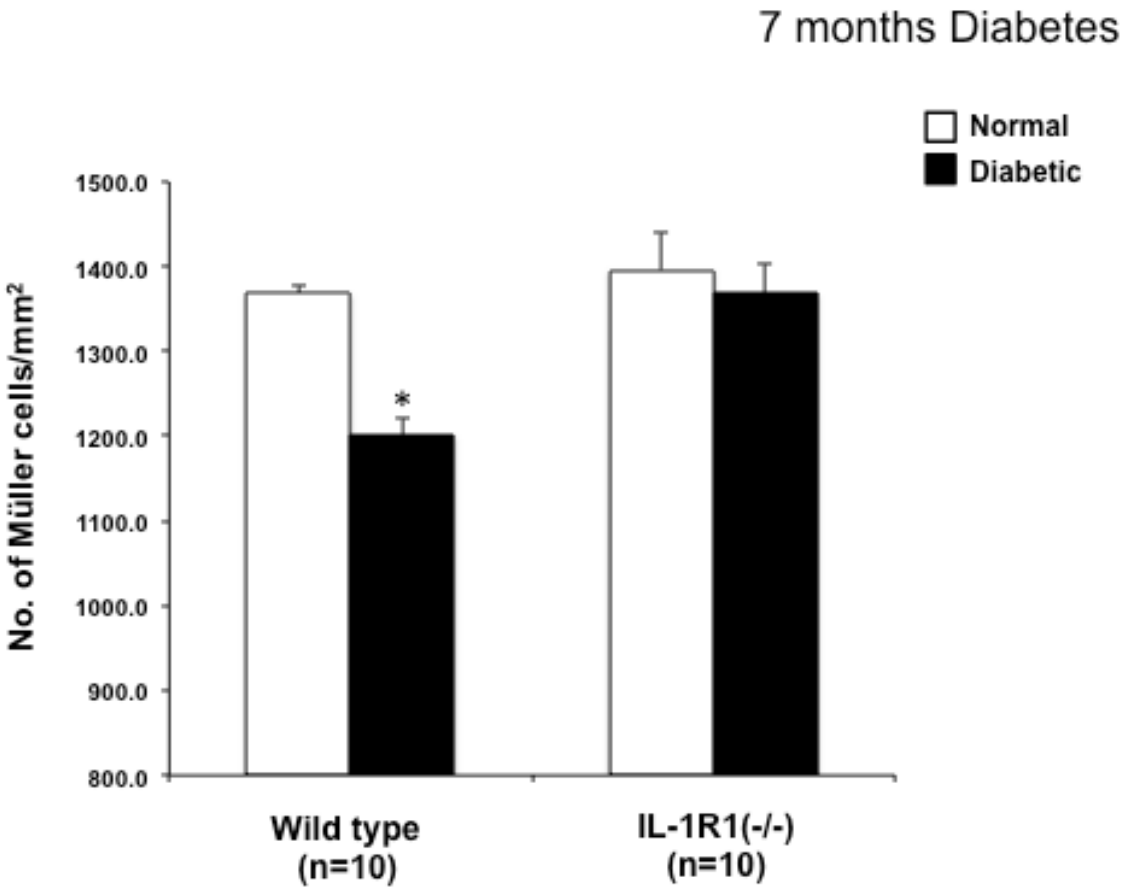
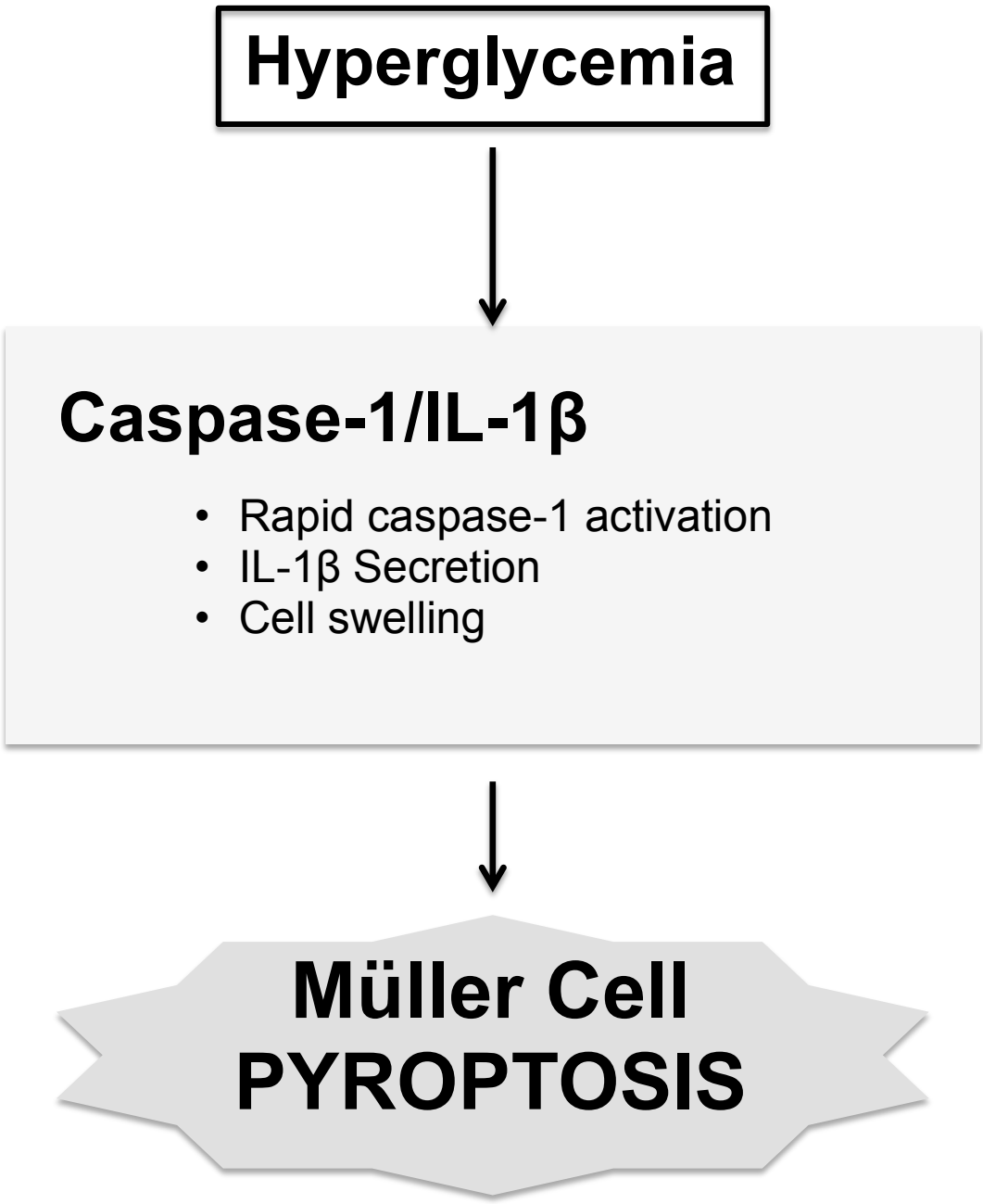


Figure 16. Scheme of pyroptosis in Müller cells.



3.7 Conclusion

In conclusion, cell death seems to be a prominent feature in the progression of diabetic retinopathy. Several retinal cell types have been shown to undergo various forms of cell death (Table 2). A lot of emphasis has been given to study apoptosis of retinal cells during the progression of the disease. Based on the studies available, it is fair to say that endothelial cells are predominantly dying by an apoptotic process, however the type of apoptosis has yet to be determined. Several studies point to a caspase-dependent mechanism involving mitochondrial damage but more studies are needed to fully determine an intrinsic or extrinsic apoptotic pathway. Other cell types suggested to undergo apoptosis are ganglion cells, amacrine cells, and S-cones. However, due to the limited number of studies on cell death in these cell types, a conclusion as to whether these cells are truly dying of apoptosis cannot be made at this time. The picture is not clear for pericytes; both apoptosis as well as necrosis have been suggested as modes of cell death. Given the new research on necrosis that indicates necrosis can also be a regulated process like apoptosis, more studies are needed to determine precise mechanisms of pericyte cell death in diabetic retinopathy. The type of cell death might depend on the phenotype of pericytes and the microenvironment surrounding these cells. Inflammation-driven pyroptosis is an emerging form of cell death that is receiving a lot of attention right now. This type of cell death is intriguing to study in the context of diabetic retinopathy since new understandings of the disease suggest that diabetic retinopathy is a chronic inflammatory disease. Müller cells are prime candidates for this form of cell death due to the fact that diabetes-induced cell death

is dependent on the activation of the caspase-1/IL-1β pathway. More studies are needed to fully understand the mechanism underlying the process of pyroptosis and to determine whether glial cells including macro and micro glial cells are undergoing pyroptosis in diabetes. Loss of other retinal cell types such as astrocytes has been reported but identification of the type of cell death has not been made(166).

Other types of cell death such as anoikis, entosis, parthanatos, netosis, cornification, and mitotic catastrophe, have yet to be identified in the course of diabetic retinopathy but cannot be excluded from the process of disease development. Further development of tools capable of assessing these modes of cell death is needed to determine whether these cell death modalities are present in diabetic retinopathy.

A better understanding of how retinal cells are dying during the development and progression of diabetic retinopathy will allow for a more targeted approach to intervene in this process. Although the general consensus is that inhibition of cell death is beneficial for disease prognosis, timing at which intervention should be started and targeting of specific retinal cell types will be crucial for a successful outcome of treatments aiming to inhibit cell death. Therefore, the more knowledge that is attained on which cell types undergo what type of cell death, the more therapeutic strategies can be developed.

Table 2. Characteristics of modes of cell death and potential retinal cell types undergoing cell death in diabetic retinopathy.

Retinal cell types were assigned into a cell death category based on identification of at least two characteristics of the respective mode of cell death. Abbreviations: AIF, apoptosis-inducing factor; ENDOG, endonuclease G; TNF, tumor necrosis factor; LC3/Atg8, microtubule-associated protein 1 light chain 3; SQSTM1, sequestosome 1; DAMP, danger associated molecular pattern; PAMP, pathogen associated molecular pattern.

	Cell Death Characteristics	**Retinal Cell Types**
Extrinsic apoptosis	• Triggered by binding of lethal ligands (FAS/CD95, TNF, TRAIL) to respective death receptor • Caspase-8 activation leading to eventual caspase-9 and caspase-3 activation as the executioner caspase • DNA fragmentation	• Endothelial cells • Pericyte • Ganglion cells • Amacrine • S-cones
Intrinsic apoptosis	• Triggered by intracellular stress (DNA damage, oxidative stress, excitoxicity) • Increased mitochondrial outer membrane permeability • Release of proteins from mitochondria (cytochrome c) • AIF and ENDOG nuclear translocation • DNA fragmentation • Caspase-9 activation leading to caspase-3 activation as the executioner caspase	• Endothelial cells • Pericytes • Ganglion cells • Amacrine • S-cones
Necrosis	• Triggered by excitotoxicity, DNA damage, or binding of lethal ligands (TNF, FasL) • Ubiquitination of RIP1 and subsequent RIP3 activation • Cell death even in the absence of caspase activity	• Pericytes
Autophagic cell death	• Presence of autophagy markers (lipidation of LC3/Atg8) • Increased degradation of autophagic substrates (SQSTM1) • Prevented by inhibition of autophagy	• ?
Pyroptosis	• Triggered by either DAMPs or PAMPs • Assembly of either inflammasome or pyroptosome complex • Caspase-1 activation and subsequent IL-1β or IL-18	• Müller cells

Chapter 4. Caspase-1 Activation in Galactosemia

4.1 Introduction

Diabetic retinopathy is the leading cause of acquired blindness in adults. Currently there is no cure for the disease and the few treatments that are available have limited success, work on a case-by-case basis, and are both a burden to the life quality of the diabetic patient and a financial burden to the health care system. The most common therapies are designed to treat the vascular changes seen in diabetic retinopathy. Research has shown however, that inflammation plays a key role in the development of diabetic retinopathy and may preclude the vascular changes seen in disease progression (61,62,111–113,115,189). Recently, a study identified at least 19 cytokines are elevated in the vitreous of patients with proliferative diabetic retinopathy compared to healthy individuals (132). Therefore, understanding inflammation in the progression of diabetic retinopathy may help to identify new targets for therapies.

One of the major concerns in diabetic retinopathy research is the lack disease-relevant of animal models. Mice have an inherently different retinal structure as they have no macula, and disease pathology doesn't progress to proliferative diabetic retinopathy as it does in humans. One interesting model is the galactosemia model. The galactosemia model was first identified in a study by the Kern lab and showed that dogs fed a 30% D-galactose diet showed retinal pathologies similar to those in the diabetic retina including microaneurysms, hemorrhages, acellular capillaries, vascular lesions and pericyte dropout(236).

Interestingly, these pathologies developed even though the animals lacked many of the systemic abnormalities typically associated with diabetes as they had normal blood glucose, free fatty acids and serum insulin(236). Since then, the galactosemia model has been used extensively as a tool to study retinal diabetic-like retinopathy without systemic diabetes-related changes(237–244). Mice and rats fed galactose-enriched diet show similar retinal pathologies to dogs including increased retinal capillary width and microaneurysms at 8 months of age, and increased retinal microaneurysms, acellular capillary formation, pericyte ghosts and capillary basement membrane thickening as duration of galactosemia is extended(244–246,246–251). Previous reports show that there is chronic, low-grade inflammation in the retinas of galactosemic mice(61,62,248). Several studies indicate there may be multiple sources contributing to this inflammation, however all reports conclude that reducing this inflammation leads to protection from disease development and may be a useful strategy in the treatment of diabetic retinopathy. Studies using galactosemic CD18 and ICAM-1 deficient mice to inhibit leukocyte adherence show there is decreased retinal inflammation which results in decreased breakdown of blood-retina barrier, decreased endothelial cell death and decreased formation of acellular capillaries(248). Previously, we have reported increased caspase-1 activity and IL-1β in the retinas of galactosemic mice(61,62). Furthermore, administration of the drug minocycline inhibited caspase-1 activity and IL-1β production in the retinas of galactosemic mice and this resulted in decreased formation of acellular capillaries(61). In addition to increased inflammation, studies have shown abnormalities in metabolism in the retinas of galactosemic mice and this may be a

driver of inflammation(247,252–258). Interestingly, these studies show there are key differences between the experimental-diabetes and galactosemia models with regards to metabolic abnormalities. In diabetic mice there were increases in both systemic and retinal oxidative stress, however in galactosemic mice oxidative stress increased only in the retinas, but was normal in the plasma(252). Furthermore, there are differential effects of antioxidants in the retinas of these two models(252,256).

Caspase-1, formerly known as Interleukin-1β Converting Enzyme (ICE), is an enzyme produced as an inactive zymogen that can be activated to convert inactive pro-IL-1β into biologically active IL-1β (75,259–262). Caspase-1 activation has been linked to increased production of reactive oxygen species (ROS) and upregulation of TXNIP, a protein which can bind and inhibit thioredoxin leading to ROS generation(137,263). Studies show that TXNIP plays a significant role in diabetic retinopathy and that inhibition of TXNIP prevents diabetes-induced inflammation, gliosis, pericyte loss and ganglion cell injury(137,197,264). Although many studies have focused on the differences in metabolism, inflammation and retinopathy pathology between high glucose and high galactose models, relatively few studies have been performed examining the effects of high galactose at the cellular level. In the present study, we examine differences between hyperglycemia and galactosemia and how they affect oxidative stress and caspase-1 activity in the retina and retinal Müller cells. Furthermore, we explore whether potential differences in caspase-1 activity can be linked to differences in oxidative stress between hyperglycemic and galactosemic conditions.

4.2 Materials and Methods

Materials: Caspase-1 substrate YVAD-AFC and caspase-1 inhibitor YVAD-FMK were purchased from Calbiochem (San Diego, CA). Mouse anti-TXNIP antibody was purchased from MBL International (Woburn, MA). Gradient-Tris-SDS-PAGE (4-20%) gels were from BioRad (Hercules, CA). 7-amino-4-trifluoro-methylcoumarin (AFC) standards came from Sigma (St. Louis, MO).

Animal Models: Male mice (C57BL/6) weighing 20 g were randomly assigned to be made diabetic, galactosemic, or left as normal controls. Diabetes was induced by streptozotocin injections (60 mg/kg body wt i.p. on 5 consecutive days) as described previously (13). Insulin was given to diabetic animals as needed to achieve slow weight gain without preventing hyperglycemia and glycosuria (0.1–0.2 units of NPH insulin subcutaneously, two to three times a week). Galactosemia was induced by feeding normal mice a diet enriched with 30% galactose.

Tissue Culture: The transformed rat retinal Müller cell line (rMC-1) has been established previously as a relevant cell type for retinal Müller cell studies (54,62,163,265). rMC-1 cells were maintained in normal (5mmol/L) glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) in a humidified incubator kept at 37 degrees Celsius with 5% CO_2.

High Glucose and Galactose Treatment: For high glucose studies rMC-1 were treated in DMEM supplemented with glucose for final glucose concentration of 25 mmol/L. For high galactose treatment rMC-1 were treated in DMEM containing standard 5 mmol/L glucose and supplemented with 20 mmol/L galactose. Studies using the

caspase-1 inhibitor (YVAD-fmk; 100μM) were performed as described in previous studies(54).

Preparation of Cytosolic Lysates: Following respective treatments, rMC-1 were lysed in cytosolic lysate buffer [CHAPS Buffer (100mM HEPES, pH 7.5 containing 10% sucrose, 0.1% CHAPS), 1 mmol/L EDTA, 1 mmol/L PMSF and leupeptin (10μg/ml) as described previously (54,88).

Caspase-1 Activity assays: Caspase-1 activity was measured as described previously(54,62,88,117,139,163). Equal amounts of sample (15 μg) were incubated in the presence of the caspase-1 specific substrate (YVAD-AFC; 2.5 μmol/L) for 1 hour at 32 degrees C. AFC fluorescence was detected by a Tecan Spectra FluorPlus fluorescence plate reader (excitation: 400 nm, emission: 510 nm). The release of AFC after cleavage by active caspase-1 was calculated against AFC standard curve and expressed as pmol AFC/mg protein/min.

Western Blot Analysis: Protein (40ug) was separated in 4-20% SDS gradient gels and blotted on nitrocellulose membrane. Membranes were incubated against TXNIP (1:1000 dilution in PBS/0.05% Tween 20) overnight at 4 degrees C followed by incubation with anti-mouse secondary antibody (1:5,000 dilution) for 1 hour at RT. Membranes were imaged using LICOR Biosciences Odyssey Imaging System. Membranes were reprobed using anti-β-actin antibody and normalized by the relative density of TXNIP/β-actin.

ROS Measurement: ROS was measured using cell permeable 2'-7'-dichlorofluroescin diacetate (H_2DCFDA) (Invitrogen, Burlington, Ontario). rMC-1 were plated on Costar 96 well plates, black with a clear bottom (Corning, NY) at 100,000 cells per

well. After treatment, media was removed, and 100 µl of 10 µM H_2DCFDA diluted in serum free media was added to each well for 15 minutes, after which H_2DCFDA was aspirated out of well and replaced with serum free media. ROS was measured on TECAN Infinite m1000 plate reader at an excitation of 494 nm and emission of 525 nm. Following measurement of ROS, fluorescence was normalized using cell permeable Hoechst 33342 nucleic acid stain and normalized to a standard curve to determine cell number.

Trypan Blue Cell Death Assay: Cell death was determined as described previously(44,54,88,163,266). Briefly, following treatment rMC-1 samples were masked and assessed for trypan blue inclusion indicating cell death. Cell death was quantified and expressed as fold change increase compared to cells cultured in normal glucose.

Statistical Analysis: Analysis of data was performed using the One-Way Anova (correlated samples; $p<0.05$) test followed by Tukey's post analysis or Kruskal-Wallis test (ordinal data; $p<0.05$) followed by Dunn's post analysis to determine statistical significance between groups. For details in statistical analysis see VassarStats, Statistical computational website. All data are presented as mean ±SDEV.

4.3 Results

4.3.1 Galactosemia Increases Caspase-1 Activity in Müller Cells

It is known that there is chronic low-grade inflammation in the retinas of galactosemic mice. Previously we have shown that there is increased caspase-1

activity in the retinas of hyperglycemic and galactosemic mice and that inhibition of caspase-1 prevents the formation of acellular capillaries in these animals. To identify the cellular source of this caspase-1 we treated rMC-1 cells with either 5 mmol/L glucose, 25 mmol/L glucose (high glucose) or 5 mmol/L glucose and 20 mmol/L glucose (high galactose). We found that treatment with either high glucose or high galactose led to a significant increase in caspase-1 activity in rMC-1 cells (Figure 17).

4.3.2 High Glucose, But Not High Galactose Generates Reactive Oxygen Species

Caspase-1 activity has been linked to mitochondrial dysfunction and increases in ROS. In order to determine whether the observed increases in caspase-1 activity were due to generation of ROS we treated cells measure ROS production in rMC-1 cells over the course of 96 hours. Interestingly, we observed increased ROS production in rMC-1 cells treated with high glucose, however high galactose had no effect on ROS production (Figure 18A,B).

4.3.3 Glucose Specific Increases in TXNIP

TXNIP is one of the primary targets of studies involving increased ROS accumulation. Interestingly, numerous reports have shown that TXNIP also plays a role in caspase-1 activition, and TXNIP is shown to be increased in studies relating to diabetic retinopathy. We observed that there are increased protein levels of TXNIP in rMC-1 cells treated with high glucose for 24 hours (Figure 19A). Interestingly, in the same manner that ROS generation was specific to glucose and not galactose, there was no increase in TXNIP protein in rMC-1 cells treated with high galactose (Figure 19B). Interestingly, we observed two bands in our Western

Blot analysis of TXNIP, the lower band was consistent the molecular weight of TXNIP, however the upper band was found around 90 kDA. More interestingly, in high glucose treated samples; the upper band appeared to decrease in intensity as the lower band increased. Next, we measured TXNIP levels in rMC-1 over the course of 96 hours to determine whether TXNIP was increased at all time points of increased ROS production. We observed significant increases in TXNIP levels at 24, 48, 72 and 96 hours of high glucose treatment (Figure 19C)

4.3.4 Caspase-1 Inhibition Prevents Galactosemia-Induced Müller Cell Death and Acellular Capillary Formation

Previously we have shown that inhibition of caspase-1 can prevent Müller cell death and acellular capillary formation in diabetic animals. To determine whether galactosemia can also lead to Müller cell death in a caspase-1 dependent manner we measured cell death in rMC-1 treated with high galactose in the presence or absence of a caspase-1 inhibitor. We found that high galactose led to a significant increase in cell death, and that treatment with a caspase-1 inhibitor significantly decreased cell death (Figure 20). To examine whether inhibition of caspase-1 affected disease progression *in vivo*, acellular capillary formation was counted in either non-diabetic or galactosemic mice. We observed increased acellular capillary formation in galactosemic animals, however inhibition of caspase-1 prevented increased acellular capillary formation in galactosemic cas-$1^{-/-}$ mice (Figure 21).

Figure 17. High Glucose and Galactose-Induced Caspase-1 Activity.

rMC-1 were treated with either normal or high galactose for 24 hours and measured for caspase-1 activity. Data expressed as mean ± SDEV. n=5, $p<0.05$.

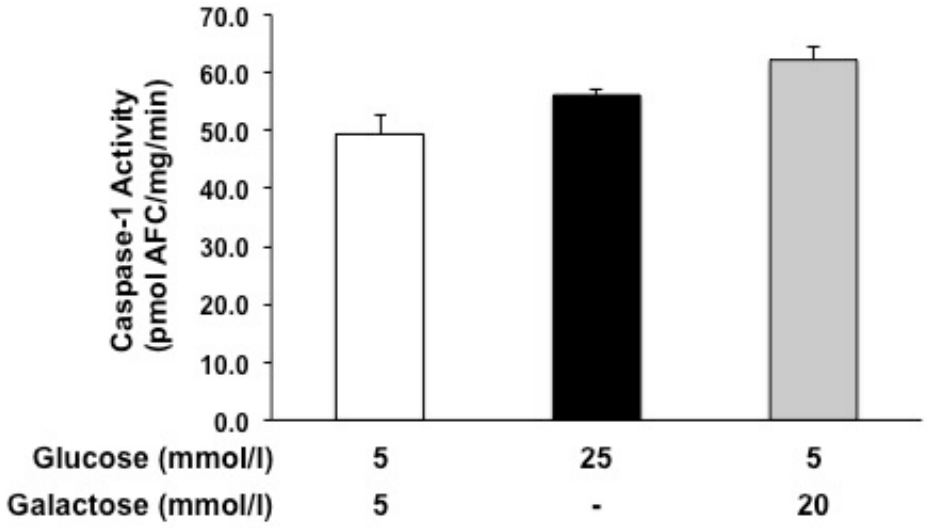


Figure 18. ROS induced by high glucose but not high galactose.

rMC-1 were treated with either normal glucose, A) high glucose, or B) high galactose for up to 72 hours. ROS production was measured and expressed as mean AFU ± SDEV. n=5, $p<0.05$.

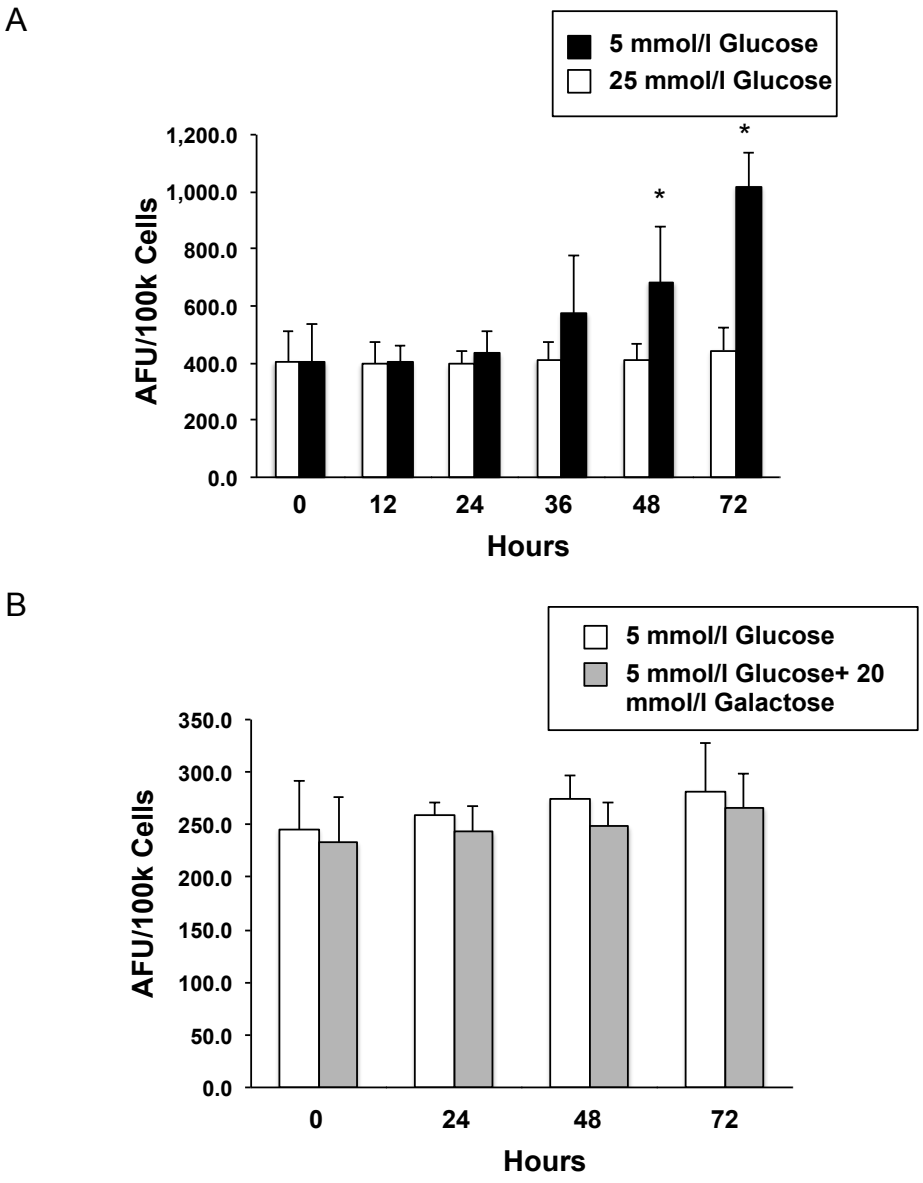
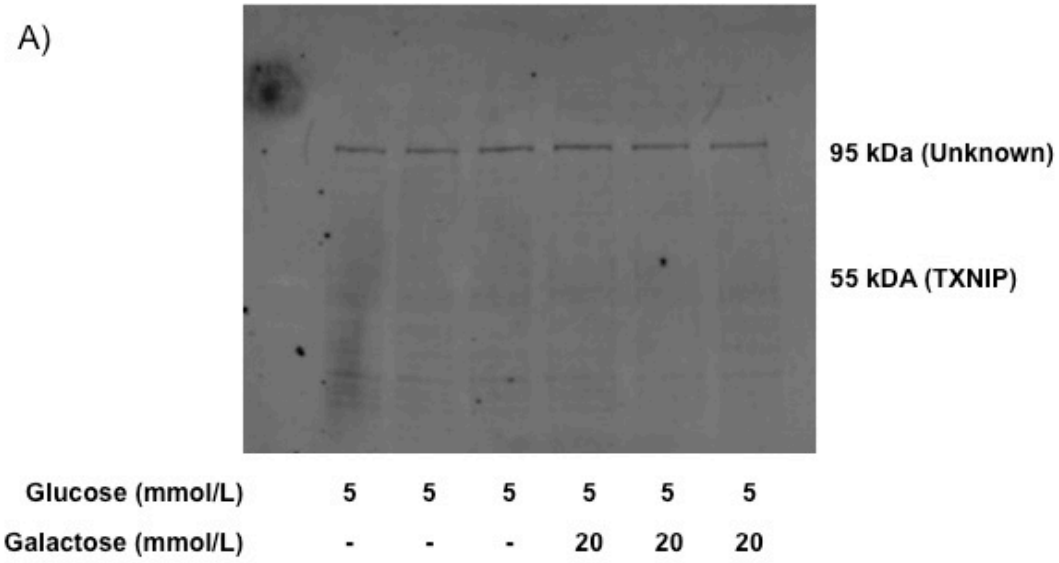


Figure 19. TXNIP protein induction is glucose specific. (Cont. on next page)

rMC-1 were treated with either normal glucose, A) high galactose, or B) high glucose. TXNIP protein levels were determined by Western Blot analysis. C) protein levels were determined by Western Blot analysis, normalized to β-actin, and expressed as mean ± SDEV (n=5) with (*) = p< 0.05 compared to 5 mmol/L glucose.



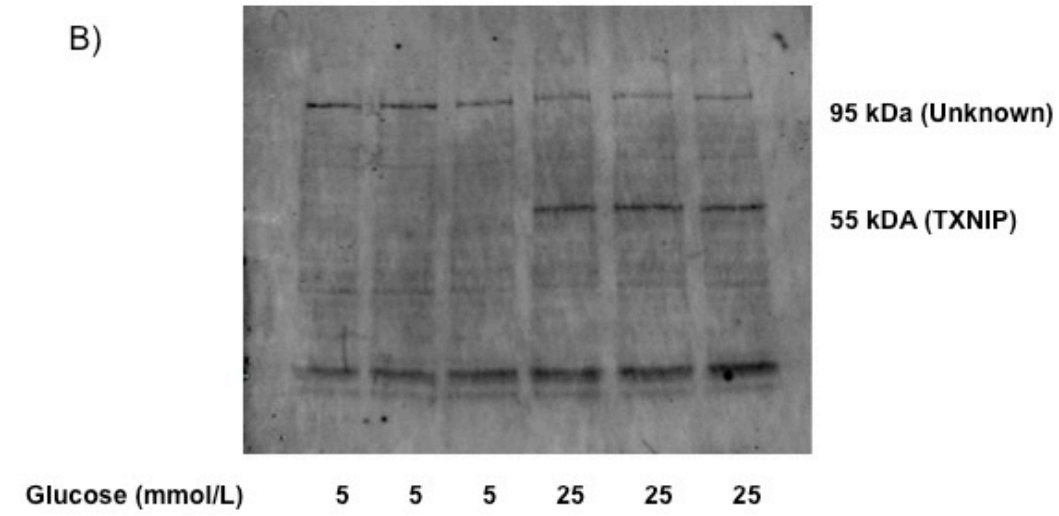


Figure 19 cont..

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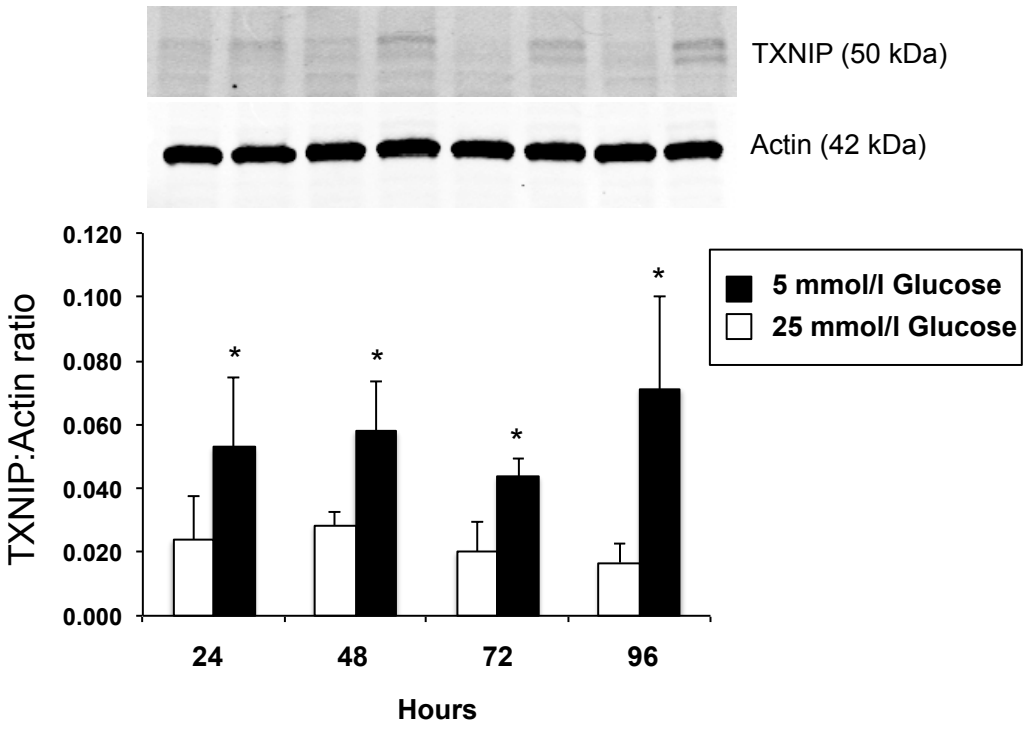


Figure 20. High Galactose leads to caspase-1 dependent Müller cell death.

rMC-1 were treated with normal or high galactose in the presence or absence of a caspase-1 specific inhibitor (YVAD-FMK). Cell death was measured using trypan blue exclusion method.

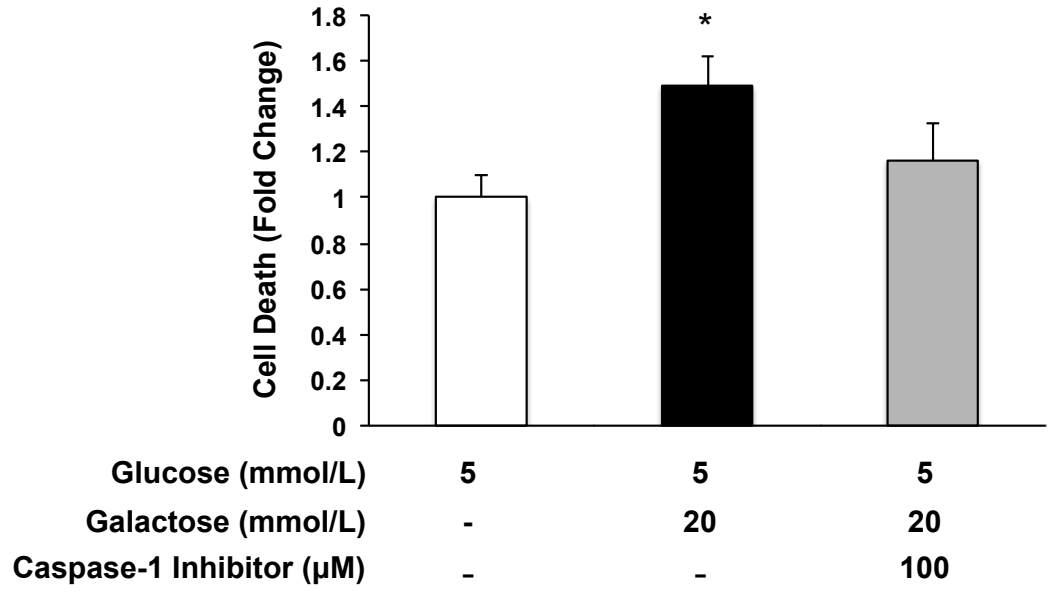
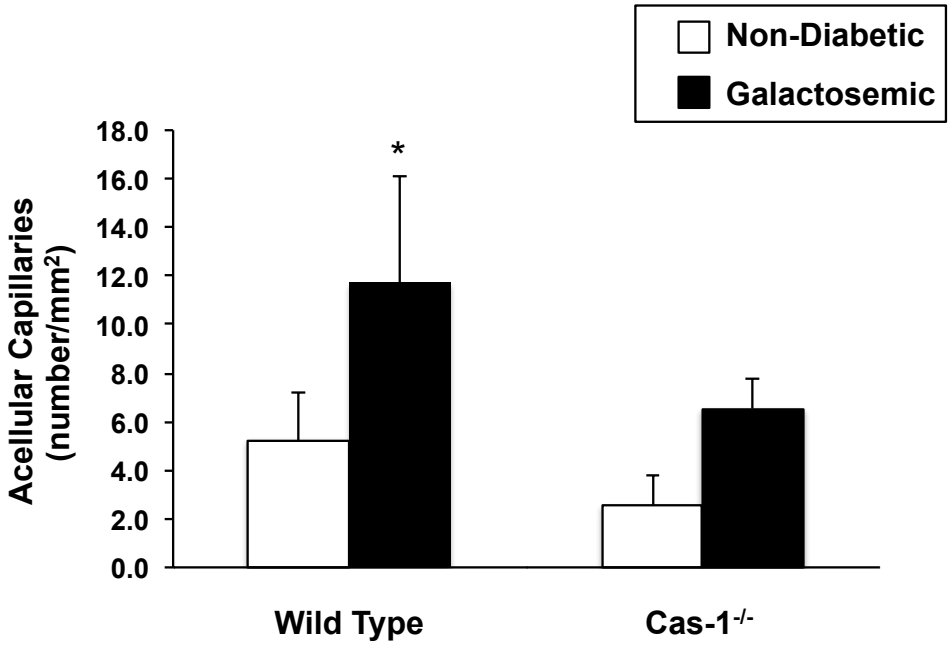


Figure 21. Acellular capillary formation in non-diabetic and galactosemic mice.

Retinas of non-diabetic (n=6) and galactosemic (n=10) wild type mice, and non-diabetic (n=6) and galactosemic (n=6) *casp1*$^{-/-}$ mice were isolated at 7 months of diabetes. Number of acellular capillaries was determined and expressed as mean ± SDEV.



4.4 Conclusion

Previous studies have shown that the galactosemia model induces diabetic-like retinopathy with similar retinal pathologies to experimental diabetes *in vivo*. Relatively few studies have been performed examining the effects of high galactose at the cellular level. It is known that diabetes leads to increased oxidative stress and inflammation, and that one cellular source of this is Müller cells. Studies have linked increased oxidative stress and inflammation in high glucose to increases in TXNIP. Thioredoxin, along with glutathione and catalase, represents one of the three primary antioxidant pathways used to eliminate ROS from the environment(267). TXNIP functions as negative regulator of thioredoxin and inhibits it from scavenging ROS, leading to ROS accumulation. In the present study, we compared the effects of high glucose and high galactose on Müller cells specifically on oxidative stress, TXNIP regulation, caspase-1 activity and Müller cell death.

First, reports indicate that high glucose leads to increased caspase-1 activation and IL-1β production in Müller cells(62,88,137). We observed that there was increased caspase-1 activity in rMC-1 cells treated with either high galactose or high glucose. This indicates that some of the retinal pathologies observed in galactosemic mice, such as increased inflammation may be due to increased caspase-1 activity and subsequent IL-1β production by Müller cells.

Next, we examined differences in ROS production in rMC-1 under high glucose or high galactose conditions. Studies indicate that there are abnormalities in retinal metabolism and oxidative stress in galactosemic mice, however there are significant differences in retinal metabolism and antioxidant response between

diabetic and galactosemic mice. We observed there is significantly increased ROS production in rMC-1 beginning at 36 hours of high glucose treatment and that it increases progressively at all time points to 96 hours. Interestingly, there were no significant difference in ROS production in rMC-1 treated with high galactose at any time point between 24 and 96 hours. This indicates that caspase-1 activity may actually be independent of ROS production in galactosemic conditions. Furthermore, under high glucose conditions, increases in caspase-1 activity are detectable as early as 24 hours, which is prior to increased ROS production, indicating that caspase-1 activity may be independent of ROS production under high glucose conditions as well, at least initially.

Since increased caspase-1 activity and oxidative stress have both been linked to increases in TXNIP expression we determined if there are similar changes in TXNIP protein levels. Interestingly, when comparing TXNIP protein levels in either high glucose to high galactose we observed similar differences between the two models to what was observed in ROS production. There is increased TXNIP protein levels in rMC-1 under high glucose conditions and it is observed at all time points between 24 and 96 hours. Consistent with TXNIP's function as a negative regulator of thioredoxin, TXNIP protein levels increase just before the accumulation of ROS in rMC-1 cells. There was no observed increase in TXNIP levels in the high galactose samples. Interestingly, in western blot images analysis we observed two bands, one at 55 kDa molecular weight corresponding to TXNIP, and another near 95 kDa. In the high glucose treated samples, as there was increased intensity of the lower band for TXNIP, there was a decrease in the upper band as if it was a bound form that was

decreasing in high glucose conditions. This change in the upper band was not observed and remained consistent in high galactose treated samples.

Previously, we have found that inhibition of caspase-1 prevents Müller cell death in rMC-1 treated with high glucose(88). Numerous studies in other systems have also shown that caspase-1 activity contributes to pyroptotic cell death(268–270). Interestingly, we observed that just as there is increased caspase-1 activity in rMC-1 treated with high galactose, there is also increased cell death after prolonged exposure. Inhibition of caspase-1 with a caspase-1 inhibitor (YVAD-fmk) prevented this cell death. The importance of caspase-1 to the progression of retinopathy in galactosemic mice was illustrated in cas-1-/- mice fed a galactose enriched diet. Retinopathy progression was prevented in these animals indicating that the caspase-1 pathway is activated not only in the STZ diabetic model as reported previously, but also in the galactosemic model of retinopathy.

Collectively this indicates that there are many similarities and differences in rMC-1 treated with either high glucose or high galactose. While both treatments led to significantly increased inflammation as observed by increased caspase-1 activity, only high glucose led to increased TXNIP levels and ROS accumulation. This indicates that caspase-1 may be activated independently of oxidative stress in rMC-1. Furthermore, both models lead to a similar eventual out come of caspase-1-dependent cell death.

Chapter 5. The Role of Interleukin-1α in Caspase-1 Activation and Cell Death in Müller Cells

5.1 Introduction

Previously we have demonstrated that IL-1β plays a crucial role in sustained caspase-1 activity and cell death in Müller cells exposed to high glucose conditions and also in the progression of diabetic retinopathy. Therefore, it was important to examine the role of another IL-1 family member, IL-1α, in these events.

There are a number similarities and differences between IL-1α and IL-1β. In terms of similarities, IL-1α and IL-1β are both synthesized as a 31 kDa precursor (pro-form) that can be cleaved into 17 kDa mature forms. Additionally, both of these cytokines function as ligands of IL-1R1 and exert similar inflammatory responses(271).

There are many differences between IL-1α and IL-1β however, for example they have unique amino acid sequences and have very different bioavailability(99,272). Whereas IL-1β is only synthesized and secreted after a cell encounters harmful stimuli, IL-1α is constitutively expressed in many cell types even under normal conditions(273). Furthermore, IL-1β is a secreted cytokine, whereas IL-1α can be secreted or membrane bound(274–278). Another key difference is that unlike IL-1β that is cleaved by caspase-1 and requires this processing to be biologically active, IL-1α is cleaved instead by calpain, however it does not require cleavage in order to be active(271). Both the precursor and cleaved

forms of IL-1α are agonists of IL-1R1 and produce identical biological responses(271,279).

In addition to its functions as a proinflammatory cytokine, IL-1α is unique in that it has a nuclear localization signal (NLS) on its N-terminal pro-piece (NTP)(280,281). This allows either the uncleaved pro-IL-1α or the cleaved IL-1α-NTP to translocate to the nucleus. The exact function of IL-1α in the nucleus is largely unknown, however studies indicate that it can bind to the chromatin and directly regulate transcription of proinflammatory genes(282–284).

5.2 Materials and Methods

Tissue Culture. Human retinal Müller cells (hMC): Handling of human tissue conformed to the tenets of Declaration of Helsinki for research involving human tissue. Human Müller cells were isolated from retinal tissue of healthy donors with no history of diabetes and chronic inflammatory diseases as previously described(127,128).

Treatment: hMC ($1x10^6$) were treated with either 5 mmol/L glucose DMEM or 25 mmol/L glucose DMEM supplemented with 2% FBS, 1% P/S for either 48 or 96 hours. Cells treated with 5 mmol/L glucose medium served as controls. For IL-1β studies, recombinant human IL-1β (1-5ng/ml) was used. For IL-1ra studies, following pretreatment with 100ng/ml IL-1ra for 1 hour, hMC were incubated in 25 mmol/L glucose DMEM for 48 or 96 hours, or in 5 mmol/L glucose DMEM plus recombinant human IL-1β (2ng/ml) for 24 hours.

Caspase-1 Activity Assay. Caspase-1 activities were measured as described previously (62,117,126–129). Briefly, equal amounts of sample protein (15 μg) were incubated in the presence of the specific caspase-1 substrate (YVAD-AFC; 2.5μmol/L) for 1 hour at 32°C. AFC fluorescence was detected by a Tecan Spectra FluorPlus fluorescence plate reader (excitation: 400 nm, emission: 510 nm). Release of AFC by active caspase-1 was calculated against an AFC standard curve and expressed as pmol AFC/mg protein/min.

Müller Cell Death In vitro: Following treatment, cells were suspended and 100μL of cell suspension was mixed with 100μL of trypan blue solution. Cell death was quantified using hemocytometer.

Immunofluorescence. Cells were plated on cover slips in a 6 well plate with 35k cells/well. After treatment cells were washed, permeabilized and stained for IL-1α using anti-IL-1α antibody (Abcam, ab9875), and nuclei was stained for DAPI. Following staining, samples were imaged using confocal microscope.

Statistical Analysis. Analysis of data was performed using the Anova One-Way (correlated samples, $p<0.05$) test followed by Tukey's post analysis or Kruskal-Wallis test (ordinal data; $p<0.05$) followed by Dunn's post analysis to determine statistical significance among groups. For details in statistical analysis see VasserStats Statistical Computation Web Site. All data are presented as mean ± SDEV.

5.3 Results

In order to examine determine if IL-1α plays a role in high glucose-induced caspase-1 activity in Müller cells, we first treated hMCs with either normal or high glucose for 96 hours and used an anti-IL-1α antibody to block the effects of IL-1α. We observed that treatment with anti-IL-1α prevented high glucose induced caspase-1 activity at 96 hours, similar to what we previously observed using an IL-1 receptor antagonist (Figure 22). To determine whether this blockage of caspase-1 activity prevented subsequent Müller cell death we measured cell death in the aforementioned experiment. Interestingly, we saw that anti-IL-1α also prevented high glucose induced Müller cell death (Figure 23).

Next, since IL-1α has a nuclear localization sequence we determined whether there could be any nuclear translocation in Müller cells. Here we treated hMCs with normal or high glucose and used immunofluorescence to examine cellular localization (Figure 24A). Interestingly, we observed an increase in the cells positive for nuclear IL-1α after 96 hours of high glucose treatment (Figure 24B).

Figure 22. IL-1α blockage decreases caspase-1 activity in Müller Cells Under High Glucose Conditions.

Caspase-1 activity after treatment with 5mmol/l or 25 mmol/l glucose in the presence or absence of anti-IL-1α. N=5, $p<0.05$.

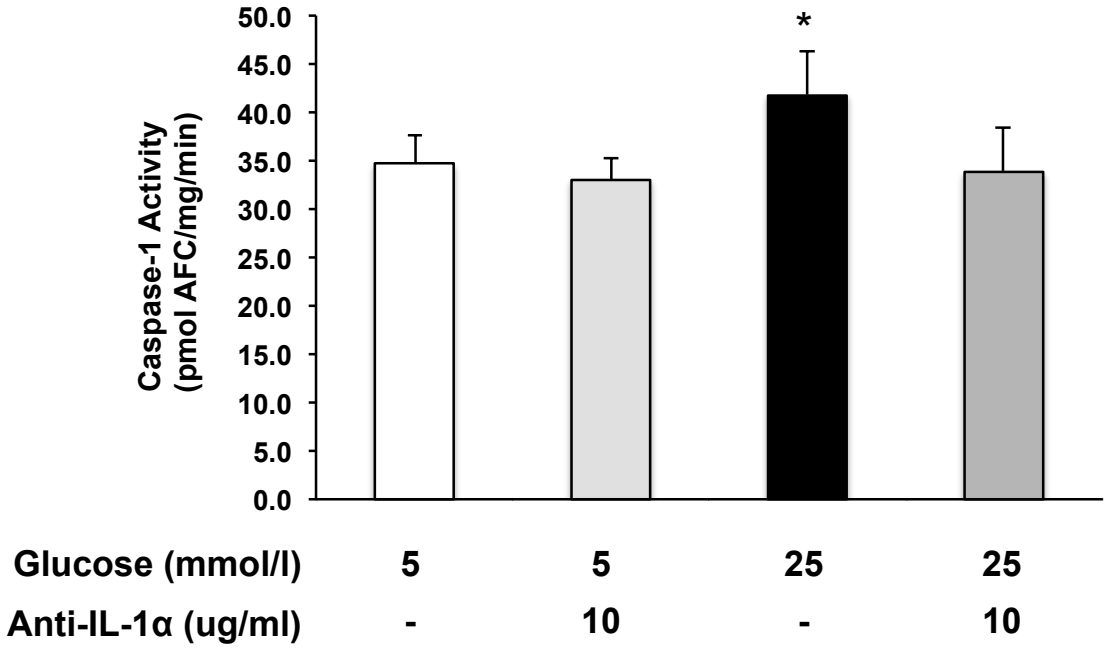


Figure 23. High glucose-induced Müller cell death inhibited by anti-IL-1α.

Müller cell death measured by Trypan Blue exclusion after treatment with 5mmol/l or 25 mmol/l glucose in the presence or absence of anti-IL-1α. N=5, $p<0.05$.

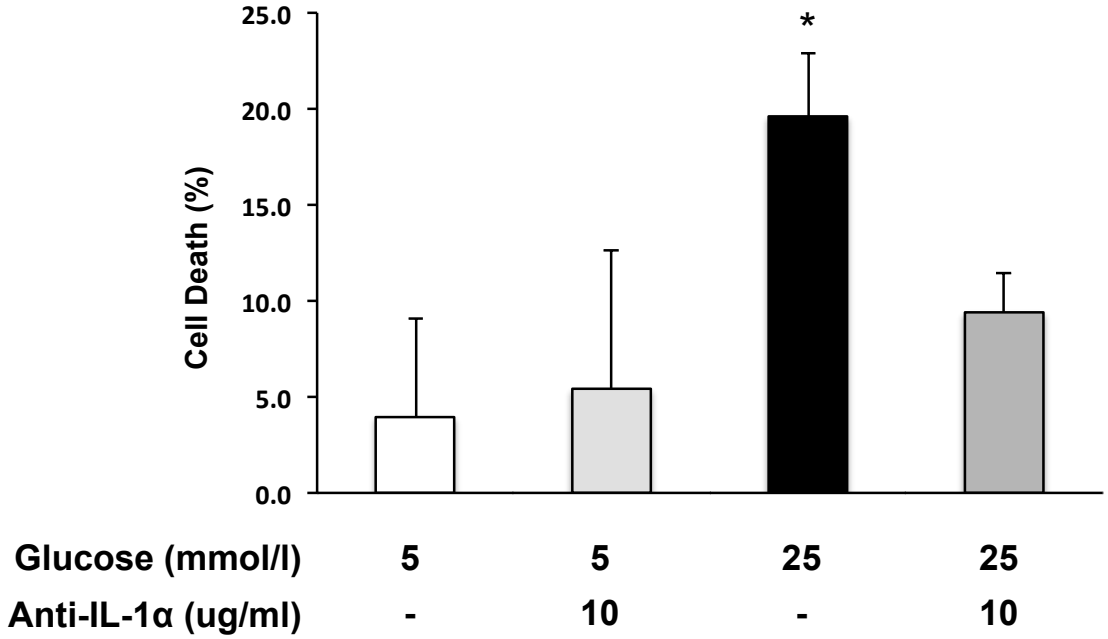
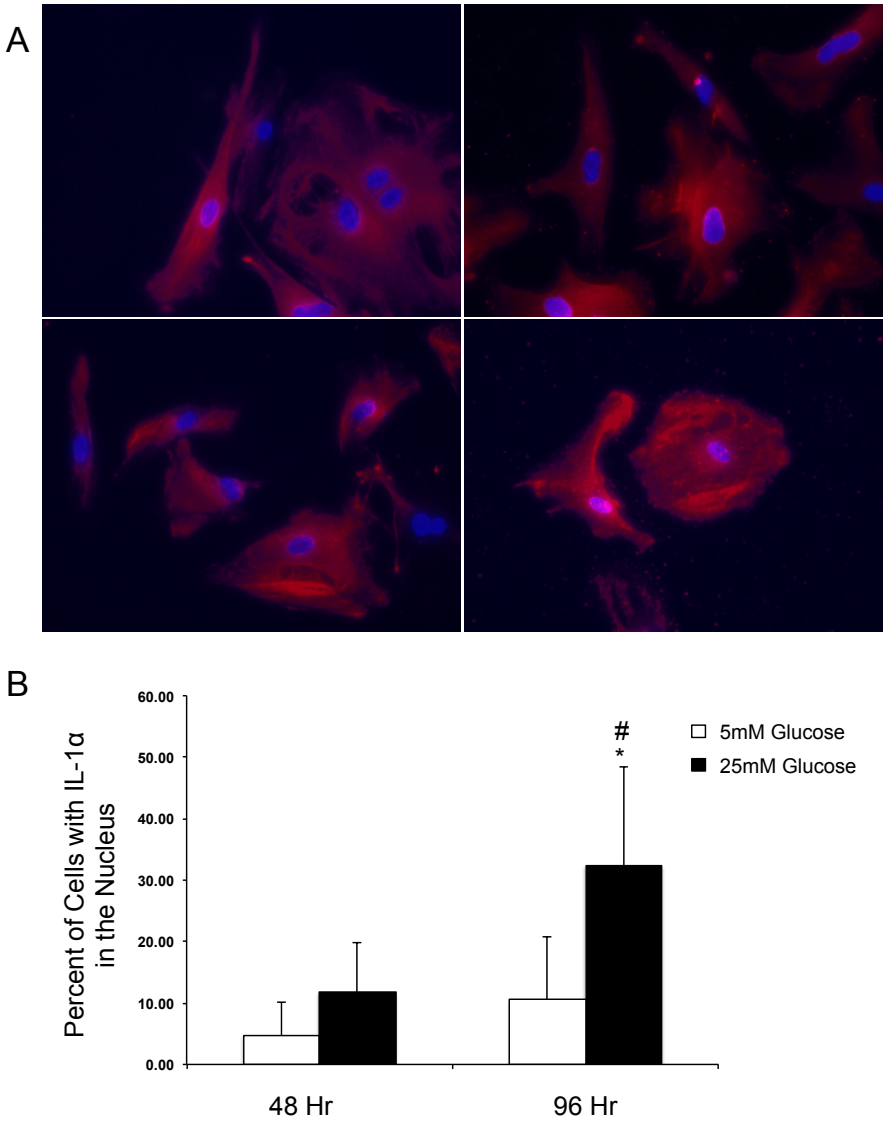


Figure 24. Nuclear Accumulation of IL-1α In Müller Cells Under High Glucose Conditions.

(A) Immunofluroescence staining of Müller cells for IL-1α (red) or DAPI (blue). Cells were treated for 48 hours- 5mM glucose (upper left), 48 hours- 25mM glucose (upper right), 96 hours- 5mmol/l glucose (lower left), or 96 hours- 25 mmol/l glucose (lower right). (B) Cells positive for nuclear IL-1α were counted and expressed as mean ± SDEV. n=5, $p<0.05$.



5.4 Conclusion

In previous chapters, we examined the caspase-1/IL-1β/IL-1R1 pathway and its role in sustained caspase-1 activation in Müller cells under high glucose conditions. In this study we examined another IL-1 family member, IL-1α, and its potential role caspase-1 activation. Interestingly, we found that blockage of IL-1α prevents sustained caspase-1 activity and subsequent cell death, similar to treatment with an IL-1 receptor antagonist. Collectively, this indicates that IL-1α may act in combination with IL-1β in driving caspase-1 activation through activation of IL-1R1. Furthermore, we have shown that high glucose induces nuclear translocation of IL-1α. It remains to be determined the exact role of IL-1α in the nucleus of Müller cells under these conditions, however it appears to be localized at focal points, consistent with previous reports of IL-1α binding to specific locations on the nuclear chromatin(283).

Chapter 6. Summary and Future Outlook

6.1 Summary of data regarding caspase-1/IL-1β feedback signaling in the retina of diabetic mice

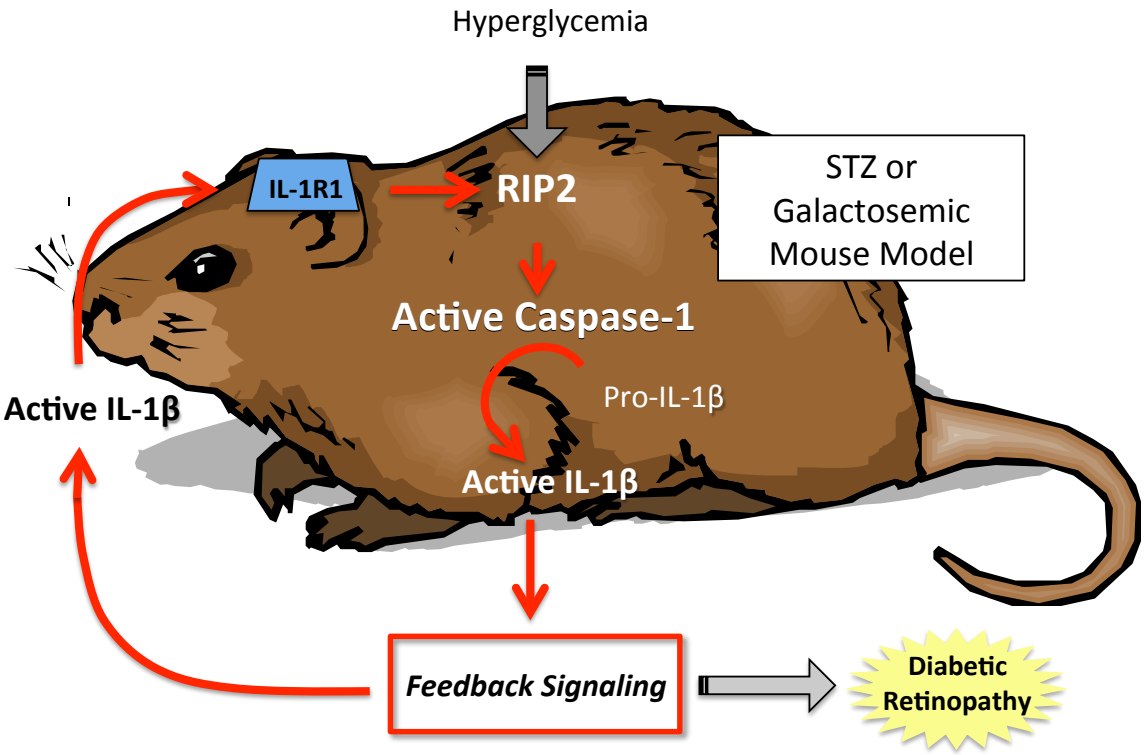
The importance of inflammation in the progression of diabetic retinopathy has become increasingly apparent. A variety of pro-inflammatory cytokines have been identified in the vitreous of patients compared to healthy individuals, among them IL-1β. Our previous studies have shown that caspase-1 activity is consistently increased in the retinas of diabetic animals and patients. New data from this thesis project provide novel mechanistic insights into the process of prolonged caspase-1 activation and IL-β production in diabetic retinopathy. Our results indicate:

- Using caspase-$1^{-/-}$ mice we demonstrated that caspase-1 activation is indeed crucial for the development of diabetic retinopathy.
- Furthermore, using IL-$1R1^{-/-}$ mice we identified that diabetes-induced caspase-1 activity progresses from an IL-1R1 independent mechanism to an IL-1R1 dependent mechanism throughout disease progression.
- These data provide for the first time an indication of a caspase-1/IL-1β/IL-1R1 feedback signaling mechanism that keeps caspase-1 active in the diabetic retina.
- Using Müller cells known to produce active caspase-1 and IL-1β under hyperglycemic conditions we confirmed that prolonged exposure to hyperglycemia leads to caspase-1/IL-1β/IL-1R1 feedback signaling causing sustained caspase-1 activity.

- Hyperglycemia increased RIP2 protein levels in Müller cells after 24 hours. Most importantly, siRNA against RIP2 decreases caspase-1 activation and subsequent IL-1β secretion under hyperglycemic conditions.
- Consistent with a decrease in caspase-1 activation and IL-1β secretion, siRNA against RIP2 prevented hyperglycemia-induced Müller cell death after 96 hours of hyperglycemia exposure.
- Interestingly, IL-1β also increased RIP2 protein levels. siRNA against RIP2 also attenuates caspase-1 activity induced by exogenous IL-1β indicating that RIP2 is not only activating caspase-1 under hyperglycemic conditions, but also by IL-1β feedback signaling.
- Diabetic and galactosemic caspase-1-/- mice were protected from Müller cell loss and the formation of acellular capillaries, confirming that caspase-1 activation plays a significant role in Müller cell loss *in vivo* and is crucial for disease progression.

Future outlook: Future studies are requires to determine whether interfering in caspase-1 activation by targeting RIP2 represents a potential therapeutic target for treatment of diabetic retinopathy. In addition, it would be valuable to test drugs that target caspase-1 or IL-1β directly which are already on the market for other diseases in the context of diabetic retinopathy. This would increase possibilities of drug choices dramatically.

Figure 25: Schematic outlining the caspase-1/IL-1β feedback signaling the diabetic mouse.



6.2 Summary of data regarding the role of IL-1α in hyperglycemia-induced caspase-1 activation in Müller cells

Hyperglycemia leads to sustained activation of caspase-1 and IL-1β production in the retinas of diabetic animals and patients *in vivo* and in Müller cells *in vitro.* Interference in this signaling by knockout of caspase-1 or the IL-1R1 prevents diabetic retinopathy indicating that the IL-1 pathway is important for the development of the disease. The IL-1 family is comprised of multiple members including IL-1α. This study was focused on identifying the role of IL-1α in hyperglycemia-induced chronic inflammation in human Müller cells. Data show that:

- Hyperglycemia led to a decrease in cytosolic IL-1α in Müller cells at 24 hours.
- Treatment with anti-IL-1α inhibited hyperglycemia-induced chronic caspase-1 activity and subsequent cell death in Müller cells suggesting that IL-1α plays a role in caspase-1/IL-1β inflammatory feedback signaling.
- Cytosolic localization of IL-1α became increasingly localized to the cytoskeleton during high glucose treatment compared to control.
- There was a significant increase in nuclear IL-1α at 96 hours indicating that IL-1α might function on a transcriptional level to sustain inflammation at prolonged high glucose exposure.
- Nuclear IL-1α appeared punctated rather than diffusely spread throughout the nucleus.

Future outlook: This study has shown that IL-1α seems to participate in hyperglycemia-induced chronic caspase-1/IL-1β/IL-1R1-mediated inflammation.

Further studies are necessary to identify the role of IL-1α in the development of diabetic retinopathy and whether IL-1α can be targeted for drug development. Furthermore, the role of IL-1α in the nucleus remains to be examined.

6.3 Implications of the data collected during this thesis project to the field of Müller cells and diabetic retinopathy

Hyperglycemia promotes release of (1) growth factors, such as vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF), and (2) cytokines and chemokines including interleukin-1β (IL-β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and chemokine ligand-2 (CCL2)(54–60,285) (61–63) from Müller cells. *In vitro* studies have provided ample evidence that Müller cells are a potential source for growth factors and cytokines when stimulated with elevated glucose levels. Considering that most of the growth factors, cytokines, and chemokines released by Müller cells have been identified in the vitreous of diabetic patients it is fair to assume that Müller cells contribute to the overall synthesis of these factors *in vivo*(231,286–288).

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

conditional VEGF knockout (KO) mice. The diabetic conditional VEGF KO mice exhibited an overall decrease in parameters associated with the pathology of diabetic retinopathy such as leukostasis, expression of inflammatory biomarkers, depletion of tight junction proteins, numbers of acellular capillaries, and vascular leakage compared to diabetic control mice(59,289,290). Additional studies focusing on altering known regulators of VEGF production such as HIF-1 (hypoxia inducible factor 1)(291) and the Wnt signaling pathway(292) specifically in Müller cells have supported the notion that Müller cell derived VEGF is actually a major component in the process of retinal angiogenesis and pathology in diabetic retinopathy. Besides VEGF, Müller cell derived PEDF has also been suggested to have its part in diabetes-induced retinal angiogenesis(42). Taken together, it seems that Müller cell derived growth factors contribute heavily to pathological vascular events in diabetic retinopathy.

Although Müller cell derived VEGF contributes to detrimental effects on the microvasculature in the diabetic retina, the intent of such growth factor production by Müller cells in the first place might have been to protect itself and the retinal neurons from a diabetic insult. This idea is supported by a study using mice that carry a disrupted VEGFR2 specifically in Müller cells. Loss of VEGFR2 caused a gradual reduction in Müller glial density, decreased of scotopic and photopic electroretinography amplitudes, and accelerated loss of photoreceptors, ganglion cells, and inner nuclear layer neurons in the diabetic retina(293). More studies are needed to fully explore and understand the beneficial effects of Müller cell derived growth factors on Müller cells itself and retinal neurons in the context of disease.

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

Besides growth factors, Müller cells release a variety of cytokines and chemokines under hyperglycemic conditions. For example, Müller cells are a major source of retinal interleukin-1beta (IL-1β) production as discussed in this dissertation(62,126,128,294,295). Caspase-1, originally named interleukin-1β converting enzyme (ICE), produces the active cytokines IL-1β and IL-18 by cleavage of their inactive proform(259,260,296,297). In Müller cells, hyperglycemia strongly induces the activation of the caspase-1/IL-1β signaling pathway as we have previously shown(62,128). Increased caspase-1 activation and elevated IL-1β levels have also been identified in the retinas of diabetic mice and retinal tissue and vitreous fluid of diabetic patients(62,113,134,295,298). We have identified that targeting this pathway by knocking down caspase-1 or the IL-1 receptor (IL-1R1) or by pharmacological intervention protects against the development of diabetic retinopathy in diabetic rats and mice(126,135). Prolonged IL-1β production by Müller cells has been shown to affect endothelial cell viability in a paracrine fashion(295). Endothelial cells are extremely susceptible to IL-1β and rapidly progress to cell death in response to this pro-inflammatory cytokine(295). Endothelial cell death is detectable in the retinal microvasculature of diabetic animals and isolated retinal blood vessels of diabetic donors and has been associated with the formation of acellular capillaries, a hallmark of retinal pathology in diabetic retinopathy(156). Besides IL-1β, Müller cells produce other well-known

pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6)(126,128,135,140,162,299,300). Anti-TNFα therapy has been proposed as a strategy to treat diabetic retinopathy in diabetic animals(111,112,115,189). Detrimental effects of IL-6 have been associated with vascular dysfunction and promotion of angiogenesis(301–303) which is why IL-6 recently has become a new therapeutical target of interest to prevent diabetes-induced vascular damage. The production and release of pro-inflammatory cytokines by Müller cells strongly contributes to the chronic inflammatory environment detected in the diabetic retina that over time promotes drop-out of a retinal cells.

From a vascular perspective, other cytokines such as IL-6 has been solely associated with detrimental effects(301–303). However, we have previously shown that IL-6 prevents hyperglycemia-induced Müller cell dysfunction and loss clearly supporting a beneficial and protective nature of IL-6(128). This observation is well in line with reports that in the retina IL-6 is an important cytokine responsible for maintaining proper neuronal function as well as stimulating neuroprotective effects(128,304–307). Treatment with IL-6 has been shown to protect retinal ganglion cells from pressure-induced cell death(304). Additionally, in an experimental model of retinal detachment, genetic ablation or neutralization of IL-6 led to a significant increase in photoreceptor cell death. However, treatment with exogenous IL-6 resulted in a significant increase in photoreceptor density in the outer nuclear layer(307). These different effects of IL-6 can potentially be attributed to the two distinct signaling pathways IL-6 acts through. Classical IL-6 signaling -

thought to be the anti-inflammatory and protective pathway - is mediated by the membrane-bound form of the IL-6 receptor (IL-6R) and the ubiquitously expressed glycoprotein 130 (gp130). Only cells such as Müller cells (but not endothelial cells) that express IL-6R are able to signal through classical IL-6 signaling. Conversely, IL-6 trans-signaling, which is mediated by binding of IL-6 to the soluble form of the IL-6 receptor (sIL-6R) and gp130, is thought to be the more pro-inflammatory and pro-angiogenic pathway(128,302,305,308–318). In diabetic patients, correlations between increased levels of IL-6 and the development of complications in the eye have been made(319–324). However, whether IL-6 levels are increased in diabetes as an attempt to protect from a pro-inflammatory environment or whether high levels of IL-6 synergistically exaggerate diabetes-induced inflammation has yet to be determined.

Whether Müller cells die in diabetic retinopathy has long been a matter of debate. It is easy to see that Müller cells are “sturdy” cells taking into account how well equipped these cells are to produce fair amounts of protective factors that shield them at least in the beginning from a chronic diabetic insult as discussed above. However, newer studies indicate that over time Müller cells actually do begin to die the longer diabetic retinopathy progresses. Frequency of Müller cell death in the diabetic retina rapidly accelerates when protective growth factors are blunted(293).

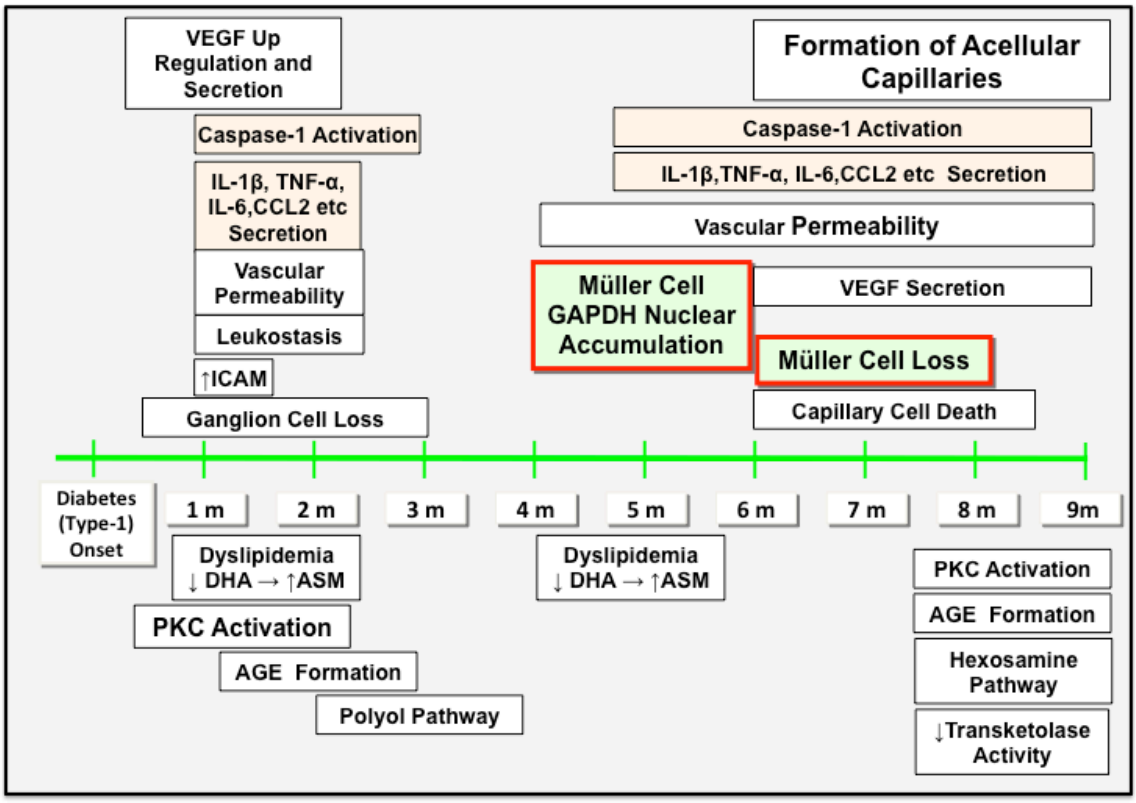
Better understanding of types of cell deaths has furthered studies to look for mechanisms other than apoptosis by which Müller cells can die in a diabetic environment. In this dissertation (Chapter 3) we have identified one particular

mechanism of cell death that stands out and can explain histological features described for Müller cells in the diabetic retina. Pyroptosis is an inherently inflammatory mediated mechanism of cell death, defined as being caspase-1-dependent(73,142,220). Müller cells show increased caspase-1 activity and IL-1β production following exposure to hyperglycemic conditions and cells die as a consequence(116,232). Although it is now very well established that initiation of pyroptosis is caspase-1 and IL-1β driven, the execution phase of pyroptosis is not yet completely understood. It has been shown that pyroptosis shares traits with both apoptosis and necrosis in the execution phase(141,227). Since execution of pyropototic cell death lacks specific marker, identifying retinal cells dying by pyroptosis *in vivo* is a difficult task. Markers such as TUNEL staining used to detect apoptotic cell death may not adequately detect pyroptosis. Therefore, we have performed a study actually counting Müller cells in the healthy and diabetic retina and determined roughly 15% cell death at 7 months of diabetes(140). Even more important, inhibition of the caspase-1/IL-1β pathway inhibited diabetes-induced Müller cell death *in vivo* as we had previously shown *in vitro*(126,128,140). Several other studies are in line with our observation that Müller cells die in a hyperglycemic environment. The first study to describe dying Müller cells in diabetic retinopathy was done using EM analysis(138). Dying Müller cells are described as being hypertrophic consistent with the notion that during pyroptosis, cells swell rather than shrink as observed in apoptotic cell death(53). To collect more evidence for Müller cells death in the diabetic retina we looked at earlier markers of cell death and we have identified that GAPDH (glyceraldehyde-3-

phosphate dehydrogenase) accumulates in the nucleus of Müller cells in the retinas of diabetic rats(163). Nuclear accumulation of GAPDH has been closely associated with cell death induction(233–235,325). Consistent with our finding that Müller cells die by pyroptotic cell death, hyperglycemia-induced nuclear accumulation of GAPDH depends on the activation of the caspase-1/IL-1β pathway(54,139). The consequences of dying Müller cells are multi faceted. On the bad side – Müller cell death will promote loss of retinal blood barrier integrity, increased vascular permeability, and loss of neuroprotection affecting both neurons and vascular cells. Loss of Müller cells in diabetes has also been associated with aneurysm formation, a clinical characteristic of diabetic retinopathy(138). However, one can also argue that on the good side – removal of activated and pro-inflammatory Müller cells might be a "shut off" mechanism to deal with an increasing inflammatory environment in the diabetic retina. A lot more studies are needed to determine the full pathway of Müller cells death and to identify whether all Müller cells are equally affected by hyperglycemia.

Figure 26: Timeline of Diabetic Retinopathy In Mice and Rats.

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



6.4 Conclusion

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

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

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

pathway(326,327). Whether these studies materialize into effective therapy strategies has to be seen in the future.

Chapter 7. Internship Experience at Hoffmann-La Roche

I spent the final year of the graduate program working at Hoffmann La-Roche at their campus in Basel, Switzerland. I was accepted for a one year internship to the Roche internship for Scientific Exchange (RiSE) program and my appointment was in the ophthalmology department of pharmaceutical Research and Early Development (pRED). The RiSE program is designed to give graduate students an opportunity to work at a pharmaceutical company with the goal of learning new techniques and learn what it is like to work in industry.

The ophthalmology department at Hoffmann La-Roche focuses on translational research with a goal of identifying new therapeutic targets in a number of vision related diseases. My experience in the field of diabetic retinopathy and inflammation was extremely useful and allowed me to be a valuable member to the research team. I was given my own research project and it allowed me to lead a project and design the experiments necessary to complete the project. Additionally, I worked with many of the other team members and it allowed me to collaborate with a team on portfolio related projects. During this work I learned many new techniques and how to incorporate them into research projects. One of the new techniques that I have used in the internship is RNA sequencing and how to analyze the data obtained. This technique has been useful in helping to identify new targets for potential therapies in a number of ocular diseases. In addition to RNA sequencing, I have also become more familiar with confocal and two photon microscopy and luminex magnetic bead cytokine assays. I have learned to use a

number of new devices including the Roche MagnaPure for RNA extraction and purification, Heidelberg for Optical Coherence Tomography (OCT), and the Phoenix Micron IV for fundus imaging and laser photocoagulation. Furthermore, I have increased my skills *in vitro* by continuing to work with the STZ diabetic mouse model and have begun to work on the Choroidal Neovascularization (CNV) mouse model.

Collectively, the experience of the RiSE internship was extremely valuable and further confirmed that I would like to pursue a career at a pharmaceutical company.

REFERENCES

REFERENCES

1. Klein R, Knudtson MD, Lee KE, Gangnon R, Klein BEK. The Wisconsin Epidemiologic Study of Diabetic Retinopathy: XXII the twenty-five-year progression of retinopathy in persons with type 1 diabetes. Ophthalmology. 2008 Nov;115(11):1859–68.

2. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care. 2010 Jan;33(Suppl 1):S62–9.

3. Antonetti DA, Klein R, Gardner TW. Diabetic Retinopathy. New England Journal of Medicine. 2012;366(13):1227–39.

4. Bek T. Clinical Presentations and Pathological Correlates of Retinopathy [Internet]. Vol. 20. Karger Publishers; 2009 [cited 2017 Jan 22]. Available from: http://www.karger.com/Article/Abstract/262659

5. Barber AJ, Antonetti DA, Gardner TW. Altered Expression of Retinal Occludin and Glial Fibrillary Acidic Protein in Experimental Diabetes. IOVS. 2000 Oct 1;41(11):3561–8.

6. Antonetti DA, Barber AJ, Khin S, Lieth E, Tarbell JM, Gardner TW. Vascular permeability in experimental diabetes is associated with reduced endothelial occludin content: vascular endothelial growth factor decreases occludin in retinal endothelial cells. Penn State Retina Research Group. Diabetes. 1998 Dec;47(12):1953–9.

7. Stamer WD, Bok D, Hu J, Jaffe GJ, McKay BS. Aquaporin-1 channels in human retinal pigment epithelium: role in transepithelial water movement. Invest Ophthalmol Vis Sci. 2003 Jun;44(6):2803–8.

8. Iandiev I, Pannicke T, Reichel MB, Wiedemann P, Reichenbach A, Bringmann A. Expression of aquaporin-1 immunoreactivity by photoreceptor cells in the mouse retina. Neurosci Lett. 2005 Nov 11;388(2):96–9.

9. Bringmann A, Reichenbach A, Wiedemann P. Pathomechanisms of cystoid macular edema. Ophthalmic Res. 2004 Oct;36(5):241–9.

10. Nagelhus EA, Horio Y, Inanobe A, Fujita A, Haug FM, Nielsen S, et al. Immunogold evidence suggests that coupling of K+ siphoning and water transport in rat retinal Müller cells is mediated by a coenrichment of Kir4.1 and AQP4 in specific membrane domains. Glia. 1999 Mar;26(1):47–54.

11. Nagelhus EA, Veruki ML, Torp R, Haug FM, Laake JH, Nielsen S, et al. Aquaporin-4 water channel protein in the rat retina and optic nerve: polarized expression in Müller cells and fibrous astrocytes. J Neurosci. 1998 Apr 1;18(7):2506–19.

12. Patil RV, Saito I, Yang X, Wax MB. Expression of aquaporins in the rat ocular tissue. Exp Eye Res. 1997 Feb;64(2):203–9.

13. Reichenbach A, Wurm A, Pannicke T, Iandiev I, Wiedemann P, Bringmann A. Müller cells as players in retinal degeneration and edema. Graefes Arch Clin Exp Ophthalmol. 2007 May;245(5):627–36.

14. Pannicke T, Iandiev I, Uckermann O, Biedermann B, Kutzera F, Wiedemann P, et al. A potassium channel-linked mechanism of glial cell swelling in the postischemic retina. Mol Cell Neurosci. 2004 Aug;26(4):493–502.

15. Pannicke T, Iandiev I, Wurm A, Uckermann O, vom Hagen F, Reichenbach A, et al. Diabetes alters osmotic swelling characteristics and membrane conductance of glial cells in rat retina. Diabetes. 2006 Mar;55(3):633–9.

16. Fine BS, Zimmerman LE. Muller's cells and the "middle limiting membrane" of the human retina. An electron microscopic study. Invest Ophthalmol. 1962 Jun;1:304–26.

17. Spitznas M. Current Research in Ophthalmic Electron Microscopy. Springer Science & Business Media; 2012. 265 p.

18. Matsui K, Hosoi N, Tachibana M. Active Role of Glutamate Uptake in the Synaptic Transmission from Retinal Nonspiking Neurons. J Neurosci. 1999 Aug 15;19(16):6755–66.

19. Derouiche A, Rauen T. Coincidence of L-glutamate/L-aspartate transporter (GLAST) and glutamine synthetase (GS) immunoreactions in retinal glia: Evidence for coupling of GLAST and GS in transmitter clearance. J Neurosci Res. 1995 Sep 1;42(1):131–43.

20. Barbour B, Brew H, Attwell D. Electrogenic glutamate uptake in glial cells is activated by intracellular potassium. Nature. 1988 Sep 29;335(6189):433–5.

21. Biedermann B, Bringmann A, Reichenbach A. High-affinity GABA uptake in retinal glial (Müller) cells of the guinea pig: Electrophysiological characterization, immunohistochemical localization, and modeling of efficiency. Glia. 2002 Sep 1;39(3):217–28.

22. Harada T, Harada C, Watanabe M, Inoue Y, Sakagawa T, Nakayama N, et al. Functions of the two glutamate transporters GLAST and GLT-1 in the retina. Proc Natl Acad Sci U S A. 1998 Apr 14;95(8):4663–6.

23. Rauen T, Taylor WR, Kuhlbrodt K, Wiessner M. High-affinity glutamate transporters in the rat retina: a major role of the glial glutamate transporter GLAST-1 in transmitter clearance. Cell Tissue Res. 1998 Jan;291(1):19–31.

24. Poitry S, Poitry-Yamate C, Ueberfeld J, MacLeish PR, Tsacopoulos M. Mechanisms of Glutamate Metabolic Signaling in Retinal Glial (Müller) Cells. J Neurosci. 2000 Mar 1;20(5):1809–21.

25. Orkand RK, Nicholls JG, Kuffler SW. Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia. J Neurophysiol. 1966 Jul;29(4):788–806.

26. Karwoski CJ, Lu H-K, Newman EA. Spatial Buffering of Light-Evoked Potassium Increases by Retinal Müller (Glial) Cells. Science. 1989 May 5;244(4904):578–80.

27. Mata NL, Radu RA, Clemmons RC, Travis GH. Isomerization and oxidation of vitamin a in cone-dominant retinas: a novel pathway for visual-pigment regeneration in daylight. Neuron. 2002 Sep 26;36(1):69–80.

28. Trevino SG, Villazana-Espinoza ET, Muniz A, Tsin ATC. Retinoid cycles in the cone-dominated chicken retina. J Exp Biol. 2005 Nov;208(Pt 21):4151–7.

29. Kanan Y, Kasus-Jacobi A, Moiseyev G, Sawyer K, Ma J-X, Al-Ubaidi MR. Retinoid processing in cone and Müller cell lines. Exp Eye Res. 2008 Feb;86(2):344–54.

30. Edwards RB, Adler AJ, Dev S, Claycomb RC. Synthesis of retinoic acid from retinol by cultured rabbit Müller cells. Exp Eye Res. 1992 Apr;54(4):481–90.

31. Palczewski K, Jäger S, Buczyłko J, Crouch RK, Bredberg DL, Hofmann KP, et al. Rod outer segment retinol dehydrogenase: substrate specificity and role in phototransduction. Biochemistry. 1994 Nov 22;33(46):13741–50.

32. Mata NL, Ruiz A, Radu RA, Bui TV, Travis GH. Chicken retinas contain a retinoid isomerase activity that catalyzes the direct conversion of all-trans-retinol to 11-cis-retinol. Biochemistry. 2005 Sep 6;44(35):11715–21.

33. Winkler BS, Arnold MJ, Brassell MA, Puro DG. Energy Metabolism in Human Retinal Müller Cells. Invest Ophthalmol Vis Sci. 2000 Sep;41(10):3183–90.

34. Kuwabara T, Cogan DG. Retinal Glycogen. Trans Am Ophthalmol Soc. 1961;59:106–10.

35. Poitry-Yamate CL, Tsacopoulos M. Glucose metabolism in freshly isolated Müller glial cells from a mammalian retina. J Comp Neurol. 1992 Jun 8;320(2):257–66.

36. Poitry-Yamate CL, Poitry S, Tsacopoulos M. Lactate released by Müller glial cells is metabolized by photoreceptors from mammalian retina. J Neurosci. 1995 Jul;15(7 Pt 2):5179–91.

37. Metea MR, Newman EA. Glial cells dilate and constrict blood vessels: a mechanism of neurovascular coupling. J Neurosci. 2006 Mar 15;26(11):2862–70.

38. Newman EA. Glial cell regulation of neuronal activity and blood flow in the retina by release of gliotransmitters. Phil Trans R Soc B. 2015 Jul 5;370(1672):20140195.

39. Tout S, Chan-Ling T, Holländer H, Stone J. The role of müller cells in the formation of the blood-retinal barrier. Neuroscience. 1993 Jul;55(1):291–301.

40. Abukawa H, Tomi M, Kiyokawa J, Hori S, Kondo T, Terasaki T, et al. Modulation of retinal capillary endothelial cells by Müller glial cell-derived factors. Mol Vis. 2009 Feb 27;15:451–7.

41. Shen W, Fruttiger M, Zhu L, Chung SH, Barnett NL, Kirk JK, et al. Conditional Müller Cell Ablation Causes Independent Neuronal and Vascular Pathologies in a Novel Transgenic Model. J Neurosci. 2012 Nov 7;32(45):15715–27.

42. Eichler W, Yafai Y, Keller T, Wiedemann P, Reichenbach A. PEDF derived from glial Müller cells: a possible regulator of retinal angiogenesis. Exp Cell Res. 2004 Sep 10;299(1):68–78.

43. Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, et al. Müller cells in the healthy and diseased retina. Prog Retin Eye Res. 2006 Jul;25(4):397–424.

44. Li Q, Puro DG. Diabetes-Induced Dysfunction of the Glutamate Transporter in Retinal Müller Cells. Invest Ophthalmol Vis Sci. 2002 Sep 1;43(9):3109–16.

45. Lieth E, Barber AJ, Xu B, Dice C, Ratz MJ, Tanase D, et al. Glial reactivity and impaired glutamate metabolism in short-term experimental diabetic retinopathy. Penn State Retina Research Group. Diabetes. 1998 May 1;47(5):815–20.

46. Lieth E, LaNoue KF, Antonetti DA, Ratz M. Diabetes reduces glutamate oxidation and glutamine synthesis in the retina. The Penn State Retina Research Group. Exp Eye Res. 2000 Jun;70(6):723–30.

47. Ambati J, Chalam KV, Chawla DK, D'Angio CT, Guillet EG, Rose SJ, et al. Elevated gamma-aminobutyric acid, glutamate, and vascular endothelial growth factor levels in the vitreous of patients with proliferative diabetic retinopathy. Arch Ophthalmol. 1997 Sep;115(9):1161–6.

48. O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW. 1,026 experimental treatments in acute stroke. Ann Neurol. 2006 Mar;59(3):467–77.

49. Sloan SA, Barres BA. The detrimental role of glial acidification during ischemia. Neuron. 2014 Jan 22;81(2):221–3.

50. Bringmann A, Pannicke T, Uhlmann S, Kohen L, Wiedemann P, Reichenbach A. Membrane conductance of Müller glial cells in proliferative diabetic retinopathy. Can J Ophthalmol. 2002 Jun;37(4):221–7.

51. Curtis TM, Hamilton R, Yong P-H, McVicar CM, Berner A, Pringle R, et al. Müller glial dysfunction during diabetic retinopathy in rats is linked to accumulation of advanced glycation end-products and advanced lipoxidation end-products. Diabetologia. 2011 Mar;54(3):690–8.

52. Rungger–Brändle E, Dosso AA, Leuenberger PM. Glial Reactivity, an Early Feature of Diabetic Retinopathy. IOVS. 2000 Jun 1;41(7):1971–80.

53. Mizutani M, Gerhardinger C, Lorenzi M. Müller cell changes in human diabetic retinopathy. Diabetes. 1998 Mar 1;47(3):445–9.

54. Yego ECK, Vincent JA, Sarthy V, Busik JV, Mohr S. Differential regulation of high glucose-induced glyceraldehyde-3-phosphate dehydrogenase nuclear accumulation in Müller cells by IL-1beta and IL-6. Invest Ophthalmol Vis Sci. 2009 Apr;50(4):1920–8.

55. Mu H, Zhang X-M, Liu J-J, Dong L, Feng Z-L. Effect of high glucose concentration on VEGF and PEDF expression in cultured retinal Müller cells. Mol Biol Rep. 2009 Nov 1;36(8):2147–51.

56. Wang L-L, Chen H, Huang K, Zheng L. Elevated histone acetylations in Müller cells contribute to inflammation: a novel inhibitory effect of minocycline. Glia. 2012 Dec;60(12):1896–905.

57. Liu X, Ye F, Xiong H, Hu D-N, Limb GA, Xie T, et al. IL-1β induces IL-6 production in retinal Müller cells predominantly through the activation of p38 MAPK/NF-κB signaling pathway. Exp Cell Res. 2015 Feb 1;331(1):223–31.

58. Du Y, Sarthy VP, Kern TS. Interaction between NO and COX pathways in retinal cells exposed to elevated glucose and retina of diabetic rats. Am J Physiol Regul Integr Comp Physiol. 2004 Oct;287(4):R735-741.

59. Wang J, Xu X, Elliott MH, Zhu M, Le Y-Z. Müller Cell-Derived VEGF Is Essential for Diabetes-Induced Retinal Inflammation and Vascular Leakage. Diabetes. 2010;59(9):2297–305.

60. Yu Y, Chen H, Su SB. Neuroinflammatory responses in diabetic retinopathy. Journal of Neuroinflammation. 2015;12:141.

61. Vincent JA, Mohr S. Inhibition of caspase-1/interleukin-1beta signaling prevents degeneration of retinal capillaries in diabetes and galactosemia. Diabetes. 2007 Jan;56(1):224–30.

62. Mohr S, Xi X, Tang J, Kern TS. Caspase Activation in Retinas of Diabetic and Galactosemic Mice and Diabetic Patients [Internet]. [cited 2012 Jun 18]. Available from: http://diabetes.diabetesjournals.org.proxy1.cl.msu.edu

63. Lei X, Zhang J, Shen J, Hu L-M, Wu Y, Mou L, et al. EPO attenuates inflammatory cytokines by Muller cells in diabetic retinopathy. Front Biosci (Elite Ed). 2011;3:201–11.

64. McIlwain DR, Berger T, Mak TW. Caspase Functions in Cell Death and Disease. Cold Spring Harb Perspect Biol. 2013 Apr 1;5(4):a008656.

65. Salvesen GS, Dixit VM. Caspase activation: the induced-proximity model. Proc Natl Acad Sci USA. 1999 Sep 28;96(20):10964–7.

66. Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, et al. Mice deficient in IL-1β-converting enzyme are defective in production of mature IL-1β and resistant to endotoxic shock. Cell. 1995 Feb 10;80(3):401–11.

67. Wilson KP, Black J-AF, Thomson JA, Kim EE, Griffith JP, Navia MA, et al. Structure and mechanism of interleukin-lβ converting enzyme. Nature. 1994 Jul 28;370(6487):270–5.

68. Yamin T-T, Ayala JM, Miller DK. Activation of the Native 45-kDa Precursor Form of Interleukin-1-converting Enzyme. J Biol Chem. 1996 May 31;271(22):13273–82.

69. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, et al. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature. 1992 Apr 30;356(6372):768–74.

70. Walker NPC, Talanian RV, Brady KD, Dang LC, Bump NJ, Ferenza CR, et al. Crystal structure of the cysteine protease interleukin-1β-converting enzyme: A (p20/p10)2 homodimer. Cell. 1994 Jul 29;78(2):343–52.

71. Shamaa OR, Mitra S, Gavrilin MA, Wewers MD. Monocyte Caspase-1 Is Released in a Stable, Active High Molecular Weight Complex Distinct from the Unstable Cell Lysate-Activated Caspase-1. PLOS ONE. 2015 Nov 24;10(11):e0142203.

72. Drive ADA 2451 C, Arlington S 900, 1-800-Diabetes V 22202. Statistics About Diabetes [Internet]. American Diabetes Association. [cited 2016 Nov 2]. Available from: http://www.diabetes.org/diabetes-basics/statistics/

73. Denes A, Lopez-Castejon G, Brough D. Caspase-1: is IL-1 just the tip of the ICEberg? Cell Death Dis. 2012 Jul;3(7):e338.

74. Yu HB, Finlay BB. The Caspase-1 Inflammasome: A Pilot of Innate Immune Responses. Cell Host & Microbe. 2008 Sep 11;4(3):198–208.

75. Dinarello CA. Interleukin-1 beta, interleukin-18, and the interleukin-1 beta converting enzyme. Ann N Y Acad Sci. 1998 Sep 29;856:1–11.

76. Fantuzzi G, Dinarello CA. Interleukin-18 and interleukin-1 beta: two cytokine substrates for ICE (caspase-1). J Clin Immunol. 1999 Jan;19(1):1–11.

77. Sansonetti PJ, Phalipon A, Arondel J, Thirumalai K, Banerjee S, Akira S, et al. Caspase-1 Activation of IL-1β and IL-18 Are Essential for Shigella flexneri–Induced Inflammation. Immunity. 2000 May 1;12(5):581–90.

78. Kim YM, Talanian RV, Li J, Billiar TR. Nitric oxide prevents IL-1beta and IFN-gamma-inducing factor (IL-18) release from macrophages by inhibiting caspase-1 (IL-1beta-converting enzyme). J Immunol. 1998 Oct 15;161(8):4122–8.

79. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity. 2005 Nov;23(5):479–90.

80. Joosten LAB, Netea MG, Fantuzzi G, Koenders MI, Helsen MMA, Sparrer H, et al. Inflammatory arthritis in caspase 1 gene-deficient mice: contribution of proteinase 3 to caspase 1-independent production of bioactive interleukin-1beta. Arthritis Rheum. 2009 Dec;60(12):3651–62.

81. Sanchez Mejia RO, Friedlander RM. Caspases in Huntington's disease. Neuroscientist. 2001 Dec;7(6):480–9.

82. Wang X, Wang H, Figueroa BE, Zhang W, Huo C, Guan Y, et al. Dysregulation of Receptor Interacting Protein-2 and Caspase Recruitment Domain Only Protein Mediates Aberrant Caspase-1 Activation in Huntington's Disease. J Neurosci. 2005 Dec 14;25(50):11645–54.

83. Chen M, Ona VO, Li M, Ferrante RJ, Fink KB, Zhu S, et al. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. Nat Med. 2000 Jul;6(7):797–801.

84. Li M, Ona VO, Guégan C, Chen M, Jackson-Lewis V, Andrews LJ, et al. Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. Science. 2000 Apr 14;288(5464):335–9.

85. Rohn TT, Head E. Caspases as Therapeutic Targets in Alzheimer's Disease: Is It Time to "Cut" to the Chase? Int J Clin Exp Pathol. 2008 Jun 10;2(2):108–18.

86. Ambati J, Atkinson JP, Gelfand BD. Immunology of age-related macular degeneration. Nat Rev Immunol. 2013 Jun;13(6):438–51.

87. Ona VO, Li M, Vonsattel JPG, Andrews LJ, Khan SQ, Chung WM, et al. Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. Nature. 1999 May 20;399(6733):263–7.

88. Vincent JA, Mohr S. Inhibition of Caspase-1/Interleukin-1β Signaling Prevents Degeneration of Retinal Capillaries in Diabetes and Galactosemia. Diabetes. 2007 Jan 1;56(1):224–30.

89. Vezzani A, Balosso S, Maroso M, Zardoni D, Noé F, Ravizza T. ICE/caspase 1 inhibitors and IL-1beta receptor antagonists as potential therapeutics in epilepsy. Curr Opin Investig Drugs. 2010 Jan;11(1):43–50.

90. Randle JC, Harding MW, Ku G, Schönharting M, Kurrle R. ICE/Caspase-1 inhibitors as novel anti-inflammatory drugs. Expert Opin Investig Drugs. 2001 Jul;10(7):1207–9.

91. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol. 2009;27:519–50.

92. Garlanda C, Dinarello CA, Mantovani A. The Interleukin-1 Family: Back to the Future. Immunity. 2013 Dec;39(6):1003–18.

93. Sims JE, Smith DE. The IL-1 family: regulators of immunity. Nat Rev Immunol. 2010 Feb;10(2):89–102.

94. Sharma S, Kulk N, Nold MF, Gräf R, Kim S-H, Reinhardt D, et al. The IL-1 family member 7b translocates to the nucleus and down-regulates proinflammatory cytokines. J Immunol. 2008 Apr 15;180(8):5477–82.

95. Werman A, Werman-Venkert R, White R, Lee J-K, Werman B, Krelin Y, et al. The precursor form of IL-1alpha is an intracrine proinflammatory activator of transcription. Proc Natl Acad Sci USA. 2004 Feb 24;101(8):2434–9.

96. Carriere V, Roussel L, Ortega N, Lacorre D-A, Americh L, Aguilar L, et al. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. Proc Natl Acad Sci USA. 2007 Jan 2;104(1):282–7.

97. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood. 2011 Apr 7;117(14):3720–32.

98. Cameron P, Limjuco G, Rodkey J, Bennett C, Schmidt JA. Amino acid sequence analysis of human interleukin 1 (IL-1). Evidence for biochemically distinct forms of IL-1. J Exp Med. 1985 Sep 1;162(3):790–801.

99. March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, et al. Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature. 1985 Jun 20;315(6021):641–7.

100. Auron PE, Webb AC, Rosenwasser LJ, Mucci SF, Rich A, Wolff SM, et al. Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. Proc Natl Acad Sci USA. 1984 Dec;81(24):7907–11.

101. Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, Ju G. Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. J Biol Chem. 1995 Jun 9;270(23):13757–65.

102. Holzinger D, Kessel C, Omenetti A, Gattorno M. From bench to bedside and back again: translational research in autoinflammation. Nat Rev Rheumatol. 2015 Oct;11(10):573–85.

103. Aksentijevich I, Masters SL, Ferguson PJ, Dancey P, Frenkel J, van Royen-Kerkhoff A, et al. An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. N Engl J Med. 2009 Jun 4;360(23):2426–37.

104. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat Genet. 2001 Nov;29(3):301–5.

105. Hoffman HM, Throne ML, Amar NJ, Sebai M, Kivitz AJ, Kavanaugh A, et al. Efficacy and safety of rilonacept (interleukin-1 Trap) in patients with cryopyrin-associated periodic syndromes: results from two sequential placebo-controlled studies. Arthritis Rheum. 2008 Aug;58(8):2443–52.

106. Pascual V, Allantaz F, Arce E, Punaro M, Banchereau J. Role of interleukin-1 (IL-1) in the pathogenesis of systemic onset juvenile idiopathic arthritis and clinical response to IL-1 blockade. J Exp Med. 2005 May 2;201(9):1479–86.

107. Fitzgerald AA, Leclercq SA, Yan A, Homik JE, Dinarello CA. Rapid responses to anakinra in patients with refractory adult-onset Still's disease. Arthritis Rheum. 2005 Jun;52(6):1794–803.

108. Stitt AW, Lois N, Medina RJ, Adamson P, Curtis TM. Advances in our understanding of diabetic retinopathy. Clinical Science. 2013 Jul 1;125(1):1–17.

109. Abcouwer SF. Neural inflammation and the microglial response in diabetic retinopathy. J Ocul Biol Dis Infor. 2012 Apr 24;4(1–2):25–33.

110. Adamis AP. Is diabetic retinopathy an inflammatory disease? Br J Ophthalmol. 2002 Apr;86(4):363–5.

111. Yoshida S, Yoshida A, Ishibashi T. Induction of IL-8, MCP-1, and bFGF by TNF-α in retinal glial cells: implications for retinal neovascularization during post-ischemic inflammation. Graefe's Arch Clin Exp Ophthalmol. 2004 May 1;242(5):409–13.

112. Sfikakis PP, Markomichelakis N, Theodossiadis GP, Grigoropoulos V, Katsilambros N, Theodossiadis PG. Regression of Sight-Threatening Macular Edema in Type 2 Diabetes Following Treatment With the Anti–Tumor Necrosis Factor Monoclonal Antibody Infliximab. Dia Care. 2005 Feb 1;28(2):445–7.

113. Demircan N, Safran BG, Soylu M, Ozcan AA, Sizmaz S. Determination of vitreous interleukin-1 (IL-1) and tumour necrosis factor (TNF) levels in proliferative diabetic retinopathy. Eye (Lond). 2006 Dec;20(12):1366–9.

114. Joussen AM, Doehmen S, Le ML, Koizumi K, Radetzky S, Krohne TU, et al. TNF-α mediated apoptosis plays an important role in the development of early diabetic retinopathy and long-term histopathological alterations. Mol Vis. 2009 Jul 25;15:1418–28.

115. Li J, Wang JJ, Yu Q, Wang M, Zhang SX. Endoplasmic reticulum stress is implicated in retinal inflammation and diabetic retinopathy. FEBS Letters. 2009 May 6;583(9):1521–7.

116. Trueblood KE, Mohr S, Dubyak GR. Purinergic regulation of high-glucose-induced caspase-1 activation in the rat retinal Müller cell line rMC-1. Am J Physiol, Cell Physiol. 2011 Nov;301(5):C1213-1223.

117. Tang J, Mohr S, Du Y-D, Kern TS. Non-uniform distribution of lesions and biochemical abnormalities within the retina of diabetic humans. Curr Eye Res. 2003 Jul;27(1):7–13.

118. Gross O, Thomas CJ, Guarda G, Tschopp J. The inflammasome: an integrated view. Immunological Reviews. 2011;243(1):136–151.

119. Humke EW, Shriver SK, Starovasnik MA, Fairbrother WJ, Dixit VM. ICEBERG: A Novel Inhibitor of Interleukin-1β Generation. Cell. 2000 Sep 29;103(1):99–111.

120. Thome M, Hofmann K, Burns K, Martinon F, Bodmer J-L, Mattmann C, et al. Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. Current Biology. 1998 Jul 16;8(15):885–9.

121. McCarthy JV, Ni J, Dixit VM. RIP2 Is a Novel NF-κB-activating and Cell Death-inducing Kinase. J Biol Chem. 1998 Jul 3;273(27):16968–75.

122. Gong C-X, Liu F, Grundke-Iqbal I, Iqbal K. Dysregulation of Protein Phosphorylation/Dephosphorylation in Alzheimer's Disease: A Therapeutic Target. Journal of Biomedicine and Biotechnology. 2006;2006:1–12.

123. Lee SH, Stehlik C, Reed JC. COP, a Caspase Recruitment Domain-containing Protein and Inhibitor of Caspase-1 Activation Processing. J Biol Chem. 2001 Sep 14;276(37):34495–500.

124. Kayagaki N, Warming S, Lamkanfi M, Walle LV, Louie S, Dong J, et al. Non-canonical inflammasome activation targets caspase-11. Nature. 2011 Nov 3;479(7371):117–21.

125. Bordon Y. Innate immunity: A new path uncovers a wrongful conviction. Nature Reviews Immunology. 2011 Dec 1;11(12):801–801.

126. Vincent JA, Mohr S. Inhibition of caspase-1/interleukin-1beta signaling prevents degeneration of retinal capillaries in diabetes and galactosemia. Diabetes. 2007 Jan;56(1):224–30.

127. Kusner LL, Sarthy VP, Mohr S. Nuclear Translocation of Glyceraldehyde-3-Phosphate Dehydrogenase: A Role in High Glucose-Induced Apoptosis in Retinal Müller Cells. Investigative Ophthalmology & Visual Science. 2004 May 1;45(5):1553–61.

128. Yego ECK, Vincent JA, Sarthy V, Busik JV, Mohr S. Differential Regulation of High Glucose–Induced Glyceraldehyde-3-Phosphate Dehydrogenase Nuclear Accumulation in Müller Cells by IL-1β and IL-6. Investigative Ophthalmology & Visual Science. 2009 Apr 1;50(4):1920–8.

129. Yego ECK, Mohr S. siah-1 Protein Is Necessary for High Glucose-induced Glyceraldehyde-3-phosphate Dehydrogenase Nuclear Accumulation and Cell Death in Müller Cells. Journal of Biological Chemistry. 2010 Jan 29;285(5):3181–90.

130. Dinarello CA, Ikejima T, Warner SJ, Orencole SF, Lonnemann G, Cannon JG, et al. Interleukin 1 induces interleukin 1. I. Induction of circulating interleukin 1

in rabbits in vivo and in human mononuclear cells in vitro. J Immunol. 1987 Sep 15;139(6):1902–10.

131. Hoffman HM, Rosengren S, Boyle DL, Cho JY, Nayar J, Mueller JL, et al. Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. Lancet. 2004 Nov 13;364(9447):1779–85.

132. Bromberg-White JL, Glazer L, Downer R, Furge K, Boguslawski E, Duesbery NS. Identification of VEGF-independent cytokines in proliferative diabetic retinopathy vitreous. Invest Ophthalmol Vis Sci. 2013;54(10):6472–80.

133. Vincent JA, Mohr S. Inhibition of caspase-1/interleukin-1beta signaling prevents degeneration of retinal capillaries in diabetes and galactosemia. Diabetes. 2007 Jan;56(1):224–30.

134. Liu Y, Biarnés Costa M, Gerhardinger C. IL-1β Is Upregulated in the Diabetic Retina and Retinal Vessels: Cell-Specific Effect of High Glucose and IL-1β Autostimulation. PLoS ONE. 2012 May 16;7(5):e36949.

135. Krady JK, Basu A, Allen CM, Xu Y, LaNoue KF, Gardner TW, et al. Minocycline reduces proinflammatory cytokine expression, microglial activation, and caspase-3 activation in a rodent model of diabetic retinopathy. Diabetes. 2005 May;54(5):1559–65.

136. Liu Y, Biarnes M, Gerhardinger C. Effect of Diabetes Duration and High Glucose on Retinal Interleukin-1β Expression. Invest Ophthalmol Vis Sci. 2009 Apr 28;50(13):4984–4984.

137. Devi TS, Lee I, Hüttemann M, Kumar A, Nantwi KD, Singh LP. TXNIP Links Innate Host Defense Mechanisms to Oxidative Stress and Inflammation in Retinal Muller Glia under Chronic Hyperglycemia: Implications for Diabetic Retinopathy. Experimental Diabetes Research. 2012;2012:1–19.

138. Hori S, Mukai DN. Ultrastructural lesions of retinal pericapillary Müller cells in streptozotocin-induced diabetic rats. Albrecht v Graefes Arch klin exp Ophthal. 1980 Mar 1;213(1):1–9.

139. Yego ECK, Mohr S. Siah-1 Protein Is Necessary for High Glucose-Induced Glyceraldehyde-3-Phosphate Dehydrogenase Nuclear Accumulation and Cell Death in Müller Cells. J Biol Chem. 2010 Jan 29;285(5):3181–90.

140. Feenstra DJ, Yego ECK, Mohr S. Modes of Retinal Cell Death in Diabetic Retinopathy. J Clin Exp Ophthalmol. 2013;4:298.

141. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ. 2009 Jan;16(1):3–11.

142. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ. 2012 Jan;19(1):107–20.

143. Busik JV, Mohr S, Grant MB. Hyperglycemia-Induced Reactive Oxygen Species Toxicity to Endothelial Cells Is Dependent on Paracrine Mediators. Diabetes. 2008 Jul 1;57(7):1952–65.

144. Salam A, Mathew R, Sivaprasad S. Treatment of proliferative diabetic retinopathy with anti-VEGF agents. Acta Ophthalmologica. 2011;89(5):405–411.

145. Al-Latayfeh M, Silva PS, Sun JK, Aiello LP. Antiangiogenic Therapy for Ischemic Retinopathies. Cold Spring Harbor Perspectives in Medicine. 2012 Feb 28;2(6):a006411–a006411.

146. Bandello F, Berchicci L, La Spina C, Battaglia Parodi M, Iacono P. Evidence for Anti-VEGF Treatment of Diabetic Macular Edema. Ophthalmic Research. 2012;48(s1):16–20.

147. Tremolada G, Del Turco C, Lattanzio R, Maestroni S, Maestroni A, Bandello F, et al. The Role of Angiogenesis in the Development of Proliferative Diabetic Retinopathy: Impact of Intravitreal Anti-VEGF Treatment. Experimental Diabetes Research. 2012;2012:1–8.

148. Gupta N. Diabetic Retinopathy and VEGF. The Open Ophthalmology Journal. 2013 Feb 1;7(1):4–10.

149. Tanaka T, Kanai H, Sekiguchi K, Aihara Y, Yokoyama T, Arai M, et al. Induction of VEGF gene transcription by IL-1 beta is mediated through stress-activated MAP kinases and Sp1 sites in cardiac myocytes. J Mol Cell Cardiol. 2000 Nov;32(11):1955–67.

150. Solà-Villà D, Camacho M, Solà R, Soler M, Diaz J-M, Vila L. IL-1beta induces VEGF, independently of PGE2 induction, mainly through the PI3-K/mTOR pathway in renal mesangial cells. Kidney Int. 2006 Dec;70(11):1935–41.

151. Jung YD, Liu W, Reinmuth N, Ahmad SA, Fan F, Gallick GE, et al. Vascular endothelial growth factor is upregulated by interleukin-1 beta in human vascular smooth muscle cells via the P38 mitogen-activated protein kinase pathway. Angiogenesis. 2001;4(2):155–62.

152. Hammes H-P, Lin J, Renner O, Shani M, Lundqvist A, Betsholtz C, et al. Pericytes and the Pathogenesis of Diabetic Retinopathy. Diabetes. 2002 Oct 1;51(10):3107–12.

153. Lorenzi M, Gerhardinger C. Early cellular and molecular changes induced by diabetes in the retina. Diabetologia. 2001 Jul;44(7):791–804.

154. Joussen AM, Murata T, Tsujikawa A, Kirchhof B, Bursell S-E, Adamis AP. Leukocyte-Mediated Endothelial Cell Injury and Death in the Diabetic Retina. The American Journal of Pathology. 2001 Jan;158(1):147.

155. Leal EC, Manivannan A, Hosoya K-I, Terasaki T, Cunha-Vaz J, Ambrósio AF, et al. Inducible Nitric Oxide Synthase Isoform Is a Key Mediator of Leukostasis and Blood-Retinal Barrier Breakdown in Diabetic Retinopathy. IOVS. 2007 Nov 1;48(11):5257–65.

156. Mizutani M, Kern TS, Lorenzi M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. Journal of Clinical Investigation. 1996 Jun 15;97(12):2883–90.

157. Romeo G, Liu W-H, Asnaghi V, Kern TS, Lorenzi M. Activation of nuclear factor-kappaB induced by diabetes and high glucose regulates a proapoptotic program in retinal pericytes. Diabetes. 2002 Jul;51(7):2241–8.

158. Podestà F, Romeo G, Liu W-H, Krajewski S, Reed JC, Gerhardinger C, et al. Bax Is Increased in the Retina of Diabetic Subjects and Is Associated with Pericyte Apoptosis in Vivo and in Vitro. The American Journal of Pathology. 2000 Mar;156(3):1025–32.

159. Asnaghi V, Gerhardinger C, Hoehn T, Adeboje A, Lorenzi M. A role for the polyol pathway in the early neuroretinal apoptosis and glial changes induced by diabetes in the rat. Diabetes. 2003 Feb;52(2):506–11.

160. Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW. Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. J Clin Invest. 1998 Aug 15;102(4):783–91.

161. Barber AJ. A new view of diabetic retinopathy: a neurodegenerative disease of the eye. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 2003 Apr;27(2):283–90.

162. Mohr S, Xi X, Tang J, Kern TS. Caspase Activation in Retinas of Diabetic and Galactosemic Mice and Diabetic Patients. Diabetes. 2002 Apr 1;51(4):1172–9.

163. Kusner LL, Sarthy VP, Mohr S. Nuclear Translocation of Glyceraldehyde-3-Phosphate Dehydrogenase: A Role in High Glucose-Induced Apoptosis in Retinal Müller Cells. IOVS. 2004 May 1;45(5):1553–61.

164. Chan-Ling T, Stone J. Degeneration of astrocytes in feline retinopathy of prematurity causes failure of the blood-retinal barrier. IOVS. 1992 Jun 1;33(7):2148–59.

165. Barber AJ, Antonetti DA, Kern TS, Reiter CEN, Soans RS, Krady JK, et al. The Ins2Akita Mouse as a Model of Early Retinal Complications in Diabetes. IOVS. 2005 Jun 1;46(6):2210–8.

166. Ly A, Yee P, Vessey KA, Phipps JA, Jobling AI, Fletcher EL. Early inner retinal astrocyte dysfunction during diabetes and development of hypoxia, retinal stress, and neuronal functional loss. Invest Ophthalmol Vis Sci. 2011;52(13):9316–26.

167. Hammes HP, Martin S, Federlin K, Geisen K, Brownlee M. Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. PNAS. 1991 Dec 15;88(24):11555–8.

168. Engerman RL, Kern TS. Progression of Incipient Diabetic Retinopathy During Good Glycemic Control. Diabetes. 1987 Jul 1;36(7):808–12.

169. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972 Aug;26(4):239–57.

170. Negoescu A, Guillermet C, Lorimier P, Brambilla E, Labat-Moleur F. Importance of DNA fragmentation in apoptosis with regard to TUNEL specificity. Biomed Pharmacother. 1998;52(6):252–8.

171. Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, et al. In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. J Histochem Cytochem. 1996 Sep;44(9):959–68.

172. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol. 1992 Nov 1;119(3):493–501.

173. Wajant H. The Fas Signaling Pathway: More Than a Paradigm. Science. 2002 May 31;296(5573):1635–6.

174. Boldin MP, Mett IL, Varfolomeev EE, Chumakov I, Shemer-Avni Y, Camonis JH, et al. Self-association of the Death Domains of the p55 Tumor Necrosis Factor (TNF) Receptor and Fas/APO1 Prompts Signaling for TNF and Fas/APO1 Effects. J Biol Chem. 1995 Jan 6;270(1):387–91.

175. Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME. Apoptosis signaling by death receptors. European Journal of Biochemistry. 1998;254(3):439–459.

176. Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, et al. FLICE, A Novel FADD-Homologous ICE/CED-3–like Protease, Is Recruited to the CD95 (Fas/APO-1) Death-Inducing Signaling Complex. Cell. 1996 Jun 14;85(6):817–27.

177. Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Litwack G, Alnemri ES. Molecular ordering of the Fas-apoptotic pathway: The Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. Proc Natl Acad Sci U S A. 1996 Dec 10;93(25):14486–91.

178. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, et al. Cytochrome c and dATP-Dependent Formation of Apaf-1/Caspase-9 Complex Initiates an Apoptotic Protease Cascade. Cell. 1997 Nov 14;91(4):479–89.

179. Tait SWG, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol. 2010 Sep;11(9):621–32.

180. Brenner C, Grimm S. The permeability transition pore complex in cancer cell death. Oncogene. 2006;25(34):4744–56.

181. Kroemer G, Galluzzi L, Brenner C. Mitochondrial Membrane Permeabilization in Cell Death. Physiol Rev. 2007 Jan 1;87(1):99–163.

182. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a Human Protein Homologous to C. elegans CED-4, Participates in Cytochrome c–Dependent Activation of Caspase-3. Cell. 1997 Aug 8;90(3):405–13.

183. Joza N, Susin SA, Daugas E, Stanford WL, Cho SK, Li CYJ, et al. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature. 2001 Mar 29;410(6828):549–54.

184. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature. 1999 Feb 4;397(6718):441–6.

185. Büttner S, Eisenberg T, Carmona-Gutierrez D, Ruli D, Knauer H, Ruckenstuhl C, et al. Endonuclease G Regulates Budding Yeast Life and Death. Molecular Cell. 2007 Jan 26;25(2):233–46.

186. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature. 2001 Jul 5;412(6842):95–9.

187. Behl Y, Krothapalli P, Desta T, DiPiazza A, Roy S, Graves DT. Diabetes-Enhanced Tumor Necrosis Factor-α Production Promotes Apoptosis and the Loss of Retinal Microvascular Cells in Type 1 and Type 2 Models of Diabetic Retinopathy. The American Journal of Pathology. 2008 May;172(5):1411–8.

188. Barber AJ, Gardner TW, Abcouwer SF. The Significance of Vascular and Neural Apoptosis to the Pathology of Diabetic Retinopathy. Invest Ophthalmol Vis Sci. 2011 Feb;52(2):1156–63.

189. Joussen AM, Doehmen S, Le ML, Koizumi K, Radetzky S, Krohne TU, et al. TNF-α mediated apoptosis plays an important role in the development of early diabetic retinopathy and long-term histopathological alterations. Mol Vis. 2009 Jul 25;15:1418–28.

190. Al-Shabrawey M, Ahmad S, Megyerdi S, Othman A, Baban B, Palenski TL, et al. Caspase-14: a novel caspase in the retina with a potential role in diabetic retinopathy. Mol Vis. 2012;18:1895–906.

191. Joussen AM, Poulaki V, Mitsiades N, Cai W, Suzuma I, Pak J, et al. Suppression of Fas-FasL-induced endothelial cell apoptosis prevents diabetic blood-retinal barrier breakdown in a model of streptozotocin-induced diabetes. FASEB J. 2003 Jan;17(1):76–8.

192. Trudeau K, Muto T, Roy S. Downregulation of Mitochondrial Connexin 43 by High Glucose Triggers Mitochondrial Shape Change and Cytochrome c Release in Retinal Endothelial Cells. IOVS. 2012 Sep 1;53(10):6675–81.

193. Kern TS, Du Y, Miller CM, Hatala DA, Levin LA. Overexpression of Bcl-2 in Vascular Endothelium Inhibits the Microvascular Lesions of Diabetic Retinopathy. Am J Pathol. 2010 May;176(5):2550–8.

194. Behl Y, Krothapalli P, Desta T, Roy S, Graves DT. FOXO1 Plays an Important Role in Enhanced Microvascular Cell Apoptosis and Microvascular Cell Loss in Type 1 and Type 2 Diabetic Rats. Diabetes. 2009 Apr 1;58(4):917–25.

195. El-Remessy AB, Rajesh M, Mukhopadhyay P, Horváth B, Patel V, Al-Gayyar MMH, et al. Cannabinoid 1 receptor activation contributes to vascular inflammation and cell death in a mouse model of diabetic retinopathy and a human retinal cell line. Diabetologia. 2011 Mar 4;54(6):1567–78.

196. Feit-Leichman RA, Kinouchi R, Takeda M, Fan Z, Mohr S, Kern TS, et al. Vascular Damage in a Mouse Model of Diabetic Retinopathy: Relation to Neuronal and Glial Changes. IOVS. 2005 Nov 1;46(11):4281–7.

197. Devi TS, Hosoya K-I, Terasaki T, Singh LP. Critical role of TXNIP in oxidative stress, DNA damage and retinal pericyte apoptosis under high glucose: Implications for diabetic retinopathy. Experimental Cell Research. 2013 Apr 15;319(7):1001–12.

198. Trudeau K, Molina AJA, Roy S. High glucose induces mitochondrial morphology and metabolic changes in retinal pericytes. Invest Ophthalmol Vis Sci. 2011 Nov;52(12):8657–64.

199. Gastinger MJ, Singh RSJ, Barber AJ. Loss of Cholinergic and Dopaminergic Amacrine Cells in Streptozotocin-Diabetic Rat and Ins2Akita-Diabetic Mouse Retinas. IOVS. 2006 Jul 1;47(7):3143–50.

200. Cho NC, Poulsen GL, Ver Hoeve JN, Nork TM. Selective loss of S-cones in diabetic retinopathy. Arch Ophthalmol. 2000 Oct;118(10):1393–400.

201. Kroemer G, El-Deiry WS, Golstein P, Peter ME, Vaux D, Vandenabeele P, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. Cell Death Differ. 2005;12(S2):1463–7.

202. Vandenabeele P, Galluzzi L, Berghe TV, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nature Reviews Molecular Cell Biology. 2010 Oct 1;11(10):700–14.

203. Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell. 2009 Jun 12;137(6):1112–23.

204. He S, Wang L, Miao L, Wang T, Du F, Zhao L, et al. Receptor Interacting Protein Kinase-3 Determines Cellular Necrotic Response to TNF-α. Cell. 2009 Jun 12;137(6):1100–11.

205. Zhang D-W, Shao J, Lin J, Zhang N, Lu B-J, Lin S-C, et al. RIP3, an Energy Metabolism Regulator That Switches TNF-Induced Cell Death from Apoptosis to Necrosis. Science. 2009 Jul 17;325(5938):332–6.

206. Zong W-X, Ditsworth D, Bauer DE, Wang Z-Q, Thompson CB. Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev. 2004 Jun 1;18(11):1272–82.

207. Bano D, Young KW, Guerin CJ, Lefeuvre R, Rothwell NJ, Naldini L, et al. Cleavage of the plasma membrane Na+/Ca2+ exchanger in excitotoxicity. Cell. 2005 Jan 28;120(2):275–85.

208. Babel J, Leuenberger P. A long term study on the ocular lesions in streptozotocin diabetic rats. Albrecht von Graefes Arch Klin Ophthalmol. 1974 Sep 1;189(3):191–209.

209. Bloodworth JMB, Molitor DL. Ultrastructural Aspects of Human and Canine Diabetic Retinopathy. IOVS. 1965 Dec 1;4(6):1037–48.

210. Addison DJ, Garner A, Ashton N. Degeneration of Intramural Pericytes in Diabetic Retinopathy. Br Med J. 1970 Jan 31;1(5691):264–6.

211. Berry DL, Baehrecke EH. Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. Cell. 2007 Dec 14;131(6):1137–48.

212. Grandér D, Kharaziha P, Laane E, Pokrovskaja K, Panaretakis T. Autophagy as the main means of cytotoxicity by glucocorticoids in hematological malignancies. Autophagy. 2009 Nov;5(8):1198–200.

213. Laane E, Tamm KP, Buentke E, Ito K, Kharaziha P, Khahariza P, et al. Cell death induced by dexamethasone in lymphoid leukemia is mediated through initiation of autophagy. Cell Death Differ. 2009 Jul;16(7):1018–29.

214. Boya P, González-Polo R-A, Casares N, Perfettini J-L, Dessen P, Larochette N, et al. Inhibition of Macroautophagy Triggers Apoptosis. Mol Cell Biol. 2005 Feb 1;25(3):1025–40.

215. Lum JJ, Bauer DE, Kong M, Harris MH, Li C, Lindsten T, et al. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. Cell. 2005 Jan 28;120(2):237–48.

216. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell. 2006 Jul;10(1):51–64.

217. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, et al. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes Dev. 2007 Jul 1;21(13):1621–35.

218. Neufeld TP, Baehrecke EH. Eating on the fly. Autophagy. 2008 Jul 1;4(5):557–62.

219. Fu D, Wu M, Zhang J, Du M, Yang S, Hammad SM, et al. Mechanisms of modified LDL-induced pericyte loss and retinal injury in diabetic retinopathy. Diabetologia. 2012 Nov 1;55(11):3128–40.

220. Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. Nat Rev Microbiol. 2009 Feb;7(2):99–109.

221. Fernandes-Alnemri T, Yu J-W, Juliana C, Solorzano L, Kang S, Wu J, et al. The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. Nat Immunol. 2010 May;11(5):385–93.

222. Fernandes-Alnemri T, Yu J-W, Datta P, Wu J, Alnemri ES. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature. 2009 Jan 21;458(7237):509–13.

223. Schroder K, Tschopp J. The inflammasomes. Cell. 2010 Mar 19;140(6):821–32.

224. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. Nature. 2012 Jan 19;481(7381):278–86.

225. Proell M, Gerlic M, Mace PD, Reed JC, Riedl SJ. The CARD plays a critical role in ASC foci formation and inflammasome signalling. Biochemical Journal. 2013 Feb 1;449(3):613–21.

226. Fernandes-Alnemri T, Wu J, Yu J-W, Datta P, Miller B, Jankowski W, et al. The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. Cell Death Differ. 2007 Sep;14(9):1590–604.

227. Labbé K, Saleh M. Cell death in the host response to infection. Cell Death Differ. 2008 Jun 20;15(9):1339–49.

228. Antonetti DA, Barber AJ, Bronson SK, Freeman WM, Gardner TW, Jefferson LS, et al. Diabetic retinopathy: seeing beyond glucose-induced microvascular disease. Diabetes. 2006 Sep;55(9):2401–11.

229. Carmo A, Cunha-Vaz JG, Carvalho AP, Lopes MC. L-arginine transport in retinas from streptozotocin diabetic rats: correlation with the level of IL-1 beta and NO synthase activity. Vision Res. 1999 Nov;39(23):3817–23.

230. Kowluru RA, Odenbach S. Role of Interleukin-1β in the Development of Retinopathy in Rats: Effect of Antioxidants. IOVS. 2004 Nov 1;45(11):4161–6.

231. Abu el Asrar AM, Maimone D, Morse PH, Gregory S, Reder AT. Cytokines in the vitreous of patients with proliferative diabetic retinopathy. Am J Ophthalmol. 1992 Dec 15;114(6):731–6.

232. Küser-Abalı G, Ozcan F, Ugurlu A, Uysal A, Fuss SH, Bugra-Bilge K. SIK2 Is Involved in the Negative Modulation of Insulin-Dependent Müller Cell Survival and Implicated in Hyperglycemia-Induced Cell Death. IOVS. 2013 May 1;54(5):3526–37.

233. Hara MR, Agrawal N, Kim SF, Cascio MB, Fujimuro M, Ozeki Y, et al. S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. Nature Cell Biology. 2005;7(7):665–74.

234. Leisner TM, Moran C, Holly SP, Parise LV. CIB1 prevents nuclear GAPDH accumulation and non-apoptotic tumor cell death via AKT and ERK signaling. Oncogene. 2012 Sep 10;

235. Nakajima H, Amano W, Kubo T, Fukuhara A, Ihara H, Azuma Y-T, et al. GAPDH aggregate formation participates in oxidative stress-induced cell death. J Biol Chem [Internet]. 2009 Oct 16 [cited 2013 Aug 8]; Available from: http://www.jbc.org/content/early/2009/10/16/jbc.M109.027698

236. Engerman RL, Kern TS. Experimental galactosemia produces diabetic-like retinopathy. Diabetes. 1984 Jan;33(1):97–100.

237. Kern TS, Engerman RL. Vascular lesions in diabetes are distributed non-uniformly within the retina. Experimental Eye Research. 1995 May;60(5):545–9.

238. Engerman RL, Kern TS. Aldose reductase inhibition fails to prevent retinopathy in diabetic and galactosemic dogs. Diabetes. 1993 Jun;42(6):820–5.

239. Kador PF, Akagi Y, Terubayashi H, Wyman M, Kinoshita JH. Prevention of pericyte ghost formation in retinal capillaries of galactose-fed dogs by aldose reductase inhibitors. Arch Ophthalmol. 1988 Aug;106(8):1099–102.

240. Kador PF, Akagi Y, Takahashi Y, Ikebe H, Wyman M, Kinoshita JH. Prevention of retinal vessel changes associated with diabetic retinopathy in galactose-fed dogs by aldose reductase inhibitors. Arch Ophthalmol. 1990 Sep;108(9):1301–9.

241. Frank RN. The galactosemic dog. A valid model for both early and late stages of diabetic retinopathy. Arch Ophthalmol. 1995 Mar;113(3):275–6.

242. Engerman RL, Kern TS. Retinopathy in galactosemic dogs continues to progress after cessation of galactosemia. Arch Ophthalmol. 1995 Mar;113(3):355–8.

243. Kador PF, Takahashi Y, Akagi Y, Blessing K, Randazzo J, Wyman M. Age-dependent retinal capillary pericyte degeneration in galactose-fed dogs. J Ocul Pharmacol Ther. 2007 Feb;23(1):63–9.

244. Kern TS. In Vivo Models of Diabetic Retinopathy. In: MD EJD, editor. Diabetic Retinopathy [Internet]. Humana Press; 2008 [cited 2017 Feb 12]. p. 137–56. (Contemporary Diabetes). Available from: http://link.springer.com/chapter/10.1007/978-1-59745-563-3_5

245. Robinson R, Barathi VA, Chaurasia SS, Wong TY, Kern TS. Update on animal models of diabetic retinopathy: from molecular approaches to mice and higher mammals. Dis Model Mech. 2012 Jul;5(4):444–56.

246. Kern TS, Engerman RL. Galactose-induced retinal microangiopathy in rats. IOVS. 1995 Feb 1;36(2):490–6.

247. Kowluru RA, Engerman RL, Kern TS. Abnormalities of retinal metabolism in diabetes or experimental galactosemia VIII. Prevention by aminoguanidine. Curr Eye Res. 2000 Oct;21(4):814–9.

248. Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, Janicki H, et al. A central role for inflammation in the pathogenesis of diabetic retinopathy. FASEB J [Internet]. 2004 Jul 1 [cited 2015 Sep 2]; Available from: http://www.fasebj.org/content/early/2004/08/28/fj.03-1476fje

249. Robison WG, Nagata M, Laver N, Hohman TC, Kinoshita JH. Diabetic-like retinopathy in rats prevented with an aldose reductase inhibitor. Invest Ophthalmol Vis Sci. 1989 Nov;30(11):2285–92.

250. Robison WG, Jacot JL, Glover JP, Basso MD, Hohman TC. Diabetic-like retinopathy: early and late intervention therapies in galactose-fed rats. IOVS. 1998 Sep 1;39(10):1933–41.

251. Glover JP, Jacot JL, Basso MD, Hohman TC, Robison WG Jr. Retinal capillary dilation: early diabetic-like retinopathy in the galactose-fed rat model. J Ocul Pharmacol Ther. 2000 Apr;16(2):167–72.

252. Kowluru RA, Kern TS, Engerman RL, Armstrong D. Abnormalities of retinal metabolism in diabetes or experimental galactosemia. III. Effects of antioxidants. Diabetes. 1996 Sep;45(9):1233–7.

253. Kowluru RA, Tang J, Kern TS. Abnormalities of Retinal Metabolism in Diabetes and Experimental Galactosemia VII. Effect of Long-Term Administration of Antioxidants on the Development of Retinopathy. Diabetes. 2001 Aug 1;50(8):1938–42.

254. Kowluru RA, Engerman RL, Kern TS. Abnormalities of retinal metabolism in diabetes or experimental galactosemia. VI. Comparison of retinal and cerebral cortex metabolism, and effects of antioxidant therapy. Free Radic Biol Med. 1999 Feb;26(3–4):371–8.

255. Kowluru RA, Jirousek MR, Stramm L, Farid N, Engerman RL, Kern TS. Abnormalities of retinal metabolism in diabetes or experimental galactosemia: V. Relationship between protein kinase C and ATPases. Diabetes. 1998 Mar;47(3):464–9.

256. Kowluru RA, Kern TS, Engerman RL. Abnormalities of retinal metabolism in diabetes or experimental galactosemia. IV. Antioxidant defense system. Free Radic Biol Med. 1997;22(4):587–92.

257. Kowluru R, Kern TS, Engerman RL. Abnormalities of retinal metabolism in diabetes or galactosemia. II. Comparison of gamma-glutamyl transpeptidase in retina and cerebral cortex, and effects of antioxidant therapy. Curr Eye Res. 1994 Dec;13(12):891–6.

258. Kern TS, Kowluru RA, Engerman RL. Abnormalities of retinal metabolism in diabetes or galactosemia: ATPases and glutathione. Invest Ophthalmol Vis Sci. 1994 Jun;35(7):2962–7.

259. Cohen GM. Caspases: the executioners of apoptosis. Biochem J. 1997 Aug 15;326 (Pt 1):1–16.

260. Creagh EM, Conroy H, Martin SJ. Caspase-activation pathways in apoptosis and immunity. Immunol Rev. 2003 Jun;193:10–21.

261. Howard AD, Kostura MJ, Thornberry N, Ding GJ, Limjuco G, Weidner J, et al. IL-1-converting enzyme requires aspartic acid residues for processing of the IL-1 beta precursor at two distinct sites and does not cleave 31-kDa IL-1 alpha. J Immunol. 1991 Nov 1;147(9):2964–9.

262. Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, et al. Human ICE/CED-3 Protease Nomenclature. Cell. 1996 Oct 18;87(2):171.

263. Koenen TB, Stienstra R, van Tits LJ, de Graaf J, Stalenhoef AFH, Joosten LAB, et al. Hyperglycemia Activates Caspase-1 and TXNIP-Mediated IL-1β Transcription in Human Adipose Tissue. Diabetes. 2011 Feb;60(2):517–24.

264. Perrone L, Devi TS, Hosoya K-I, Terasaki T, Singh LP. Inhibition of TXNIP expression in vivo blocks early pathologies of diabetic retinopathy. Cell Death Dis. 2010 Aug;1(8):e65.

265. Sarthy VP, Brodjian SJ, Dutt K, Kennedy BN, French RP, Crabb JW. Establishment and characterization of a retinal Müller cell line. IOVS. 1998 Jan 1;39(1):212–6.

266. Xi X, Gao L, Hatala DA, Smith DG, Codispoti MC, Gong B, et al. Chronically elevated glucose-induced apoptosis is mediated by inactivation of Akt in cultured Müller cells. Biochemical and Biophysical Research Communications. 2005 Jan 21;326(3):548–53.

267. Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. Nature Reviews Drug Discovery. 2013 Dec 1;12(12):931–47.

268. Miao EA, Leaf IA, Treuting PM, Mao DP, Dors M, Sarkar A, et al. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. Nat Immunol. 2010 Dec;11(12):1136–42.

269. Miao EA, Rajan JV, Aderem A. Caspase-1-induced pyroptotic cell death. Immunol Rev. 2011 Sep;243(1):206–14.

270. Bast A, Krause K, Schmidt IHE, Pudla M, Brakopp S, Hopf V, et al. Caspase-1-Dependent and -Independent Cell Death Pathways in Burkholderia pseudomallei Infection of Macrophages. PLOS Pathogens. 2014 Mar 13;10(3):e1003986.

271. Di Paolo NC, Shayakhmetov DM. Interleukin 1α and the inflammatory process. Nat Immunol. 2016 Aug;17(8):906–13.

272. Abcouwer SF. Neural inflammation and the microglial response in diabetic retinopathy. Journal of Ocular Biology, Diseases, and Informatics. 2012 Apr 24;4(1–2):25–33.

273. Bersudsky M, Luski L, Fishman D, White RM, Ziv-Sokolovskaya N, Dotan S, et al. Non-redundant properties of IL-1α and IL-1β during acute colon inflammation in mice. Gut. 2014 Apr;63(4):598–609.

274. Kurt-Jones EA, Beller DI, Mizel SB, Unanue ER. Identification of a membrane-associated interleukin 1 in macrophages. Proc Natl Acad Sci U S A. 1985 Feb;82(4):1204–8.

275. Kurt-Jones EA, Fiers W, Pober JS. Membrane interleukin 1 induction on human endothelial cells and dermal fibroblasts. The Journal of Immunology. 1987 Oct 1;139(7):2317–24.

276. Kurt-Jones EA, Virgin HW, Unanue ER. In vivo and in vitro expression of macrophage membrane interleukin 1 in response to soluble and particulate stimuli. J Immunol. 1986 Jul 1;137(1):10–4.

277. Kurt-Jones EA, Kiely JM, Unanue ER. Conditions required for expression of membrane IL 1 on B cells. The Journal of Immunology. 1985 Sep 1;135(3):1548–50.

278. Brody DT, Durum SK. Membrane IL-1: IL-1 alpha precursor binds to the plasma membrane via a lectin-like interaction. J Immunol. 1989 Aug 15;143(4):1183–7.

279. Kim B, Lee Y, Kim E, Kwak A, Ryoo S, Bae SH, et al. The Interleukin-1α Precursor is Biologically Active and is Likely a Key Alarmin in the IL-1 Family of Cytokines. Front Immunol [Internet]. 2013 Nov 20 [cited 2017 Mar 23];4. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3834611/

280. Wessendorf JH, Garfinkel S, Zhan X, Brown S, Maciag T. Identification of a nuclear localization sequence within the structure of the human interleukin-1 alpha precursor. J Biol Chem. 1993 Oct 15;268(29):22100–4.

281. Luheshi NM, Rothwell NJ, Brough D. The Dynamics and Mechanisms of Interleukin-1α and β Nuclear Import. Traffic. 2008 Jan;10(1):16–25.

282. Cohen I, Rider P, Carmi Y, Braiman A, Dotan S, White MR, et al. Differential release of chromatin-bound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. Proc Natl Acad Sci USA. 2010 Feb 9;107(6):2574–9.

283. Lamacchia C, Rodriguez E, Palmer G, Gabay C. Endogenous IL-1α is a chromatin-associated protein in mouse macrophages. Cytokine. 2013 Aug;63(2):135–44.

284. Pollock AS, Turck J, Lovett DH. The prodomain of interleukin 1alpha interacts with elements of the RNA processing apparatus and induces apoptosis in malignant cells. FASEB J. 2003 Feb;17(2):203–13.

285. Zhou T, Che D, Lan Y, Fang Z, Xie J, Gong H, et al. Mesenchymal marker expression is elevated in Müller cells exposed to high glucose and in animal models of diabetic retinopathy. Oncotarget. 2016 Dec 15;

286. Brooks HL Jr, Caballero S Jr, Newell CK, Steinmetz RL, Watson D, Segal MS, et al. Vitreous levels of vascular endothelial growth factor and stromal-derived factor 1 in patients with diabetic retinopathy and cystoid macular edema before and after intraocular injection of triamcinolone. Arch Ophthalmol. 2004 Dec;122(12):1801–7.

287. Demircan N, Safran BG, Soylu M, Ozcan AA, Sizmaz S. Determination of vitreous interleukin-1 (IL-1) and tumour necrosis factor (TNF) levels in proliferative diabetic retinopathy. Eye. 2005 Nov 11;20(12):1366–9.

288. Hernández C, Segura RM, Fonollosa A, Carrasco E, Francisco G, Simó R. Interleukin-8, monocyte chemoattractant protein-1 and IL-10 in the vitreous fluid of patients with proliferative diabetic retinopathy. Diabetic Medicine. 2005;22(6):719–722.

289. Bai Y, Ma J, Guo J, Wang J, Zhu M, Chen Y, et al. M��ller cell-derived VEGF is a significant contributor to retinal neovascularization. The Journal of Pathology. 2009 Dec;219(4):446–54.

290. Wang J-J, Zhu M, Le Y-Z. Functions of Müller cell-derived vascular endothelial growth factor in diabetic retinopathy. World J Diabetes. 2015 Jun 10;6(5):726–33.

291. Lin M, Chen Y, Jin J, Hu Y, Zhou KK, Zhu M, et al. Ischaemia-induced retinal neovascularisation and diabetic retinopathy in mice with conditional knockout of hypoxia-inducible factor-1 in retinal Müller cells. Diabetologia. 2011 Mar 1;54:1554–66.

292. Zhou KK, Benyajati S, Le Y, Cheng R, Zhang W, Ma J. Interruption of Wnt signaling in Müller cells ameliorates ischemia-induced retinal neovascularization. PLoS ONE. 2014;9(10):e108454.

293. Fu S, Dong S, Zhu M, Sherry DM, Wang C, You Z, et al. Müller Glia Are a Major Cellular Source of Survival Signals for Retinal Neurons in Diabetes. Diabetes. 2015 Oct;64(10):3554–63.

294. Lei X, Zhang J, Shen J, Hu L-M, Wu Y, Mou L, et al. EPO attenuates inflammatory cytokines by Muller cells in diabetic retinopathy. Front Biosci (Elite Ed). 2011;3:201–11.

295. Busik JV, Mohr S, Grant MB. Hyperglycemia-Induced Reactive Oxygen Species Toxicity to Endothelial Cells Is Dependent on Paracrine Mediators. Diabetes. 2008 Jul;57(7):1952–65.

296. Thornberry NA, Molineaux SM. Interleukin-1 beta converting enzyme: a novel cysteine protease required for IL-1 beta production and implicated in programmed cell death. Protein Sci. 1995 Jan;4(1):3–12.

297. Dinarello CA. Interleukin-1 beta, interleukin-18, and the interleukin-1 beta converting enzyme. Ann N Y Acad Sci. 1998 Sep 29;856:1–11.

298. Mohr S. Potential new strategies to prevent the development of diabetic retinopathy. Expert Opin Investig Drugs. 2004 Mar;13(3):189–98.

299. Trueblood KE, Mohr S, Dubyak GR. Purinergic regulation of high-glucose-induced caspase-1 activation in the rat retinal Müller cell line rMC-1. Am J Physiol Cell Physiol. 2011 Nov 1;301(5):C1213–23.

300. Yoshida S, Sotozono C, Ikeda T, Kinoshita S. Interleukin-6 (IL-6) production by cytokine-stimulated human Müller cells. Curr Eye Res. 2001 May;22(5):341–7.

301. Barnes TC, Anderson ME, Moots RJ. The Many Faces of Interleukin-6: The Role of IL-6 in Inflammation, Vasculopathy, and Fibrosis in Systemic Sclerosis. International Journal of Rheumatology. 2011;2011:1–6.

302. Neurath MF, Finotto S. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. Cytokine & Growth Factor Reviews. 2011 Apr;22(2):83–9.

303. Rojas M, Zhang W, Lee DL, Romero MJ, Nguyen DT, Al-Shabrawey M, et al. Role of IL-6 in Angiotensin II-Induced Retinal Vascular Inflammation. Investigative Ophthalmology & Visual Science. 2009 Oct 15;51(3):1709–18.

304. Sappington RM, Chan M, Calkins DJ. Interleukin-6 Protects Retinal Ganglion Cells from Pressure-Induced Death. IOVS. 2006 Jul 1;47(7):2932–42.

305. Zhao X-F, Wan J, Powell C, Ramachandran R, Myers MG, Goldman D. Leptin and IL-6 family cytokines synergize to stimulate Müller glia reprogramming and retina regeneration. Cell Rep. 2014 Oct 9;9(1):272–84.

306. Sims SM, Holmgren L, Cathcart HM, Sappington RM. Spatial regulation of interleukin-6 signaling in response to neurodegenerative stressors in the retina. Am J Neurodegener Dis. 2012;1(2):168–79.

307. Chong DY, Boehlke CS, Zheng Q-D, Zhang L, Han Y, Zacks DN. Interleukin-6 as a Photoreceptor Neuroprotectant in an Experimental Model of Retinal Detachment. IOVS. 2008 Jul 1;49(7):3193–200.

308. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta. 2011 May;1813(5):878–88.

309. Barnes TC, Anderson ME, Moots RJ. The Many Faces of Interleukin-6: The Role of IL-6 in Inflammation, Vasculopathy, and Fibrosis in Systemic Sclerosis. International Journal of Rheumatology. 2011;2011:1–6.

310. Naugler WE, Karin M. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends in Molecular Medicine. 2008 Mar;14(3):109–19.

311. Tilg H, Trehu E, Atkins MB, Dinarello CA, Mier JW. Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. Blood. 1994 Jan 1;83(1):113–8.

312. Ropelle ER, Flores MB, Cintra DE, Rocha GZ, Pauli JR, Morari J, et al. IL-6 and IL-10 Anti-Inflammatory Activity Links Exercise to Hypothalamic Insulin and Leptin Sensitivity through IKKβ and ER Stress Inhibition. Vidal-Puig AJ, editor. PLoS Biology. 2010 Aug 24;8(8):e1000465.

313. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, et al. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. J Clin Invest. 1998 Jan 15;101(2):311–20.

314. Yasukawa H, Ohishi M, Mori H, Murakami M, Chinen T, Aki D, et al. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. Nature Immunology. 2003;4(6):551–6.

315. Leibinger M, Müller A, Gobrecht P, Diekmann H, Andreadaki A, Fischer D. Interleukin-6 contributes to CNS axon regeneration upon inflammatory stimulation. Cell Death Dis. 2013;4:e609.

316. Atreya R, Mudter J, Finotto S, Müllberg J, Jostock T, Wirtz S, et al. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. Nat Med. 2000 May;6(5):583–8.

317. Campbell IL, Erta M, Lim SL, Frausto R, May U, Rose-John S, et al. Trans-signaling is a dominant mechanism for the pathogenic actions of interleukin-6 in the brain. J Neurosci. 2014 Feb 12;34(7):2503–13.

318. Chalaris A, Schmidt-Arras D, Yamamoto K, Rose-John S. Interleukin-6 trans-signaling and colonic cancer associated with inflammatory bowel disease. Dig Dis. 2012;30(5):492–9.

319. Koskela UE, Kuusisto SM, Nissinen AE, Savolainen MJ, Liinamaa MJ. High vitreous concentration of IL-6 and IL-8, but not of adhesion molecules in relation to plasma concentrations in proliferative diabetic retinopathy. Ophthalmic Res. 2013;49(2):108–14.

320. Funk M, Schmidinger G, Maar N, Bolz M, Benesch T, Zlabinger GJ, et al. ANGIOGENIC AND INFLAMMATORY MARKERS IN THE INTRAOCULAR FLUID OF EYES WITH DIABETIC MACULAR EDEMA AND INFLUENCE OF THERAPY WITH BEVACIZUMAB. Retina. 2010 Oct;30(9):1412–9.

321. Wegner M, Araszkiewicz A, Piorunska-Stolzmann M, Wierusz-Wysocka B, Zozulinska-Ziolkiewicz D. Association Between IL-6 Concentration and Diabetes-Related Variables in DM1 Patients with and without Microvascular Complications. Inflammation. :1–6.

322. Funatsu H, Yamashita H, Noma H, Mimura T, Yamashita T, Hori S. Increased levels of vascular endothelial growth factor and interleukin-6 in the aqueous humor of diabetics with macular edema. Am J Ophthalmol. 2002 Jan;133(1):70–7.

323. Funatsu H, Yamashita H, Shimizu E, Kojima R, Hori S. Relationship between vascular endothelial growth factor and interleukin-6 in diabetic retinopathy. Retina (Philadelphia, Pa). 2001;21(5):469–77.

324. Funatsu H, Yamashita H, Ikeda T, Mimura T, Eguchi S, Hori S. Vitreous levels of interleukin-6 and vascular endothelial growth factor are related to diabetic macular edema. Ophthalmology. 2003 Sep;110(9):1690–6.

325. Jayaguru P, Mohr S. Nuclear GAPDH: changing the fate of Müller cells in diabetes. Journal of Ocular Biology, Diseases, and Informatics. 2011;4(1):34–41.

326. Walker RJ, Steinle JJ. Role of β-Adrenergic Receptors in Inflammatory Marker Expression in Müller Cells. IOVS. 2007 Nov 1;48(11):5276–81.

327. Walker RJ, Anderson NM, Jiang Y, Bahouth S, Steinle JJ. Role of β-Adrenergic Receptor Regulation of TNF-α and Insulin Signaling in Retinal Müller Cells. Invest Ophthalmol Vis Sci. 2011 Dec 1;52(13):9527–33.