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Stem Cell Technology for Age Related Macular Degeneration Intervention

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STEM CELL TECHNOLOGY FOR AGE RELATED MACULAR DEGENERATION INTERVENTION

A Thesis

by

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ABSTRACT

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The main focus of this thesis is to study induced pluripotent stem cell derived Retinal Pigmented Epithelium (RPE) cells in various physiological conditions mimicking RPE transplantation rejection. Retinal pigmented epithelium (RPE) cells are located between the choroid and photoreceptors within the eye and are essential to provide nutrients from blood to rods and cones, as well retinoids of the visual cycle. Vision loss and various ocular diseases are attributable to the degeneration or dysfunction of the RPE cells, leading to blindness. One of the major ocular problem from RPE dysfunction is macular degeneration. Age-related macular degeneration (AMD) can be frequently diagnosed in patients over the age of 60. In the early stages of AMD, some symptoms may not be noticeable but will lead to vision loss in both eyes. Induced pluripotent stem cells (iPSC) can be derived from somatic cells and have been used in regenerative medicine, replacing cells that have been lost or damaged. iPSC culture can be derived from a 'patient-match' because these cells come from blood or skin cells. I plan to study how RPE cells can be protected from hypoxia, hyperglycemia, and proinflammatory conditions. Results from this will provide important information on the molecular pathway on RPE survival under different pathological conditions. Our long-term goal is to investigate how to protect RPE from dysfunction due to aging and explore a novel approach to preserve stem cell derived RPE for transplantation in AMD to restore vision and prevent vision loss.

DEDICATION

I want to dedicate this project to Chippy. The completion of my masters studies would not have been possible without the love and support of my family, my dogs, and Amanda Hutt. My mom Janie Valdez and my dad Raul Valdez who inspired me and motivated wholeheartedly. Thank you for all your love, patience, and never letting me let my cells die.

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CHAPTER I

INTRODUCTION

Age-Related Macular Degeneration (AMD) has been previously identified as one of the most leading cause of blindness in the elderly population and the leading cause of central visual loss. While diagnosing AMD can involve numerous factors, the most frequently used diagnostic criteria would be by the abnormal amounts of RPE pigmentation. Additional characteristics can also involve geographic atrophy, which is stimulated by the excessive drop out of RPE cells. While much research has been done in order to determine what is the underlying cause of AMD, much of it is can be determined by genetics or by environmental factors (Coleman HR 2008). An example of this can be high cholesterol in patients not only serving as an increased risk of heart disease and diabetes but can also lead to AMD. Moreover, the risk an individual has of getting AMD can be increased by their age, genetic history, body mass index and their dietary habits. While there have already been numerous studies in attempts to distinguish the cause of AMD, many environmental factor and stressors still need to be taken into consideration. As individuals start to mature and age, the melanin that can be distinguished in their eyes can no longer be found in abundance. The main hallmark of AMD can be distinguished by the senescence of RPE cells by oxidative stress (Aryan et al., 2016).

AMD can be diagnosed as either wet AMD or dry AMD. AMD naturally occurs by the deposition of drusen. These types of abnormalities take place within the RPE cells. In comparison, the drusen can also have a role in what is initiating the loss of RPE cells, ultimately leading to AMD. While AMD is considered a progressive retinal degeneration, there are currently no preventative measures that can be taken or is there any approved types of treatment therapies for early diagnosed

AMD. (Kozlowski 2012). To date, some of the only available treatment for AMD relies heavily on anti-VEGF treatments. Furthermore, that is the exact reason why RPE stem cell transplantation is the new forefront for AMD treatments. Even though stem cell transplantation is considered a fairly new concept, it has already been studied in animal models successfully and is currently going under clinical trials. (Kozlowski 2012).

Retinal Pigmented Epithelium (RPE) cells are essential for our vision and for the blood retinal barrier. RPE cells play several vital roles within the visual cycle and in the blood retinal barrier (BRB) by acting as a barrier between the retina and the bloodstream. These important cells can be identified in monolayers within the neuroretina photoreceptors and the choroids. These types of cells are required for the photoreceptors in the eye to remain viable, while also having a role within the regeneration of retinoids and the repairment of tissues (Klimanskaya I 2004) (Fisher and Reh, 2001). RPE are located between the choroid and photoreceptors in the eye and they are essential to provide nutrients from blood to rods and cones, including retinoids of the visual cycle. There are also tight junctions that can be used in order to unite RPE cells and the surrounding endothelial cells. In addition, RPE cells are very involved in protecting the eyes from photooxidation and how light is being absorbed. Additionally, RPE cells are the first line of defense for protection are essentially functioned to preserve complement-medicated inflammation or to stimulate the occurrence of inflammation. RPE cells are identified as being able to keep inflammation that is not essential at a controlled level, by facilitating the eye with an alternative means of avoiding inflammation induced damage.

Furthermore, the RPE monolayer also aids in the processing of retinoids in the visual cycle and phagocytosis (Muniz et al., 2014). With that being said, any type of RPE damage or dysfunction can lead to visual ailments or other visual deficiencies. Even though there is always a number a cell loss, unlike regenerative organs like the liver and tongue, the eye cannot regenerate or replace cells

that have already been lost. The loss of these cells act as a diagnostic criteria for AMD. Vision loss and various ocular diseases are attributable to the degeneration or dysfunction of the RPE cells, leading to blindness. One of the major ocular problems from RPE dysfunction is macular degeneration. AMD can be frequently diagnosed in patients over the age of 60 years. In the early stages of AMD, some symptoms may not be noticeable but will lead to vision loss in both eyes.

The main course of treatment for AMD has anti-VEGF treatments, in addition to photodynamic therapy (Greene et al., 2014; Hazim et al., 2017; Jim et al., 2019). Even though there have been different mechanistic approaches to compensate for the RPE loss, none of them have had a 100% success rate. Therefore, stem cell research has been used in order to grown RPE cells and use them for transplantations to compensate for the RPE cell loss in efforts to mitigate further damage. More recently, RPE cell transplantation has been used as a novel form of treatment and has worked successfully. This is done by the replacement of the damaged or degenerated RPE cells. This form of treatment can be limited because of a lack of RPE cell availability, surgical complications, or various types of immune responses, but is still considered a form of treatment due to the success rate (Hazim et al., 2017).

Muniz et al., 2014., identified the visual cycle in iPSC differentiation to RPE cells and the visual cycle with IMR90-RPE cells. RPE cells have been studied over numerous years and much research has been done in order to determine the route of passage of chemokines and how they function physiologically. The gradients involved with chemokines are being modified by multiple stimuli such as secretion and fluid absorption. Many retinal diseases arise from the degeneration of microglia, RPE, muller cells, or retinal ganglion cells. The loss and degeneration of these cells can stimulate multiple chemokine responses and upregulation. Once the chemokines have been stimulated, they will in turn assist in immune regulation (Harlene at al., 2012; Juel et al., 2012; Nagineni et al., 2015; Rutar et al., 2015; Shi et al., 2008)., While RPE cells can be located within the retina, when

RPE cells begin to associate with microglia, they stimulate the activation of cytokines. Some of the cytokines that get activated can be considered to be inflammatory precursors for the microglia. For example, a study identified CCL2, CXCL1, and CXCL10 being produced in RPE cells due to interacting with microglia (Detrick and Hooks., 2020).

1.1 Stem Cell Technology for AMD Treatment

Stem cell therapies have been studied and used in medical practices for over 20 years. The downfall of this type of regenerative medicine would be at an ethical standpoint because of how embryonic stem cells are harvested and where they are harvested from. In total, there are four types of stem cells: (1) Embryonic stem cells, which come from the embryo within in eurto before implantation occurs. (2) Induced pluripotent stem cells, which arise from genetic reprogramming of adult stem cells. (3) Tissue-specific stem cells generated by a specific organ. (4) Mesenchymal stem cells, which are taken from the stroma. The more popular and more widely known types of stem cells are embryonic stem cells and induced pluripotent stem cells. While embryonic stem cells have more ethical issues due to being harvested from embryos, induced pluripotent stem cells (iPSC) can be derived from somatic cells and have been used in regenerative medicine. Additionally, iPSC has been used to replace cells that have been lost or damaged by apoptosis or degeneration. What makes these cells innovative is that they possess the possibility of being a ‘patient-match’ because these cells can be harvested from blood or skin cells. Therefore, the use of iPSC in RPE transplantation is innovative as stem cells to replenish aging RPE cells in AMD patients. As in many regenerative medical operations and procedures, there is always a chance of organ transplantation rejection, in addition to organ failure. Furthermore, other complications can arise with these transplants such as subretinal microenvironment, possible immune rejection, complications with immunosuppression, incorrect placement, tumorigenesis, and possible contamination leading to sepsis.

1.2 Cytokines and Chemokines

Cytokines can be found in a variety of cells. They have been identified as having a numerous amount of roles within inflammatory and anti-inflammatory conditions. Cytokines play a crucial role as showing promise as treatments for disorders originating within the immune system. The topic of this study revolves around RPE cells, which has been acknowledged as having an abundant amount of cytokines. The cytokines that are shown to have pro-inflammatory responses are IL-6, IL-8, MCP-1, CXCL9, and CXCL10 (Detrick & Hooks, 2020). In comparison, cytokine IFN-b can be identified as having a rare connection with RPE cells. Thus, there have been numerous studies that can conclude various cytokines can have an increasing amount of VEGF, such as IL-1b, TNF-a, and IFN-g. The most common form of treatment for the increase of VEGF in relation to AMD is the use of anti-VEGF treatments. Different receptors are found on the surfaces of RPE cells. TLF4 is activated as a results of C5A stimulating the C5A receptor; This is just an example of how inflammation can be stimulated within the retina, as a result, various pro-inflammatory cytokines are going to be initiated.

Chemokines are secreted by cells within the body due to infections or as an anti-inflammatory response. By coinciding with inflammatory responses, chemokines can be found to interact with immune system regulators such as macrophages, lymphocytes and neutrophils (Detrick & Hooks, 2020). Chemokines are secreted in the eye as a response to various types of retinal disorders. The cytokines previously mentioned (IL-6, IL-8, MCP-1, CXCL9, and CXCL10) are secreted as a response to stress and this stress will also act as a type of chemoattractant. Moreover, IL-8 acts as a precursor as a result to an early inflammatory response. Another chemokine that has high popularity within RPE studies is going to be CCL2 (MCP-1), which is identifiable as being involved with AMD (Detrick & Hooks, 2020). Chemokines function within the ocular tissues and can be increased when visual diseases are present. Chemokines can function as a type of immune response by getting secreted by RPE cells and microglia. AMD involves the degeneration of RPE and can lead to the disruption of

photoreceptor oxidation. This in turn can lead to the stimulation of the release of IL-8, IL-6, and MCP-1, all of which can be identified as either chemokines or cytokines. Chemokine receptor-3 (CCR-3) has been previously identified to be involved in AMD and within choroidal neovascularization. Even though there has been research done to identify the cause of AMD, one of the most studied has been inflammation (Harlene at al., 2012; Juel et al., 2012; Nagineni et al., 2015; Rutar et al., 2015; Shi et al., 2008).

1.3 Problem Statement

Over the many years of research for AMD, there has not been any progress regarding any type of prevention or treatment other than anti-VEGF treatments. This study poses as a cell model for iPSC-RPE in various environments to see how TNF-a and Il-8 can act as possible cell protectants against different glucose concentrations in proinflammatory and hypoxic conditions, in addition to the various chemokines and cytokines expressed.

1.4 Research Objective

The purpose of this study address various key points in AMD treatments and possible therapeutic advancements. This study will be primarily used for identifying if other treatments can be used in order to protect the RPE monolayer after transplantation. The expected results of these experiments is to determine what chemokines and cytokines are involved within RPE cells and how cell loss and death can be reduces for less rejection after transplantation. I hypothesize IL-8 will be able to prevent the loss of iPSC-RPE in different media conditions and environmental stress and MCP-1 will be at an elevated expression. If this is the case, IL-8 can be used after RPE transplants to minimize rejection.

CHAPTER II

REVIEW OF THE LITERATURE

2.1 Overview of this Chapter

This section reviews Age-Related Macular Degeneration and the use of stem cells for therapeutic treatment for retinal pigmented epithelium dropout.

2.2 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a leading cause in visual loss in many elderly people (Chakravarthy et al., 2010). Most AMD is nonexudative, while exudative AMD, which is around 10%, will be responsible for cases with severe visual impairment and blindness. AMD is a complex disease and has been shown to not only have environmental risk factors, but also genetic ones (Yang et al., 2006). Research has shown various risk factors can include a family history of AMD and old age (Chakravarthy et al., 2010). One of the causes of AMD has been known to be diabetes. Diabetes is a disease caused by insulin impairment leading to elevated levels of glucose and abnormal metabolism in the body. Diabetes has risen from 4.7% in the late 1900s to 8.5% in 2014 (WHO, 2016). This since, has led to a rapid increase in diagnoses of this disease worldwide. It is believed that the blood retina barrier can be diminished due to the high levels of inflammation of the retina, leading to the angiogenic variances in diabetic vitreous (Maier et al., 2008). One complication of diabetes is diabetic retinopathy (DR) which can lead to severe visual impairments

in adults. Research has shown how the release of certain cytokines in intraocular tissues of DR patients may lead to the development of AMD (He, 2018). This study also illustrated the increased tendency of patients with diabetes having a greater risk of AMD compared to the control group. In patients with DR, the risk of developing AMD was further intensified. In addition, the blood-retina barrier (BRB) can degrade due to the development of the DR. Both DR and AMD are two of the most common degenerative diseases responsible for blindness due to retinal disease. Both conditions can affect the central macula and the development of both diseases is associated to the presence of an inflammatory response, which accelerates the disease progression (Cummings et al., 2008). Other complications such as retinal vasculature damage and neovascularization can be attributed to the evidence supporting inflammatory mediators in the pathogenesis of DR leading to an influx of leukocytes (He, 2018). These complications can have serious and damaging effects on the retinal pigment epithelium.

The retinal pigment epithelium (RPE) is a monolayer of pigmented cells located between the neural retina and the choroid whose role is to maintain the functional integrity of the retina. The RPE has many major roles, including nutrient regulation between the neural retina and choriocapillaris, the absorption of light, the protection against photo-oxidation, and amongst other key functions (Strauss, 2005). These functions of RPE will be deregulated in various retinal diseases such as prolific diabetic retinopathy (PDR) and AMD (Murugeswali, 2017). In terms of diabetes, several studies have illustrated induced inflammation and RPE early dysfunction concomitant with hyperglycemia as well as photoreceptor damage (Omri et al., 2011; Omri et al., 2013).

Age Related Macular Degeneration (AMD) can be diagnosed by the loss of the Retinal Pigmented Epithelium (RPE) cells. This retinal disease causes visual loss mostly common in patients over 60 years of age. While the loss of RPE cells are a common characteristic for diagnosis, drusen can also be used to diagnosis AMD, which can be either wet or dry AMD.

The first sign of AMD will fall under a patient getting initially diagnosed with dry AMD, done so by identifying a type of retinal dysfunction. This retinal dysfunction will later lead to the progression and eventually diagnosis of wet AMD. Dry AMD includes the use of drusen along with beta-amyloid peptides that can be identified within the retina. Wet AMD is also categorized as neovascularization and is diagnosed less commonly, whereas dry AMD is diagnosed more frequently on a day-to-day basis in clinics. Moreover, the presence of oxidative stress can proceed to cause inflammation when alpha-lipoc acid cannot be used as a preventive. Therefore, induced pluripotent stem cells have been used for regenerative therapeutic treatment to compensate for the RPE cell loss. While doing research with AMD, various studies have looked at interleukin-6 (IL-6) due to this cytokine being identified as AMD progresses in patients.

In comparison, wet AMD is also identified as the foundation for over 80% of blindness (Hernandez-Zimbron et al., 2017). This type of AMD is what is characterized with the increase of RPE dropout, in addition to choroidal neovascularization. Additionally, these patients who have already been previously diagnosed with wet AMD and neovascularization related to choroidal neovascularization, can eventually develop atrophy. The neovascularization also promotes the damage to the macula as a result from the Bruch's Membrane rupturing due to the choroid. As a result, this damage will then contribute to blurry and spotty vision a patient can

visualize with wet AMD. Wet AMD begins when there is the growth of abnormal blood vessels beneath the macula. Instead of these new blood vessels being ideal and in good conditions, they in turn are identified as being delicate, leading to leakage of blood and other liquids. Once the angiogenesis of these blood vessels has gotten out of hand, the macula will begin to cause the change in vision due to being out of its designated place.

While there is treatment for wet AMD such as antiangiogenic medications, which are the primary course of treatment due to being able to recover vision after only a few years, there is still nothing on the market to reverse the damage. While AMD is progressing in patients, both angiogenic and antiangiogenic factors can be identified. Antiangiogenic treatments can help aid compensating for the damage that was caused by vascular endothelial growth factor (VEGF) but there is no procedure or treatments in order to prevent the development of AMD. Treatments with anti-VEGF is predominantly used in order to act as a postponement of AMD progression. Being a type of growth factor, VEGF is categorized with ocular neovascularization and vascular permeability and a specific protein that is used in vascular proliferation. Aside from vascular proliferation, VEGF-A is indispensable for angiogenesis, in addition to being essential for endothelial cell movement. Whilst there are a variety of VEGFs, the main type that is used for therapeutic motives is going to be VEGF-A. Other treatments for AMD can fall on the use of siRNA in order to inhibit the VEGF activity by identifying the specific mRNA. When the mRNA is already identified, the expression of VEGF can be inhibited.

While many diseases are genetically passed on from parent to child, many years of research have been conducted in order to determine genes for AMD. With that being said,

Genome-Wide Association Studies have been used in order to help distinguish therapeutic targets for AMD and consider pathogenesis. As a result, these studies function by getting specific gene variations to a specific sample size with or without AMD. Consequently, these various types of studies can categorize multiple genetic loci that can locate nucleotide polymorphisms (SNPs). In turn, these SNPs were shown to have links to AMD and the more at risk a person is to get diagnosed with AMD, Dr.Patel (Patel et al., 2008) identified patients who had alternative CFH genes. In comparison, environmental factors can also contribute to the chances of getting an AMD diagnosis. For example, the chances of an individual getting diagnosed with AMD is going to be higher in someone who is an avid smoker compared to someone is a non-smoker. Chen et al., 2011 and Thornton et al., 2005 both confirmed either quitting smoking have a smaller chance of getting diagnosed with AMD or individuals who smoke are more at risk of getting an AMD diagnosis. In continuation, there are also physiological factors to take in to consideration such as various deficiencies, elucidating antioxidants essential for AMD prevention. In conclusion, while genetic testing would be considered a great advancement not only for AMD but for other degenerative diseases such as Alzheimer and Parkinson's disease. While there maybe genetic factors and contributions that can alter the likelihood of an individual's chance of getting AMD, there is going to need to be multiple loci and more environmental factors (Ambati & Fowler, 2012)

2.3 Retinal Pigmented Epithelium

Retinal pigmented epithelium (RPE) can be described as a type of epithelium with

specialized functions. The three most essential functions of the RPE cells is transporting nutrients, such as water and ions, acting as a protectant against photo-oxidatation, contributing to the visual cycle by the reisomerization of all-*trans*-retinal, keeping the retina secretion stabilized, and plays a key role in the photoreceptor membrane phagocytosis (Simo et al., 2010). While being key modulators in the visual cycle, they can be identified as part of the retina because of their neuroectodermal classification. Moreover, the cells are also part of the Blood-Retinal-Barrier (BRB) and are connected to other surrounding endothelial cells by tight junctions. Together the RPE and other endothelial cells can support the flow of fluids and solutes in the BRB while acting as a protectant and not allowing toxic components in. Moreover, RPE can also act as part of immune responses for the eye through the BRB because of the secretion of various types of immunosuppressive factors, such as major histocompatibility complex (MHC), cytokines, and adhesion molecules (Simo et al., 2010). . With that being said, RPE can be classified as an essential type of cell in the visual cycle.

While Diabetic Retinopathy (DR) is considered one of the most leading causes of blindness, having diabetes and any type of hyperglycemia can have a causation and lead to various other diseases and ailments. High glucose can lead to a downregulation of GLUT-1. This type of downregulation occurs as a result of the Akt pathway caused by oxidative stress in RPE cells. When this occurs, the retina can be changed because of IRPB, a protein. Because of this, there will be a defect in the RPE's role as a defense mechanism. This type epithelium can be located within the neural retina and the choriocapillaris and function with the blood retina barrier to inhibit the diffusion of transepithelial. Being classified as a layer of pigmented cells, this

epithelium acts as an absorption pad for the retina to absorb light. RPE is also fundamental for visual function and neuroretina survivability (Simo et al., 2010).

As previously mentioned, RPE functions in the movement and placement of ions and water. However, RPE moves these materials because of the transepithelial transport, caused by Na^+-K^+-ATPase. Aside from water and ions, RPE can also act as a vehicle from the subretinal space to the choroid for other types of electrolytes and passages needed for supplements to get to the photoreceptors from the blood. In the movement to the photoreceptors, RPE passages glucose and has an abundant amount of these glucose transporters. Due to RPE and choroidal cells being closely located to the neuroretina, they are primarily associated with not only AMD but CNV. The RPE can also secrete multiple chemokines and cytokines, in addition to various growth factors in order to help the regulation of the neuroretina and choroid's physiology and function (Simo et al., 2010).

In regards to defense mechanistic approaches within the eye, the RPE can help because of the amounts of melanin. This melanin within the RPE act with particular types of wavelengths. The next defense activation comes from the melanin working alongside to glutathione. Due to the abundance of superoxide dismutase present within RPE, zeaxanthin and lutein can be identified because of their nonenzymatic antioxidant properties.

While RPE have been identified to secrete growth factors, this secretion helps keep the retina regulated and also the choriocapillaris. Moreover, RPE stimulated the production of several molecules to help ensure the subsistence of the surrounding photoreceptors and can also aid in the distribution of the necessary nutrients. With many different growth factors being continuously

secreted the most meaningful would be Vascular Endothelial Growth Factor (VEGF) and Pigment Epithelium-Derived Factor (PEDF), aside from the less noteworthy but still essential such as Fibroblast Growth Factor (FGF), Nerve Growth Factor (NGF), Brain-Derived Growth Factor (BDGF), and lastly Platelet-Derived Growth Factor (PDGF). In addition, RPE can also secrete various chemokines.

As the most substantial secreted growth factors, VEGF and PEDF assist the retina is various ways and has been identified to be secreted from different areas of the RPE. The location of RPE is essential for the secretion of VEGF and PEDF due to PEDF interacting with neurons and VEGF associating with choroidal epithelium. For example, PEDF can serve as a type of factor with antiangiogenic origin, in addition to having neuroprotective characteristics. Lastly, PEDF can also play a role as a modulator in vascularization and is secreted. In comparison, VEGF gets secreted in both healthy and diabetic eyes; the only difference is the amount of VEGF that is getting secreted. While in healthy eyes, only a small amount of VEGF is being excreted and works by acting as a preventative insert in order to inhibit the apoptosis of endothelial cells. However, advanced glycation end products (AGEs) can lead to there to be an accumulation of VEGF and can be the leading cause of DR. Both VEGF and PEDF can be found in healthy eyes but when VEGF is overexpressed in abundance, DR is established (Murugeswari et al., 2016).

In the loss of vision, retinal and choroid angiogenesis are essential in this occurrence. They are key in both DR and AMD. Various problem arise from angiogenesis such as microvascular abnormalities and lead to an increase of permeability. In diabetes, RPE will proceed to get deregulated, which can also be found in Age Related Macular Degeneration

(AMD). DR incorporates the BRB which was protected by the surrounding endothelial cells and when this occurs, the RPE and other endothelial cells will proceed to get weaker and lead to pathophysiological edema (Murugeswari et al., 2016).

Diabetes, also known as hyperglycemia is defined as the ability to produce insulin is compromised, the human metabolism will then be uncharacteristic and result in an increased levels of glucose identified in the blood and urine. In DR, diabetes can have a stimulus effect of various pathways such as the AGEs pathways and this can ultimately result in an rise of inflammation, the cytokines expressed and within vascular dysfunction. When exposed to hyperglycemic conditions, RPE with have fundamental modifications and in turn will affect the barrier and the amount of cytokines being secreted; which is what recent research has been primarily focused on. However, various clinical trials have been done in order to determine a leakage in the RPE monolayer of fluorescein. DR is one of the leading causes to visual abnormalities and visual loss caused by diabetes mellitus. The secretome in RPE still has not been clearly described with DR or diabetes but knowing the various types of growth factors in RPE can be used for disease management and treatment. While this is still essential in understanding how RPE and DR work together with AMD and other retinal diseases, understanding how cytokines and chemokines work within these specific retinal diseases can help a new generation of treatments that are not anti-VEGF. (Murugeswari et al., 2016).

2.4 Cytokines and Chemokines: RANTES/MCP-1

With AMD, much of its causation can originate from inflammation as one of the main

mechanisms, aside from also being caused by the association inflammatory molecules (Nagineni at al., 2015). Previous research has been able to identify a relationship between neovascularization with inflammation and VEGF secretion. Inflammatory cytokines such as TNF-a can cause an influx and increase in VEGF secretion by RPE and its surrounding fibroblast cells from the choroid. Proinflammatory cytokines like TNF-a are secreted by the macrophages and can have a key part in the RPE irregularity that is correlated to AMD.

Cytokine and chemokines are secreted proteins that regulate and determine how the immune system responds, as well as other functions such as immune cell trafficking and cellular arrangement of immune cells. The type of cytokine that is produced depends on the immune response. This means different types of cytokines will be produced depending if the immune response is cytotoxic, cell-mediated, allergic, or even humoral. Usually in response to this, several cytokines will synergize together in order to optimize their function. Chemotactic cytokines (chemokines) can be secreted by numerous cells as a response to inflammatory conditions or by other signaling caused by autoimmune diseases. Chemokines can be distinguished by the amount of amino acids that fall between the initial N-terminal cysteines and identified as either C-C or C-X-C. The chemokines can be regulated by the activation of various signaling molecules and phospholipases because of transmembrane G protein coupled receptors. Multiple receptors can call for the same ligand and can produce overlapping with C-C and C-X-C. However, even though both C-C and C-X-C have overlapping binding sites, they differ in designated targets; C-C is more closely correlated with macrophages and monocytes whereas C-X-C are related to neutrophiles, and both C-C and C-X-C work closely with lymphocytes. (Nagineni at al., 2015). Some cytokines,

such as IL-6, IL-8, and MCP-1 were found to increase cultured RPE cell when stimulated with either IL1-b or TNF-b (V.M. Elner et al., 1990, S.R Planck et al., 1992). This illustrates the polarizing secretion of these cytokines is one factor in inducing these diseases. Barrier breakdown due to DR has also been implicated to be due to elevated expression of cytokines such as MCP-1, IL-8, IL-6, ICAM, and others (I.J Crane et al., 2000, M. Xie et al., 2014). One cytokine, MCP-1, is secreted from RPE suggestion that it may be implicated in inflammatory responses which, as other research has shown, may limit damage to the neurosensory retina (N. Dong et al., 2012). Other studies showed that in diabetic animal models, early DR was often shown to be associated with increased MCP-1 expression and highly correlated with advanced disease progression (N. Dong et al., 2012). Other research has revealed that the levels of both MCP-1 and IL-8 secretion due to RPE is proportional to blood glucose levels as well as inflammatory responses, suggesting that this may cause alterations in blood retinal function in patients with DR (I.J Crane et al., 2000). Furthermore, the levels of these cytokines, such as MCP-1, TNF-a, and IL-8 strongly correlate with how severe DR is in the patient (B.T. Ozturk et al., 2009).

Both MCP-1 and RANTES are classified as CC chemokines and have specialized functions. In an hypoxic retina, MCP-1 can produce monocytes as an immune response and is primarily produced by retinal endothelial cells. This can also be proven to occur within PDR (Mitamura et al., 2001). In contrast, RANTES is stimulated by inflammatory responses and inflammatory cells. In short, RANTES can also be initiated by RPE and retinal endothelial cells and in a diabetic model, can be correlated with neovascularization in the retina (Kim et al., 2003; Khodarev at al., 2003). Consequently, another study was able to identify RANTES accumulated in a diabetic retina

compared to a non-diabetic patient. A study conducted by Maier et al., 2008, elucidated there is an influx and upregulation of MCP-1 within the human vitreous samples and an upregulation of RANTES in vitreous sample serum; that same study confirmed the presence of an inflammatory and angiogenic response in diabetic patients with DR (Maier et al., 2008). Moreover, MCP-1 can be classified as a regulator of photoreceptor apoptosis in multiple retinal diseases, leading to MCP-1 serum being present in type 2 diabetes.

2.5 Induced Pluripotent Stem Cells

Stem cells have been used with more popularity within the last decade aside from the moral and ethical standpoints. In stem cell research, stem cells can be identified in various organs and tissues within the human body. Stem cells that originated within embryonic development will technically stay within our body throughout our lifetime as differentiated cells. While stem cells can come directly from embryos before implantation occurs, they can also be derived from human blood by a peripheral blood stem cell collection, bone marrow by a bone marrow harvest, by tissue-specific stem cells, or can be classified as induced pluripotent stem cells (iPSC).

Embryonic stem cells (ESC) are harvested from the blastocyst once an ovum has been successfully fertilized by a sperm after intercourse. Once the ESCs have been harvested and grown on to a cell culture dish, they will remain at ESCs. In comparison, if these cells would have remained in the ovum, they would have propagated and produced the human organs and tissues needed for a human to survive. ESC were initially created within mouse embryos, eventually leading to human studies (Kumar et al., 2020). While ESC would be highly useful in regenerative medicine as treatment for degenerative disease such as Alzheimer's and Parkinson's Disease, they

remain morally and ethically controversial due to removing a 2-6-day old embryo as a therapeutic treatment and scientific gain. While this type of regenerative medicine can be utilized in disease-specific therapies, there have been various bioethical disputes, hindering this medical advancement. For this reason, scientists turned to other forms of stem cells such as tissue specific or induced pluripotent stem cells (iPSC). iPSC can be a link for future studies in identifying the mechanistic features of diseases, in addition to pharmaceutical advances. While iPSC can be utilized instead of ESC, they do not replace them; however, this can be identified by using microarray analyses.

Tissue specific stem cells can be classified as adult stem cells. These stem cells can be derived from human blood by a peripheral blood stem cell collection or bone marrow by a bone marrow harvest. An example of this would be mesenchymal cells; they are located within bone marrow and can promote the growth of fat and bone, giving rise to other cells and tissues. This is done by removing healthy bone marrow when no cancer is identified. In comparison, induced pluripotent stem cells are manmade in a lab by utilizing another cell type. For instance, iPSC are initiated using viruses in order to add genes, resulting in tissue-specific cells. This type of stem cells are created by utilizing peripheral blood mononuclear cells or human skin fibroblast cells. This induction is exhibited by using the genetic reprograming of Oct4, Sox3, Klf4, and c-Myc and were able to successfully propagate mouse iPSC (Han & Yoon, 2011; Rodolfa, 2008).

iPSC have been used more recently because of its easy accessibility and not proving any ethical issues. These stem cells have been proven to work well with CRISPR/Cas9. While doing any type of regenerative therapies, there are always going to be risks and challenges to face along

the way. While stem cell transplants have been used widely as a therapeutic treatment for cancer, these transplants can also help with other diseases. Induced pluripotent stem cells have been used widely in various aspects on research. As previously mentioned, iPSC are derived from skin cells such as fibroblasts. Once the skin fibroblasts have been isolated, they will then be reprogrammed after being sustained successfully in culture. The cell reprogramming can be done in more than one way; by nuclear transfer, cell fusion, direct reprogramming, or done so by cell explanation.

In short, nuclear transfer involves the removal of genetic material from the female oocyte and will be replaced the new fibroblast that was differentiated prior. In comparison, cellular fusion is a type of hybrid cell that contains the capacity of both embryonic stem cell and somatic cells, resulting in tetraploid stem cell lines to be generated and used for scientific research. Nuclear transfer became more widely known after being used with Dolly, which lead to the successful generation of another adult by a cell from another (Wilmut et al., 1997). This experiment lead to *in vitro* feralization, which helps numerous individuals conceive when they cannot do so naturally. Prior to the Dolly experiment, nuclear transfer experiments occurred in various frog species and showed as the donor nucleus increased, it was possible to obtain tadpoles from terminally differentiated cells (Briggs and King, 1952; Gurdon et al., 1975). After the success of the Dolly experiment, several mouse studies were conducted concerning the properties of SCNT-derived ES (ntES) cells and that of proof-of-concept demonstrations for regenerative medicine. Research done by Egli et al. Illustrated how polyspermic zygotes can be used for successful nuclear transfer (Egli et al., 2007). Although there are many difficulties and challenges that come with nuclear transfer, it remains to be a reliable technique when not only producing

healthy clones, but also producing pluripotent stem cells identical to the fertilized embryo. Another useful cellular programming technique is cellular fusion.

Cellular fusion remains to be a useful cellular programming technique in which the fusion of somatic cells or cell lines are used in order to investigate a phenotypic trait at a cellular level (Rodolfa, 2008). Early cellular fusion studies demonstrated some cellular identities could predominate others in a hybrid (Blau et al., 1985; Boshart et al., 1993). This finding inspired other research concerning the mechanisms of cell fate and the decisions that were made in the process of development. Research done by Miller and Ruddle would form an observation that perhaps a pluripotent phenotype is able to dominate when an embryonal carcinoma was fused with murine somatic cells (Miller and Ruddle, 1976). Researchers would hope that this method might be an alternative to nuclear transfer, however, subsequent programming of ES and somatic cell fusion has proven to still be inefficient (Hochedlinger and Jaenisch, 2006; Tada et al., 2001). One major challenge with fusion is the production of patient-specific stem cells. Research has been done to try to mitigate this, but more research is necessary to ensure the removal of the embryonic stem cell genome does not cause genomic rearrangement or instability (Rodolfa, 2008).

In comparison, direct reprogramming is a concept that was initially constructed by Dr. Waddington (Waddington, 1957). Thus, when in cell culture, cell culture media can help stimulate self-renewal. This process promotes retroviral deprogramming leading to the induction of the various transcription factors that help remove the fibroblastic morphology and physiology, promoting iPSC (Rodolfa, 2008). While the differentiation of iPSC and use of embryonic stem cells have been used in previous studies, there is very little evidence stating iPSC can be

generated by explanting cells and putting them in the corresponding cell culture conditions. However, there have been a few studies done by Guan et al., 2006 and Kanatsu-Shinohara et al., 2014 that identified iPSC can be extracted from adult testes and also extracted neonatally. These investigations elucidated the possibility of iPSC being extracted directly form the organ and proceeding to propagate when placed in the appropriate cell media. When taking phenotypic characteristics in to considerations, the cells that were extracted from male testes will naturally have male imprints compared to embryonic stem cells preserving the original somatic imprints and developing in to expected adult tissues and organs.

The use of Oct4, Sox3, Klf4, and c-Myc can contribute to an individual's risk of cancer. In new research investigators used *OCT4, SOX2, NANOG,* and *LIN28* in order to successfully reprogram somatic cells to be differentiate to iPSC and share ESC characteristics (Yu et al., 2007). In order to begin the process of producing iPSC, various genes were identified to initiate the reprogramming process. The protocol to induce pluripotency through reprogramming has been used within various types of cell lines, however dermal fibroblasts have remained to be the more popular choice due to their high success rate of reprogramming (Kumar et al., 2020). Takahashi et al., aimed to use human somatic fibroblast cells in order to propagate iPSC by utilizing retroviral transduction. Once *OCT4, SOX2, NANOG,* and *LIN28* genes were identified, they were then to be duplicated within a viral vector; the virus that is commonly used is the lentivirus. This is primarily done for homologous recombination. These 4 genes were selected because of their possibility of being able to reprogram cells that have phenotypes associated with mesenchymal cells. Investigators primarily chose IMR90 because of past publications of DNA

fingerprints and reprogrammed clones, in addition to diploid human cells being characterized by the ENCODE Consortium. Characteristics of IMR90 cells have identified slow passage growth which also aids in compact colonies, ultimately leading to better iPSC reprogramming and morphology began to be visible after ~2 weeks and propagated colonies at ~3 weeks. Moreover, IMR90 cells underwent transduction with *OCT4, SOX2, NANOG,* and *LIN28,* leading to IMR90 derived iPSC.

The iPSC that were used in this Master of Biochemistry and Molecular Biology Thesis initially had the induced pluripotent markers, telomerase activity and gene expression that would be expected from human embryonic stem cells, whilst having the ability to initiate differentiation to Retinal Pigmented Epithelium by Dr. Alberto Muniz . iPSC have the potential to be used for various types of research studies, especially regenerative medicine, human disease models, new drug developments, and cell transplantation. This can be done because of the ability these cells have to be used in various organs. The main cause of concern for iPSC transplantation is transplant rejection because of immune rejection and mutations occurring. Therefore, patient derived iPSC can be used more reliably because the cells are already coming from the host, reducing the probability of transplant rejection.

CHAPTER III

Materials and Methods

3. 1 Cell Culture and Differentiation of iPSC Cells

Human iPS-RPE were cultured on Matrigel-coated six-well plates. Cell culture medium was changed daily until cells showed confluency for passaging. The enriched iPS-RPE was then seeded and cultured in fetal RPE media composed of MEM, N1 supplement, glutamine, nonessential amino acids, taurine 0.25 mg/mL, hydrocortisone 10 ng/mL, triiodothyronine 13 ng/mL, and 15% fetal bovine serum (FBS). The seeding density at each passage after enrichment was 1 x 10^5 cells/cm2, as mentioned by Muniz et al., 2014. Cells were allowed to grow until approximately 80% confluent and split accordingly in to 6 well plates after initial thawing. For experiments, iPS-RPE at passages four and five were cultured in 24 well plates containing 1mL fetal RPE media for up to 4 months prior to the experiment. The cell culture media was changed every 2 days. These cells were previously RPE initiated by Dr. Alberto Muniz and were kept in cryopreserve. After thawing, they were maintained in RPE media until ready for experiments.

3.2 ARPE19 Cell Culture

The ARPE19 cell line was commercially obtained from ATCC. These cells were cultured in Cells were then cultured for 24 hours with 1mL of Dulbecco's Modified Eagle's Medium, 15% Fetal Bovine Serum and 1% Penicillin/Steptomycin. Cells were seeded at 100,00 cells per plate until reaching confluency to be passaged at P5.

3.3 iPS-RPE Cell Culture Treatments

iPS-RPE cells were passaged and seeded as P5 on two 24 well plates at 20,000 cells per well and cultured up to four months prior to experiments. Cells were treated with Lipopolysaccharide (LPS; 10µg/mL), Cobalt Chloride (1mM/mL) 10ng/mL of TNFa or IL-8 (12.5ng/mL or 25ng/mL) with 0 mM/mL, 5.5mM/mL, or 30mM/mL of glucose conditioned complete RPE media.

3.4 Hydrogen Peroxide Treatment

Cells were trypsinized utilizing Trypsin + EDTA and seeded in to a 24 well plate. Passage 5 of ARPE19 cells were harvested from 1,000,000 in 12 wells. Cells were then cultured for 24 hours with 1mL of Dulbecco's Modified Eagle's Medium and Fetal Bovine Serum. After being incubated for 24 hours with 5% CO_2 at 37°C, the culture media was aspirated and changed to serum free media and cells were then treated with 0mM, 400mM, 600mM and 800mM of hydrogen peroxide for two hours in order to induce oxidative stress.

3.5 Immunohistochemistry

iPS-RPE cells were washed with PBS and fixed at room temperature in 4% paraformaldehyde for 10 minutes. Cell culture slides of tissue were incubated in 3% BSA in PBS for 1 hour followed by incubation in 1:250/1:100 for 5ug/mL dilution of primary antibody either 1 hour at room temperature or overnight at 4°C. Immunocytochemistry was performed using standard procedures with RPE65 antibody (1:250, MAB5428; Millipore) or ZO-1 antibody (1:100, AB-2532187; Thermo Fisher) applied overnight at 48C. Primary antibody was removed by washing 3X quickly, then 3X for 10 min each in 3% BSA in PBS. Goat anti-mouse IgG Alexa Fluor 568 from Molecular Probes (1:50, cat. No. A11031; Life Technologies), was used as the secondary antibody. Cell culture slides were imaged using an Olympus FV10i confocal microscope. Protocol for Antibody staining was provided by ThermoFisher Scientific.

3.6 Cell Viability Assay

In order to determine the iPSC-RPE cell proliferation and viability, cells prior to treatment, 12 hours after treatment and 72 hours after treatment was identified using the Trypan Blue Method. iPSC-RPE were trypsinized (trypsin + EDTA) and harvested after 12 and 72 hours; the total cell numbers were quantified using a hemocytometer and a cell counter.

3.7 Chemokine and Cytokine Quantification

At the end of treatments, cell culture media supernatants were collected and stored in -80°C. The amount of secreted MCP-1 and RANTES were identified using a multiplex ELISA (BioRad).

CHAPTER IV

RESULTS

4.1 Antibody Stain-Immunohistochemistry

Immunohistochemistry (IHC) was done in order to determine if iPSC-RPE had completely differentiated to RPE. Primary antibody such RPE65 was followed by Goat anti-mouse IgG Alexa Fluor 568 from Molecular Probes and ZO-1 was utilized as a primary conjugated antibody. While RPE65 was not be closely identified but could clearly see tight junctions present as well as morphological characteristics in cell passage 5, 3 months in to culture. ZO-1 conjugated antibody was then used to distinguish if tight junctions were present in experimental cells in cell passage 5, 4 months in to culture. Tight junctions were strongly identified and could distinguish between which cells were surrounding fibroblasts and RPE. This was utilized as a confirmatory examination of the iPSC to confidently determine the RPE differentiation.

4.2 iPSC-RPE Viability

The iPSC-RPE cells were in culture for 4 months at cell passage 5 and treated with10ng/mL and 25ng/mL of TNF-**a** and IL-8 in LPS and Cobalt (II) Chloride treated with 0mM, 5.5mM, and 30mM of glucose**.** In order to determine the iPSC-RPE cell proliferation and viability, cells prior to treatment, 12 hours after treatment and 72 hours after treatment was identified using the Trypan Blue Method. iPSC-RPE were trypsinized (trypsin + EDTA) and harvested after 12 and 72 hours; the total cell numbers were quantified using a hemocytometer and a cell counter. Instead of the expected amount of cell death in either the proinflammatory or the hypoxic conditions, various wells proceeded to propagate and continue to grow on the RPE monolayer.

Within the proinflammatory environmental cells conditioned with LPS, there was a decrease in cell viability in the wells treated with 10ng/mL of TNF-a in 5.5mM/mL and 30mM/mL of glucose. These few wells decreased about 20,000 cells after 72 hours. However, the wells that were treated with 12.5ng/mL and 25ng/mL of IL-8, all increased in either glucose conditions. One well in particular that was treated with 12.5ng/mL in 30mM of glucose went from 11,700 cells at 12 hours to 2,060,000 cells at 72 hours. In comparison, another well was treated with 25ng/mL of IL-8 and increased from 11,700 cells at 12 hours to 258,000 at the end of treatment at 72 hours.

Consequently, there was also an increase of cell viability within the hypoxic environmental conditioned cells with cobalt chloride from the start of treatment at 12 hours to the end at 72 hours. There was an increase in cell viability in all wells treated except the well that was treated with 10ng/mL of TNF-a in 5.5mM/mL, which validates the cell viability in the proinflammatory environment treated with 10ng/mL of TNF-a in 5.5mM/mL.

Based on cell viability numbers, there is conclusive data elucidating IL-8 contributing to

increased viability of iPSC-RPE cells in proinflammatory and hypoxic conditions with various glucose concentrations.

4.3 Chemokine and Cytokine Quantification

To determine the amount of MCP-1 and RANTES from both iPSC-RPE and ARPE19, cultured conditioned media from cells treated with TNF-α and IL-8. A MultiPlex from BioRad was used to determine the amount of the observed concentration of MCP-1 and RANTES was secreted before, during, and after treatment of 10ng and 25ng/mL of TNF-α and IL-8 in LPS and Cobalt (II) Chloride treated with 0mM, 5.5mM, and 30mM of glucose over 12, 24, 48, and 72 hours. The iPSC-RPE cells were in culture for 4 months at cell passage 5. After 72 hours of treatment, MCP-1 was expressed in higher concentrations over the 12 and 24 hours more than 48 and 72 hours in LPS cultured conditioned media. In comparison, the cultured conditioned media treated with Cobalt (II) Chloride had a higher concentration of RANTES secreted than RANTES in LPS. Cell samples were taken at immature iPSC-RPE 2 weeks in to cell culture, mature iPSC-RPE 1 week before treatment and 12, 24, 48, 72 hours after treatment. In the raw data, MCP-1 was expressed at an increased value compared to RANTES which was not as elevated. In order to determine the significance of the values between the expression of MCP-1 and RANTES, the means were compared using a Two Sample T-Test. This was able to statistically determine MCP-1 expression are significant in the various glucose conditions compared to those expressed by RANTES at various glucose conditions. To analyze the main and interaction effect, all different variables were assayed and time values using a Two Way ANOVA. In the various time conditions, RANTES showed significant values at 72 hours whereas MCP-1 expressed significant values at 12 hours.

CHAPTER V

DISCUSSION

This study was conducted in order to determine the expression of chemokine and cytokines MCP-1 and RANTES in different diabetic glucose conditions in iPSC derived RPE cells. This study was essential in understanding possible therapeutic treatment and models for AMD and other types of degenerative diseases. The long term goal for this project is to determine if IL-8 had any type of cell protectant against various conditions. In order to determine the susceptibility RPE cells have in various conditions, such as LPS for proinflammatory conditions and cobalt chloride for hypoxia, the cells were analyzed with an IHC and MultiPlex Panel ELISA. A Two Sample T-Test was done in order to compare the two means for the two different groups; moreover, the two means of RANTES and MCP-1 expression are significant in the various glucose conditions. In addition, the results from a Two Way ANOVA calculated the main effect and an interaction effect. Thus, with the interaction effect, all factors are considered at the same time and the MCP-1 secretions for 12 hours was significant whereas, RANTES expression was significant at 72 hours.

Due to the iPSC-RPE being previously initiated to begin RPE differentiated, IHC was needed in order to determine if the cells had been successfully differentiated. Figure 1 can elucidate the process of deafferentation the RPE underwent. When the cells were initially seeded from the cyrotank, they had no classis RPE morphology and in turn, obtained fibroblastic characteristics. Cells obtained from Dr.Muniz and previously differentiated methods described in Muniz et al., 2014 were used for these iPSC-RPE treatments. As described by Muniz et al., 2014, the hexagonal morphology was lost after passaging and there was no initial sign of pigmentation.

The anticipated and expected RPE morphology was reestablished after confluency was already reached. Figure 1 describes what morphology the cells had at different time points after the initial thawing. As described by Muniz et al., 2014, the iPSC-RPE gained fibroblast characteristics after thawing and remained elongated for some time. Figure 1A, the iPSC-RPE were already confluent but showed no signs of tight junctions or any RPE morphology. Figure 1B and 1C was taken one week after the first passaging and one month after the initial thawing. There is clearly gradual changes of morphological characteristics. The cells began to lose fibroblastic morphology but not yet gain any definitive RPE morphology such as tight junctions or hexagonal cell shapes. Once the cell culture at gained more confluency and larger colonies 6 weeks in to cell culture, RPE characteristic began to arise. In Figure 1D, there is the presence of tight junctions and some hexagonal cell shape.

Moreover IHC was done on iPSC-RPE cells after confluency was reached at passage 5. In figure 2A, the RPE65 gene transcript was not identifiable after 2 months in culture. However ZO-I was used in order to identify the tight junctions that are accompanied in RPE cells. Figure 2B can demonstrate the iPSC-RPE tight junctions. While the tight junctions can be identified by morphology on its own, ZO-I IHC was done as a confirmatory test. Figure 2 shows two different immunohistochemistry (IHC) experiments being conducted. Figure 2A shows IHC done with RPE65 after about 6 weeks of culture and Goat anti-mouse IgG Alexa Fluor 568 from Molecular Probes was used as the secondary antibody. There was nothing distinguishing the expression of RPE65, however, there was clearly expected RPE morphology, colonies, and the presence of tight junctions. In comparison, Figure 2B was done after 3 months in to culture. In comparison, figure 2A shows more uniform iPSC-RPE cells, whereas Figure 2B shoes the positive expression of ZO-1 identifying tight junctions with less uniformed RPE hexagonal cell shape.

TNF-a and IL8 treatment, cell viability was determined of iPSC-RPE in either no glucose,

low glucose or high glucose conditions, in a proinflammatory or hypoxic environment at the 12 and 72 hour marks. The cell viability determined the wells that were either treated IL8 at 12.5ng/mL or 50ng/mL continued to grow and remain viable despite being in hypoxic and proinflammatory conditions in Figures 3A and 3B. In the proinflammatory conditioned plate with LPS, it was identified the wells that were treated with 5.5mM or 30mM of glucose and 15.5ng/mL or 50ng/mL of IL-8, remained viable. After the 72 hours, 2 of the wells continued to have the iPSC grow on to the monolayer and needed to be manually detached. In comparison, the hypoxic condition had very little differences from the proinflammation conditioned plate in regards to viability and cell numbers. This can support that IL8 can be used as a possible form of treatment for AMD or as an RPE protectant against transplant rejection. Taking in to consideration the function IL-8 has within the immune system as a proinflammatory marker, IL-8 might also be able to be utilized as an anti-inflammatory in iPSC-RPE. In comparison, the wells that were treated with 10ng/mL of TNF-a, with 5.5mM or 30mM of glucose and cobalt chloride for an hypoxic environment, the cell viability either went down or had no change at all.

Moreover, in order to identify the amount of MCP-1 and RANTES being secreted and expressed by iPSC-RPE cells, MultiPlex assay was used. Multiplexing allows for multiple biomarkers and targets to be detected in a single sample. Bio-Plex speeds up the experimental time and labor costs because only one single experiment needs to be run instead of multiple single ELISAs. Unlike most traditional ELISAs, the Bio-Plex is completed in solution. The magnetic beads are covered with covalently linked antibodies which provides a greater surface area for target capture and the capture is done in a free solution environment instead of a coated surface(Houser B., 2012). Cell samples were taken at immature RPE 2 weeks in to cell culture, mature RPE 1 week before treatment and 12, 24, 48, 72 hours after treatment. In the raw data, MCP-1 was expressed at an increased value compared to RANTES which was not as elevated. Additionally,

some samples such as the wells treated with TNF-a were out of range OOR, ULOQ (upper limit of quantification) and/or greater than the closest in range standard or identified as below that standard of range such a RANTES expression in both TNF-a and IL-8 treated wells. These conditions were TNF-a in high glucose conditions in a proinflammatory environment, IL-8 12.5ng/mL and 25 ng/L with no glucose and high glucose in a hypoxic environment, respectively.

Figure 4 describes the expression of MCP-1 and RANTES over 12 hours in a proinflammatory environment treated with 10µg/mL LPS or in a hypoxic environment treated with 1mM/mL cobalt chloride. In Figure 4A, it can be inferred that the high glucose induced a higher expression and increase of MCP-1 being secreted by the iPSC-RPE cells. In comparison, 10ng/mL of TNF-a also was secreted in higher amounts with 0mM/mL and 5.5mM/mL of glucose treated media. However, the secretion of MCP-1 was different in either concentrations of IL-8. At 12.5ng/mL, the expression of MCP-1 remained consistent in all 3 glucose concentrations whereas with 25ng/mL of IL-8, the expression of MCP-1 was lowest at 5.5mM of glucose conditioned media. Figure 4B identified the expression of RANTES with 10µg/mL LPS conditioned cell culture media. There was no expression of RANTES with the cells treated with 10ng/mL of TNF-a. However, the wells that were treated with 12.5ng/mL or 25ng/mL of IL-8 had high expressions of RANTES in 0mM/mL, 5.5mM/mL, and 30mM/mL of glucose conditioned media. Figures 4 C and D elucidated the expression of MCP-1 and RANTES in hypoxia conditioned media with 1mM/mL cobalt chloride. MCP-1 was expressed in all treatments with TNF-a and IL-8 but varies in amount that were expressed. The lowest MCP-1 expression was in the 5.5mM of glucose conditioned with 10ng/mL of TNF-a. In comparison, the highest expression of MCP-1 fell under 30mM/mL glucose conditioned media also with 10ng/mL of TNF-a. Similarly, RANTES expression was also noted but at higher levels. The highest expressions was in 5.5mM/mL of glucose treated media and treated with 10ng/mL of TNF-a and 12.5ng/mL of IL-8. the results from

a Two Way ANOVA calculated the main effect and an interaction effect; all factors are considered at the same time and the MCP-1 secretions for 12 hours was significant compared to RANTES.

Figure 5 describes the expression of MCP-1 and RANTES over 24 hours in a proinflammatory environment treated with 10µg/mL LPS or in a hypoxic environment treated with 1mM/mL cobalt chloride. In comparison to figure 5, Figure 5A identifies the expression of MCP-1. All 3 glucose concentrations showed various MCP-1 activity. The highest values for figure 5A were the wells treated with either 0mM/mL or 30mM/mL of glucose, treated with 10ng/mL of TNF-a, 12.5ng/mL or 25ng/mL of IL-8. In comparison, RANTES expression in a proinflammation environment showed very little expression but similarly only had expression when treated with 5.5mM/mL or 30mM/mL of glucose conditioned media and 12.5ng/mL or 25ng/mL of IL-8, which is identified in figure 5B. When treated with 1mM/mL cobalt chloride in order to induce hypoxia, the values in figure 5C remained to be expressed at lower and decreased values. 10ng/mL of TNF-a gave the highest expression of MCP-1 at almost 10,000ng/mL whereas the second highest expression was also 10ng/mL of TNF-a, at slightly over 2,000 ng/mL. All wells treated with 12.5ng/mL or 25ng/mL of IL-8 remained to have decreased values over 24 hours in an hypoxic condition compared to the other proinflammatory environment treated wells. Moreover, the expression of RANTES was also elevated compared to the proinflammatory environment treated wells, specifically the wells treated with 10ng/mL of TNF-a. it was identified over the 24 hours, 10ng/mL of TNF-a was able to induce more expression in the hypoxic environment compared to the proinflammatory environment treated wells, which was also noted at 12 hours, but not as comparable by the expression of MCP-1 with cells treated with 10µg/mL LPS to induce a proinflammatory response.

In comparison to figures 4 and 5, the expressions of both MCP-1 and RANTES decreased significantly from both 12 and 24 hours to 48 hours. Figure 6 describes the expression of MCP-1

and RANTES over 48 hours in a proinflammatory environment treated with 10µg/mL LPS or in a hypoxic environment treated with 1mM/mL cobalt chloride. Figure 6A depicts all concentrations of 10ng/mL of TNF-a and 12.5ng/mL of IL-8 treated wells with 0mM/mL, 5.5mM/mL, and 30mM/mL of glucose conditioned media secreted MCP-1 in a proinflammatory condition. In comparison, figure 6B can clearly see a decreased expression of RANTES except in 30mM/mL of glucose conditioned media. 10ng/mL of TNF-a and 12.5ng/mL of IL-8 treated wells showed some expression of RANTES with 30mM/mL of glucose conditioned media. With the wells treated with 1mM/mL cobalt chloride to induce hypoxia, all 10ng/mL of TNF-a and 12.5ng/mL of IL-8 treated induced MCP-1 expression but saw the highest at 10ng/mL of TNF-a with 30mM/mL of glucose conditioned media, as seen with figure 6A. Lastly, figure 6D showed an increase of RANTES treated with 1mM/mL cobalt chloride to induce hypoxia compared to the wells treated with 10µg/mL LPS or in a hypoxic environment. 6D identified the wells treated with 10ng/mL of TNF-a in all 0mM/mL, 5.5mM/mL, and 30mM/mL of glucose conditioned media was higher than well treated with either 12.5ng/mL or 25ng/mL of IL-8.

At the end of treatment, cell culture conditioned media was collected. There was a significant decrease is MCP-1 expression from 12 hours to 72 hours. However the expression of MCP-1 in figure 7A caused by 10ng/mL of TNF-a in 30mM/mL of glucose conditioned media remained consistent throughout all serum collections and was only expressed in 5.5mM/mL and 30mM/mL of glucose conditioned. 7B identified RANTES being expressed by 10ng/mL of TNF-a, 12.5ng/mL or 25ng/mL of IL-8 but at different glucose concentrations. 25ng/mL of IL-8 showed expression of RANTES when in 0mM/mL of glucose conditioned media. In comparison, 10ng/mL of TNF-a in 5.5mM/mL of glucose conditioned media caused an increased amount of RANTES to be expressed. In 30mM/mL of glucose conditioned media, 12.5ng/mL of IL-8 resulted in RANTES expression. Both figured 7C and 7D identify the expression of MCP-1 and RANTES in

hypoxic conditions by treating with 1mM/mL cobalt chloride. The expression of MCP-1 in cobalt chloride was significantly higher when treated with .5ng/mL of IL-8 when treated with 0mM/mL of glucose conditioned media. In comparison, the expression of RANTES was elevated in all 0mM/mL, 5.5mM/mL and 30mM/mL of glucose conditioned when treated with 10ng/mL of TNF-a. the results from a Two Way ANOVA calculated the main effect and an interaction effect and identified RANTES expression was significant at 72 hours comparable to MCP-1 expression at 12 hours.

Both MCP-1 and RANTES was expressed but differed significantly in expression. MCP-1 was found in almost all the wells and was also elevated above range whereas RANTES was identified to be secreted below the standard ranges. In the anticipation from studies done by Maier et al., 2008, the expression of both MCP-1 and RANTES in iPSC-RPE can be comparable to the influx and upregulation of MCP-1 within the human vitreous samples and an upregulation of RANTES in vitreous sample serum; that same study confirmed the presence of an inflammatory and angiogenic response in diabetic patients with DR. In comparison, the studies done by I.J Crane et al., 2000, proposes the levels of these cytokines, such as MCP-1, TNF-a, and IL-8 strongly correlate with how severe DR is in the patient.

Standard curves are important in highlighting the relationship between two quantities, in this case the fluorescence intensity and concentration which can be described in Figure 8. Here, a standard curve was done for both MCP-1 and RANTES. The observed expression of the majority of the wells were within the standards for the exception of a few samples' RANTES expression which fell below the expected standard.

Lastly, in order to identify the significance between expressions of RANTES and MCP-1, statistical analysis was conducted. A two way ANOVA was performed comparing various glucose levels in LPS and Hypoxia conditions in the span of four time periods: 12hr, 24hr, 48hr, and 72hr.

Each ANOVA would be for a single time point and which MCP-1 or RANTES were expressed. Of the 8 ANOVAs performed, two were found to be statistically significant. The first, a 12hr ANOVA looking at MCP-1 expression had a p value of 0.014. The second, a 72hr ANOVA looking at RANTES expression had a p value of 0.0008. What this means is that in these two times looking at MCP-1 and RANTES expressions specifically, the groups differed significantly from the overall group mean.

Over the course of these experiments, this study was essential in understanding possible therapeutic treatment and models for AMD and other types of degenerative diseases with the use of possible chemokines that are naturally expressed within the human physiology. The long term goal for this project is to determine if IL-8 had any type of cell protectant against various conditions which is what can be hypothesized using the cell viability over the course of iPSC-RPE treatments. More research need to be done in order to determine the functionality of IL-8 as form of treatment for AMD models and other types of degenerative diseases

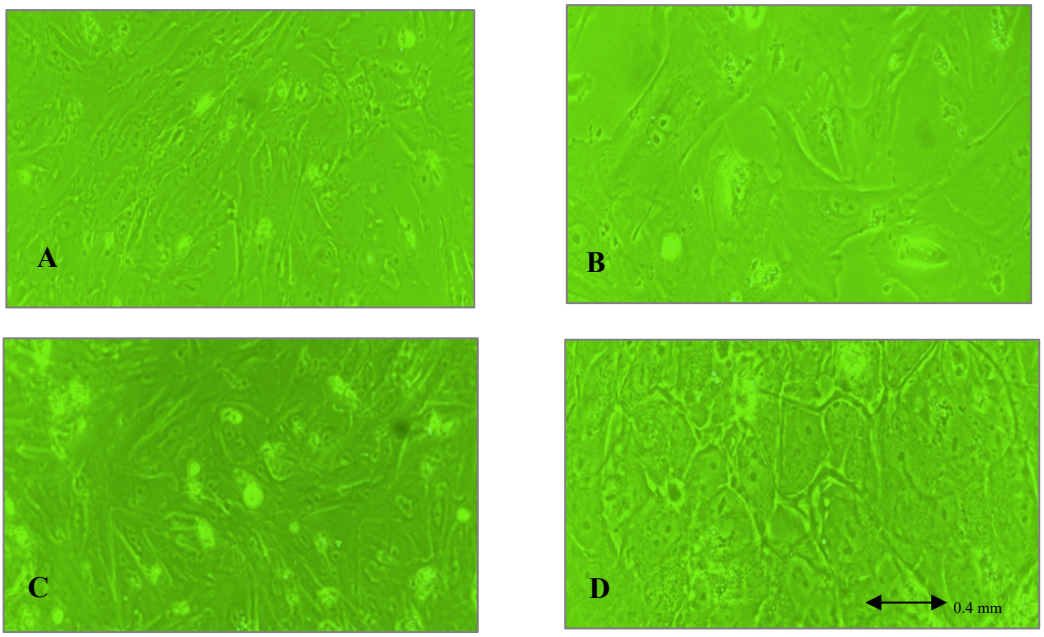


Figure 1: A: 5 days after thawing iPSC-RPE; **B:** 1 week after first passage of iPSC-RPE; **C:** one month after thawing iPSC-RPE; **D:** 6 weeks in culture showing some form of RPE morphology.

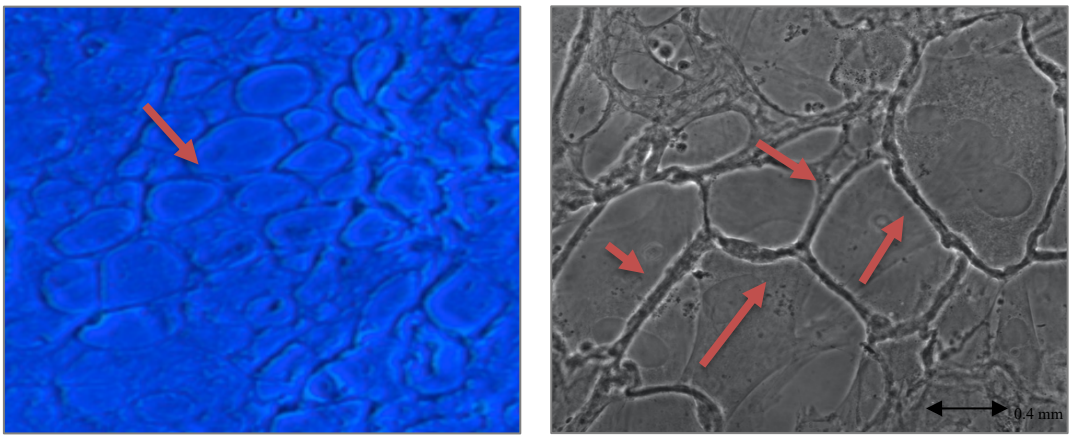
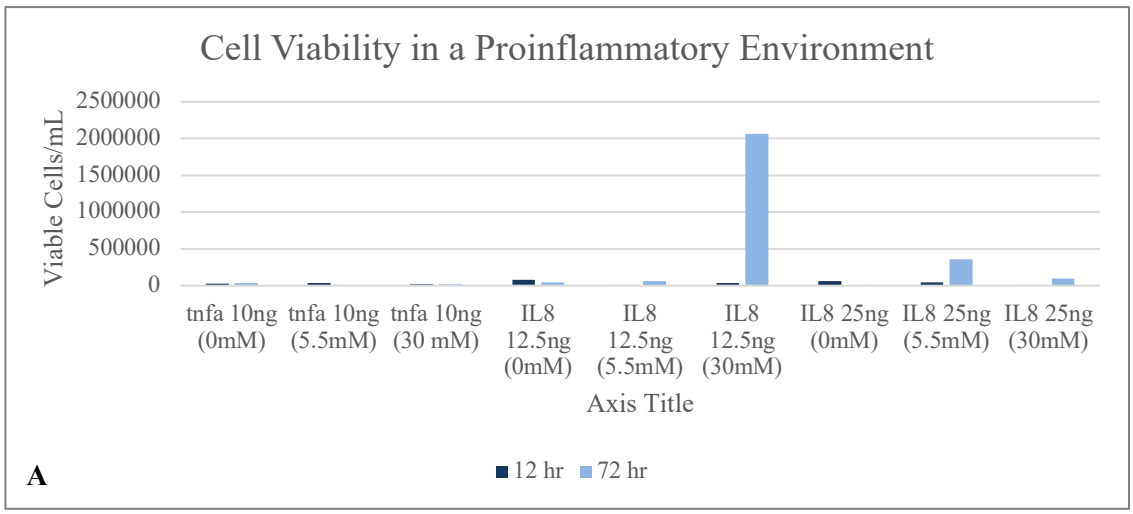


Figure 2: A: RPE65 IHC done of iPSC-RPE cell passage 5; **B:** ZO-1 IHC iPSC-RPE cell passage 5



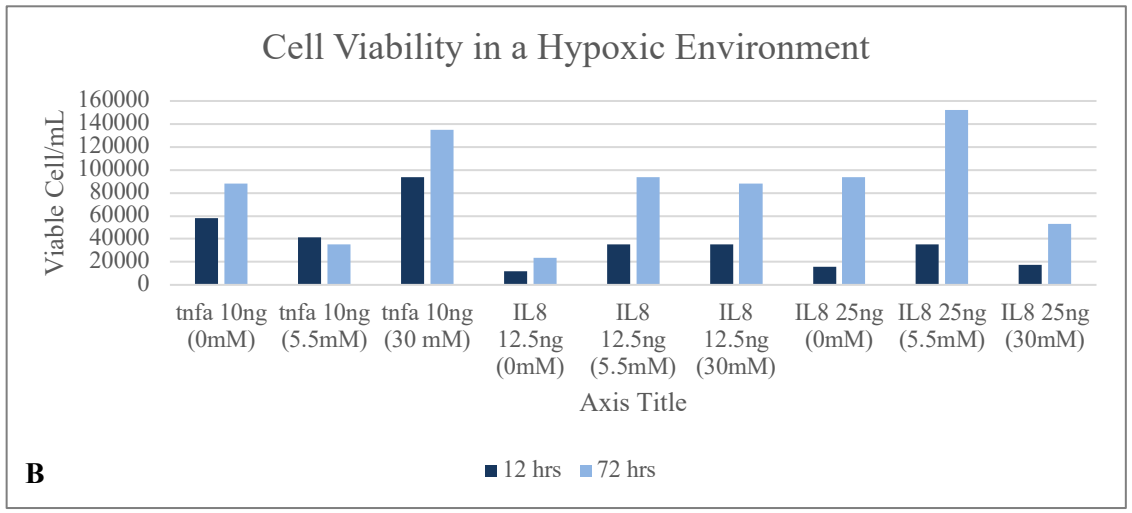


Figure 3: A: iPSC-RPE Viability in Proinflammatory Conditions after 12 and 24 hours; **B:** iPSC-RPE Viability in Hypoxic Conditions after 12 and 72 hours.

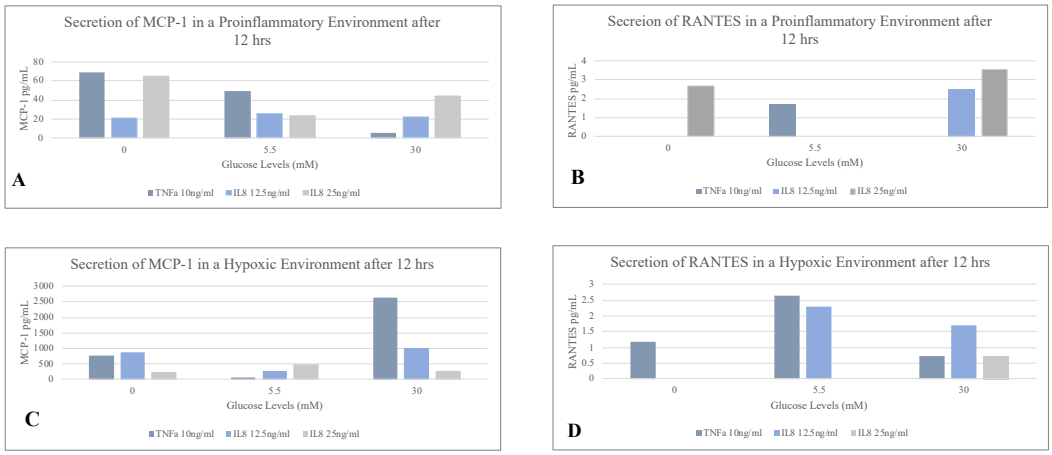


Figure 4: A: MCP-1 Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with LPS for a proinflammatory environment over 12 hours; **B:** RANTES Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with LPS for a proinflammatory environment over 12 hours; **C:** MCP-1 Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with 1mM/mL of Cobalt Chloride of for an hypoxic environment over 12 hours; **D:** RANTES Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with 1mM/mL of Cobalt Chloride for an hypoxic environment over 12 hours.

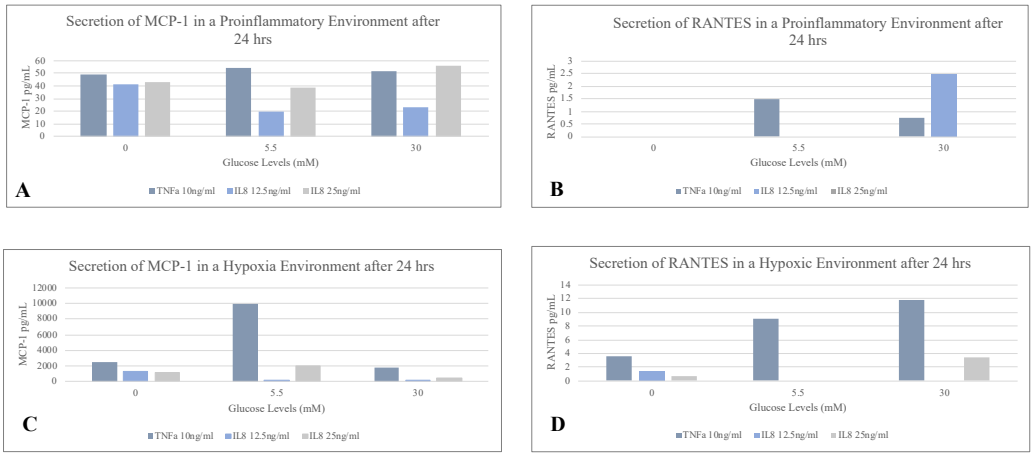


Figure 5: A: MCP-1 Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with LPS for a proinflammatory environment over 24 hours; **B:** RANTES Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with LPS for a proinflammatory environment over 24 hours; **C:** MCP-1 Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with 1mM/mL of Cobalt Chloride of for an hypoxic environment over 24 hours; **D:** RANTES Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with 1mM/mL of Cobalt Chloride for an hypoxic environment over 24 hours.

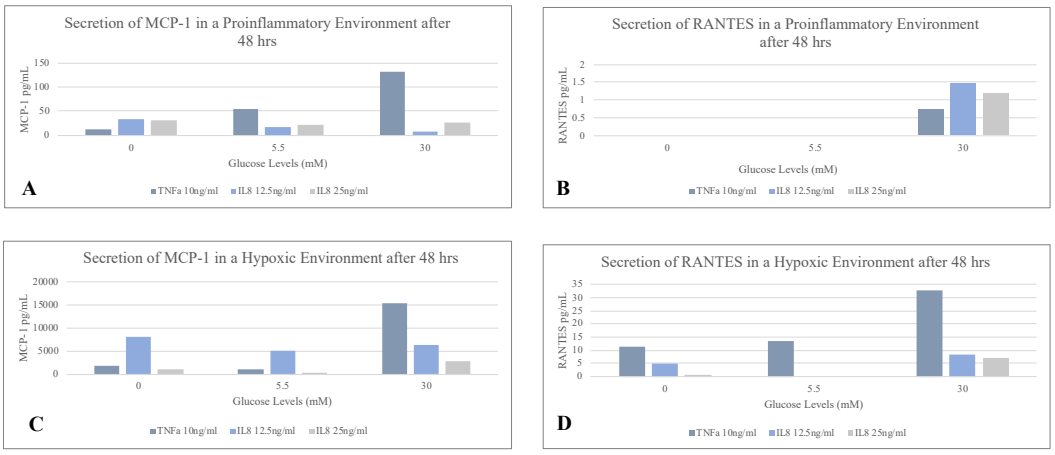


Figure 6: A: MCP-1 Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with LPS for a proinflammatory environment over 48 hours; **B:** RANTES Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with LPS for a proinflammatory environment over 48 hours; **C:** MCP-1 Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with 1mM/mL of Cobalt Chloride of for an hypoxic environment over 48 hours; **D:** RANTES Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with 1mM/mL of Cobalt Chloride for an hypoxic environment over 48 hours.

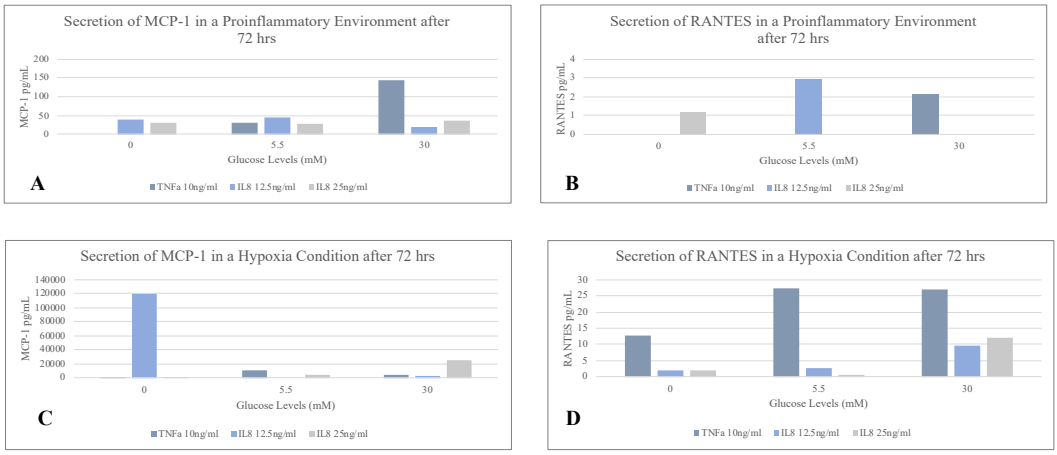


Figure 7: A: MCP-1 Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with LPS for a proinflammatory environment over 72 hours; **B:** RANTES Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with LPS for a proinflammatory environment over 72 hours; **C:** MCP-1 Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with 1mM/mL of Cobalt Chloride of for an hypoxic environment over 72 hours; **D:** RANTES Expression in iPSC-RPE cells treated with 10ng/mL of TNF-α, 12.5 ng/mL or 25ng/mL of IL-8, in either 0mM/mL, 5.5mM/mL, or 30mM/mL of media with 1mM/mL of Cobalt Chloride for an hypoxic environment over 72 hours.

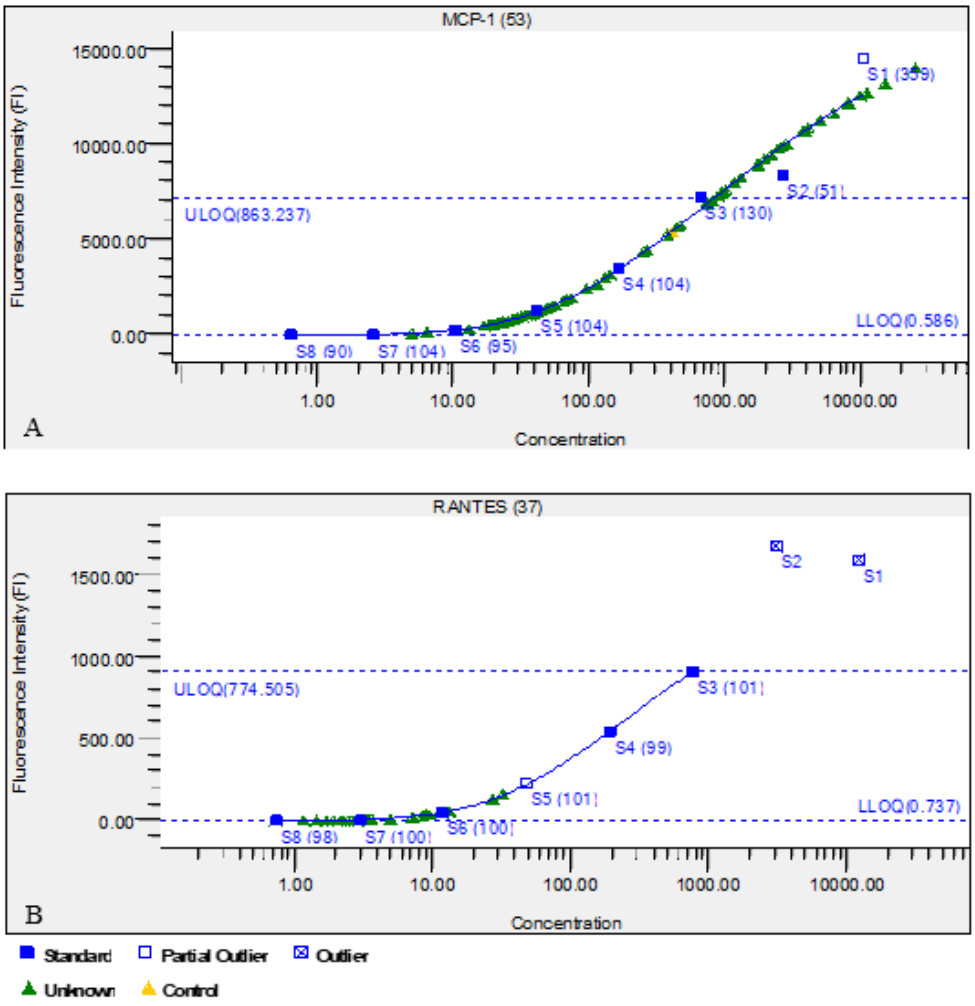


Figure 8: Standard Curves of MCP-1 and RANTES expression
A: MCP-1 Standard Curve-Regression Type: Logistic:5PL, Std. Curve: FI = 7.71204 + (18062 - 7.71204) / ((1 + (Conc / 1.97247)^-0.386148))^10, FitProb. = 0.0000, ResVar. = 10.9764;
B: RANTES Standard Curve-Regression Type: Logistic:5PL, Std. Curve: FI = 2.2546 + (1678.02 - 2.2546) / ((1 + (Conc / 3.26682)^-0.465368))^8.03738, FitProb. = 0.8546, ResVar. = 0.0336.

LPS	NG	LG	HG	Total
Count	3	3	3	9
Sum	155.49	97.86	97.86	351.21
Average	51.83	32.62	32.62	39.02333
Variance	701.2251	201.5472	201.5472	368.3359

Hypoxia	NG	LG	HG	Total
Count	3	3	3	9
Sum	1903.66	808.1	3914.65	6626.41
Average	634.5533	269.3667	1304.883	736.2678
Variance	111364.5	41896.19	1458353	609778.3

Total	NG	LG	HG
Count	6	6	6
Sum	2059.15	905.96	4012.51
Average	343.1917	150.9933	668.7517
Variance	146696.2	33653.79	1069018

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	2187674	1	2187674	8.139081	0.014544	4.747225
Columns	822006.4	2	411003.2	1.529107	0.256122	3.885294
Interaction	833730	2	416865	1.550916	0.251715	3.885294
Within	3225437	12	268786.4			
Total	7068847	17				

Table 1: MCP-1 expression ANOVA 12 hours

LPS	NG	LG	HG	Total
Count	3	3	3	9
Sum	1.17	2.95	2.13	6.25
Average	0.39	0.983333	0.71	0.694444
Variance	0.4563	2.900833	1.5123	1.283503

Hypoxia	NG	LG	HG	Total
Count	3	3	3	9
Sum	16.81	30.76	81.33	128.9
Average	5.603333	10.25333	27.11	14.32222
Variance	38.31613	216.9617	0	159.8578

Total	NG	LG	HG
Count	6	6	6
Sum	17.98	33.71	83.46
Average	2.996667	5.618333	13.91
Variance	23.66263	113.7249	209.6929

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	835.7235	1	835.7235	19.27501	0.00088	4.747225
Columns	389.4514	2	194.7257	4.491126	0.034992	3.885294
Interaction	379.3841	2	189.6921	4.375031	0.037409	3.885294
Within	520.2946	12	43.35788			
Total	2124.854	17				

Table 2: RANTES expression ANOVA 72 hours

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BIOGRAPHICAL SKETCH

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