Creation of an *in-vitro* Co-culture Model of Microglia and Retinal Pigment Epithelium Cells for Investigating the Innate Immune Mechanisms of the Retina

by

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Abstract

Background: The immune mechanisms of the retina in response to gene therapy have been previously understudied due to assumptions of ocular immune privilege. Ocular immune privilege is the concept that there is a tolerance or reduced immune response to antigen exposure in the eye. With the recent FDA and Health Canada approvals of the first ocular gene therapy, Luxturna, there has been a resurgence of interest in viral vector-based gene therapies for treating of ocular pathologies. Inherited retinal dystrophies (IRDs), in particular, of the outer retina may be treated through masking for the loss of function of the mutated gene with the introduction of the Wild Type (WT) copy. In treatment for IRDs such as choroideremia, adeno-associated virus serotype 2 (AAV-2) viral vector is delivered to the inner retina through a sub-retinal injection with the intent of targeting the retinal pigment epithelium (RPE) cells. Recent studies and clinical trials have reported poor outcomes as a result of this procedure for WT gene delivery which included inflammation causing unwanted damage to ocular tissues.

Purpose: The aim of this study was to create and test the immunological activity in an *in vitro* co-culture model using induced pluripotent stem cell (iPSC) derived RPE cells and SV-40 immortalized microglia to model interactions of two resident immune cells found in the retina. I hypothesize that the model will respond to treatment with pro-inflammatory master regulators IL-1β and TNF-α in a dose dependent matter including the secretion of pro-inflammatory cytokines.

Methods: iPSC RPE cells were grown on cell culture plates until the demonstration of pigmentation, polarization, polygonal morphology, and markers characteristic of RPE. SV-40 microglia cells were grown on permeable supports until mature. The cell types were combined by moving the permeable inserts into the cell culture plates with iPSC RPE cells to form the co-culture model. The model was treated with a series of pro-inflammatory stimulants including IL-1β, TNF-α and Poly(I:C) and inflammatory response was measured using an enzyme-linked immunosorbent assay (ELISA) and RT-qPCR to quantify secreted pro-inflammatory cytokines IL-6, IL-8 and CCL-2. Medium was sampled for ELISA analysis across 5 timepoints: at the time of treatment, 3 hours, 24 hours, 72 hours and 168 hours post treatment.

Results: Techniques were established in this study which increased the survival and maintenance of the mature monolayer of the RPE cells in both the presence and absence of microglial cells. These improvements were observed through visual inspection of cultured RPE cells using an EVOS cell Imaging System with images captured at 10x magnification. Upon testing the co-cultured RPE and microglia model with pro-inflammatory stimuli and using an ELISA assay and RT-qPCR to measure the secretion of pro-inflammatory cytokines (IL-6, IL-8 and CCL-2), no statistically significant differences were found between the untreated controls and pro-inflammatory treatments at each time point. Levels of IL-6, IL-8 and CCL-2 increased across timepoints, although treatment at all levels did not yield significant differences.

Conclusions: The model and alterations to RPE growth established in this study will aid in future iterations of study using this model. Further testing of the immunological features of this proposed model is required in order to establish whether or not it may provide insight into the immune mechanisms of the retina. It may be advisable to use co-stimulatory treatments or other pro-inflammatory stimulus to further test the immunological capabilities of this system *in vitro.*

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List of Abbreviations

AAV adeno-associated virus

CHM choroideremia (gene)

CHM Choroideremia (disorder)

CNS central nervous system

CXCL chemokine (C-X-C) motif ligand 1

DAMP damage associated molecular pattern

dsDNA double stranded DNA

dsRNA double-stranded RNA

DNA deoxyribonucleic acid

FBS fetal bovine serum

IF immunofluorescence

IFN interferon

IL interleukin

IRF interleukin regulatory factor

iPSC induced pluripotent stem cell

iPSC-RPE retinal pigment epithelium derived from induced pluripotent stem cells

IRD inherited retinal dystrophy

IRF interferon regulatory factor

MAP Mitogen-activated protein

MCP-1 Monocyte chemoattractant protein-1 (also known as CCL-2)

MHC major histocompatibility complex

MYD88 myeloid differentiation primary response 88

NF-kB nuclear factor kappa B

NSR neurosensory retina

PAMP pathogen associated molecular pattern

Poly (I:C) Polyinosinic:polycytidylic acid

RNA ribonucleic acid

RP retinitis pigmentosa

RPE retinal pigment epithelium

ssRNA single stranded RNA

TGF-β transforming growth factor β

TIR toll-interleukin-1 receptor

TIRAP TIR adaptor protein

TLR toll-like receptor

TNF-α tumor necrosis factor α

TRIF TIR-domain-containing adapter-inducing interferon-β

vg vector genomes

WT Wild Type

A note about the COVID-19 Pandemic

Beginning in March 2020, the novel coronavirus SARS-CoV-2 caused a global pandemic which affected many parts of the global supply chains and economies. In particular, due to the increase in required PCR testing and research work centered around the pandemic, a variety of essential lab supplies became increasingly difficult or impossible to acquire. Some of these supplies include disposables such as pipette tips, as well as specialized items such as permeable supports of specific types or viral vector. As a result, most experiments which were performed to completion had unexpected adaptations or blockages which were caused by the shortages of these materials. There were many examples caused by shortages of essential lab resources and equipment with the pandemic that spanned my time in the lab. I am grateful for the patience and creativity of my fellow lab members and mentors.

1. Introduction

1.1-The Retina

The neural retina is a highly organized tissue of the eye, primarily responsible for phototransduction and signal processing[1] Visual processing then occurs in the visual cortex.[1,2] The retina is derived from neuroectodermal embryonic cells and has a dual vascular supply from both the choroid and the central retinal artery to accommodate the metabolic processes which occur within the tissue.[1] The retina is composed of ten layers, which include both the retinal pigment epithelium and the photoreceptors.[2] In addition to the layered structure of the retina, there are also a variety of other support cells including glial cells such as the microglia which are located in the inner and outer plexiform layers. (Figure 1).[3]

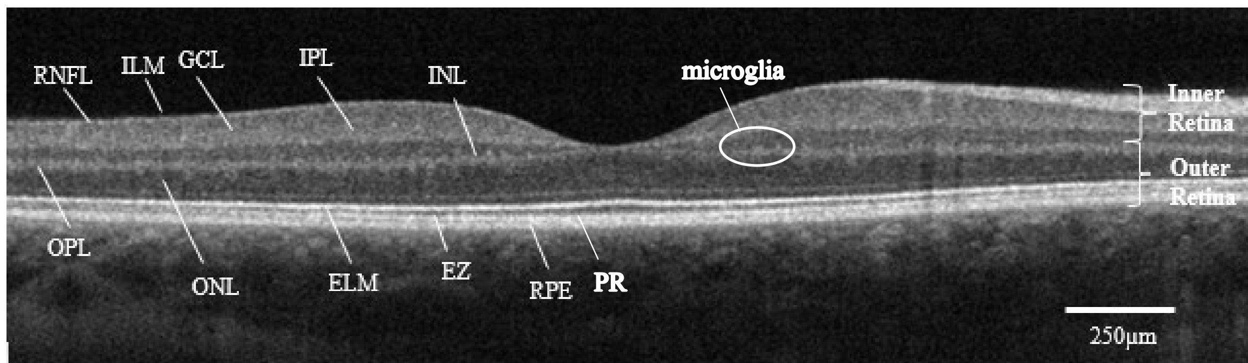

Figure 1. Normal macula optical coherence tomography image depicting the layers of the retina. RNFL = retinal nerve fiber layer; ILM = internal limiting membrane; GCL = ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; ELM = external limiting membrane; EZ = ellipsoid zone; RPE = retinal pigment epithelium. PR = photoreceptors; microglia – within INL and OPL (unseen) (Adapted from image from Wiessner et. Al)[4]

1.2 – Retinal Immunity

The immune mechanisms of the retina have been historically understudied due to the assumption that "the retina is immune privileged.[5]" This previous designation of immune privilege in the retina originated from a study which researched transplantation in the eye.[6] The transplanted graft in the study was not rejected which allowed 'immune ignorance' to be assumed. Following this study there was a significant amount of research to suggest the presence of ocular immune response. Although there are mechanisms which aim to mitigate the immune response severity within the retina, the previous designation of 'immune ignorance' may have caused the immune mechanisms of the retina to be understudied.[5,7]

The blood-retinal barrier is established from the tight junctions of the RPE and establishes a retinal micro-environment where the immune system may act independently from the rest of the human body.[8] There is a variety of unique immune functions including inflammatory and anti-inflammatory responses that are tightly regulated in order to deal with damage and infection while mitigating risk and damage to important ocular tissues.[8] The micro-environment formed by the blood-retinal barrier requires further study in particular in diseased retinas where activation of ocular immune cells may occur due to damage or disease affecting surrounding cells. There are multiple cells within the retina involved in retinal immunity including both the RPE and resident glial cells such as retinal microglia.[3,9]

1.3-The Retinal Pigment Epithelium (RPE)

The retinal pigment epithelium is a monolayer of hexagonal pigmented cells which are held together with tight junctions.[10] The RPE is the outermost layer of the retina, separating the photoreceptors from the choroid, and is responsible for the maintenance and function of ocular cells, particularly the photoreceptors.[10] The RPE are polarized cells with the basal side facing the choroid and apical side facing the photoreceptors. The RPE cells are a critical component of the visual cycle which involves the phagocytosis of spent photoreceptor outer segments by the RPE and the regeneration of 11-cis-retinal from all-trans retinol for re-use in the photoreceptors.[11] The apical side of the RPE cells have microvilli which increase the apical surface area for epithelial transport as well as for phagocytosis of photoreceptor outer segments. RPE cells are pigmented due to the presence of melanosomes and this pigmentation is essential for the absorption of light which reduces potential damage from ultraviolet light on the retina.[10] In addition, the RPE transports nutrients and waste between the choroid and the photoreceptors, and combats oxidative stress in the retina (Figure 2).[9,12–14] The RPE cells have numerous critical functions involved in the visual cycle and protection of ocular tissues. Death or degeneration of the RPE cells would result in damage and death of additional ocular tissues including the photoreceptors, leading to degraded visual function and blindness.

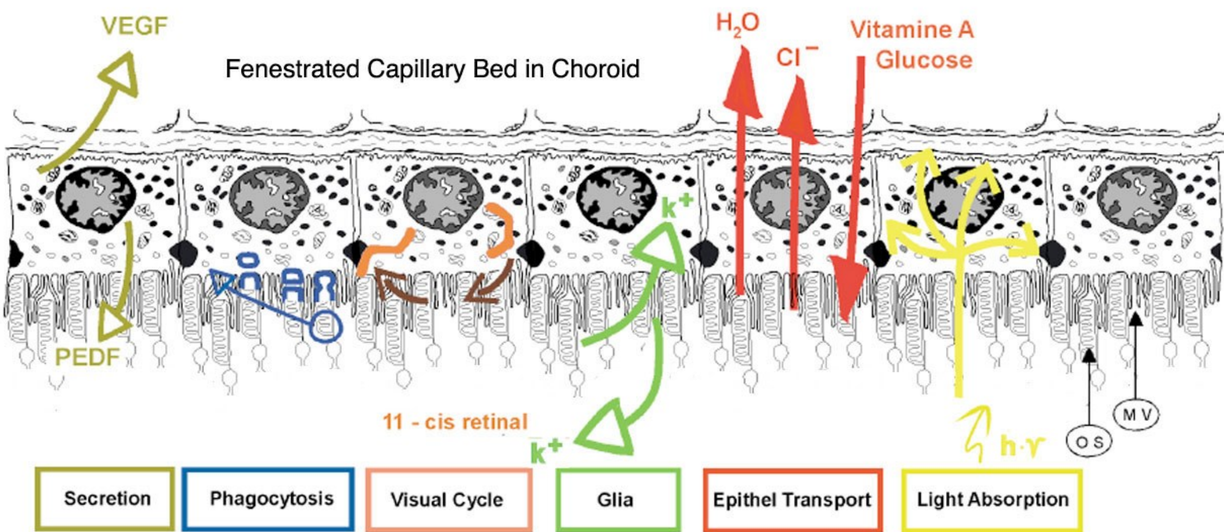

Figure 2. Summary diagram of the major functions of the retinal pigment epithelium (From Olaf Strass, 2005)

1.4- The Photoreceptors

Photoreceptors are specialized neurons responsible for sensing light. There are two main types of photoreceptors; the rod photoreceptors are highly sensitive to light and assist the eye with high acuity dim light vision, and the cones photoreceptors are activated by bright light and are able to detect color.[15,16] There are three types of cone photoreceptors in the human retina, blue, red and green, each of which are sensitive to a different wavelength of light. Incident photons of light interact with a derivative of Vitamin A bound to opsin molecules in the photoreceptor outer segments. The process of photon detection requires significant metabolic energy to support the chemical reactions which occur. The photoreceptor outer segments which detect incident photons are spent and eliminated by the RPE during the process of light detection and must later be regenerated by the photoreceptors.[17] The visual cycle is a metabolically intensive process, causing the retina to be the most metabolically active tissue in the human body.[11,18] The photoreceptors

are post-mitotic meaning they are not capable of further cell division to create more photoreceptor cells and must instead regenerate their outer segments. The post-mitotic nature of photoreceptors makes them a primary focus in many ocular diseases because death and degeneration of these cells leads to irreversible vision damage.

1.5 -Immune Capabilities of the RPE

The RPE express toll-like receptors (TLRs) which are a common surface receptor that recognizes pathogen associated molecular patterns (PAMPS), and damage associated molecular patterns (DAMPs). PAMPs are molecules with specific common motifs of pathogenic origin. These specific motifs are the ligands for pattern recognition receptors (PRRs), such as TLRs, that trigger an innate immune response. DAMPs are endogenous danger molecules released from damaged or dying host cells. DAMPs are recognized by PRRs and may also lead to an inflammatory response in addition to initiation and propagation of the cell and tissue repair processes.[19,20] Human RPE cells express nine of the ten discovered human TLRs. The TLRs are synthesized in the endoplasmic reticulum, are passed through the Golgi, and transported to the surface of the cell through endosomes. Each TLR recognizes a specific molecular pattern, which when activated, causes a signalling cascade leading to immune upregulation through the secretion of chemokines, cytokines, and the production of adhesion molecules. For example, double-stranded ribonucleic acid (dsRNA), which is characteristic of viral infection, is commonly recognized by TLR-3. The activation of TLR-3 begins a signalling cascade which leads to escalation of the immune response through signalling to other cells, increasing permeability of the blood retinal barrier, and recruiting of circulating leukocytes into the subretinal space.

The RPE also displays a variety of other surface molecules including Major Histocompatibility Complex II (MHC II). MHC II is integral to the creation of the adaptive immune response due to it's role in the display of extracellular antigens which have been endocytosed and processed within the cell prior to being mounted onto the MHC II molecule. MHC II displaying the processed extracellular antigen on the cell surface aids in communication and immune upregulation involving other immune cells including CD4+ T-Cells.[21] Stimulation of CD4+ T-Cells leads to stimulation and recruitment of a variety of other adaptive immune cells and sustained upregulation of the adaptive immune response.

1.6 – Toll-Like Receptor Signalling

The RPE displays a variety of TLRs. TLR-3 and TLR-9 are both located within the endosome and are responsible for respectively detecting viral dsRNA and bacterial DNA which contains unmethylated cytosine—guanine (CpG) motifs (CpG-DNA).[22–24] Endosomal TLRs undergo proteolytic cleavage by cathepsins; however, the N-terminal end of TLR-9 remains closely associated with the truncated TLR-9 and forms a complex, essential for the detection of bacterial DNA.[22] Upon activation of TLRs, a series of toll-interleukin-receptor (TIR) domain-containing adaptors are recruited.[25] In the case of TLR-9 both myeloid differentiation primary response 88 (MyD88), and TIR adaptor protein (TIRAP) which are responsible for signal transduction from both inside and outside of the cell are recruited.[26,27] The recruitment of these signal transduction proteins causes activation of the transcription factor nuclear factor kappa B (NF-kB) and a series of immune response specific mitogen activated protein (MAP) kinases leading to the activation of inflammatory cytokine genes.[28,29] TLR-3 when activated recruits TIR-domain-containing adaptor -inducing interferon- β (TRIF) which results in activation of interferon

regulatory factor-3 (IRF-3), NF-kB and MAP Kinases which leads to the production of inflammatory cytokines and type I interferons.[30] Both TLR-3 and TLR-9 are present on RPE cells and are critical in the detection of PAMPs in the subretinal space.[22]

1.7 – Pro-Inflammatory Cytokines and Adhesion Molecules

The triggering of TLRs results in the upregulation and secretion of pro-inflammatory cytokines including, Tumor necrosis factor-α (TNF- α), Interleukin-1 β (IL-1β), Interleukin-6 (IL-6), Interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (CCL-2).[22,31] TNF-α is produced as a result of NF-kB signalling and is critical in cell proliferation, cell survival and apoptosis. TNF-α orchestrates the pro-inflammatory cytokine cascade in many cases and is often referred to as a 'master regulator' of pro-inflammatory cytokine secretion.[22,32,33] The overproduction of TNF-α is closely associated with a variety of chronic inflammatory diseases including rheumatoid arthritis.[32] Like TNF-α, IL-1β is associated with autoimmune diseases and is upstream in pro-inflammatory signalling cascades leading to its reference as a master regulator of inflammation and the immune system.[34,35] Both TNF-α and IL-1β are attractive therapeutic targets in treatments of inflammatory disease due to their involvement in numerous pro-inflammatory processes including the downstream release of further pro-inflammatory cytokines.[32,34] These cytokines are therapeutic targets in a variety of other chronic inflammatory diseases including rheumatoid arthritis, and diseases of the heart and central nervous system.

IL-6 is a pleiotropic cytokine which activates the IL-6 receptor on other cells initiating a signalling cascade that results in the transcription and translation of cytokines, receptors, and protein kinases that are necessary to propagate the immune response.[36] In addition, IL-6 aids in generating the adaptive immune response through inducing differentiation and maturation of naïve

T cells which are important for creating the adaptive immune response and upregulating secretion of IFN-γ.[36]

IL-8 is released from several cell types in response to inflammation and is responsible for propagating the first immunological mechanism to damage or infection known as the innate immune response. Activation of the innate immune response results in the same series of actions irrespective of the stimulus, making it non-specific. IL-8 contributes to the innate immune response through its association with immune cells, more specifically, neutrophils, and recruitment and the release of antimicrobial and cytotoxic mediators from the neutrophils which is a process known as degranulation.[37]

CCL-2, also known as monocyte chemoattractant protein (MCP-1), is responsible for the recruitment of monocytes to the site of infection or inflammation.[38] IL-6, IL-8, and CCL-2 have chemoattractant properties which promote recruitment of circulating leukocytes and immune cells to the blood retinal barrier as a result of an immune response triggered in the subretinal space. In addition to the pro-inflammatory cytokines which are secreted because of TLR activation and result in chemotaxis to the blood retinal barrier, a series of adhesion molecules are upregulated and presented on the surface of RPE cells during pro-inflammatory responses. Chemokine (C-X-C motif) ligand (CXCL) 9, CXCL10, and intracellular adhesion molecule-1 (ICAM-1) are adhesion molecules which facilitate the entry of circulating leukocytes across the blood-retinal barrier allowing for upregulation of the adaptive immune response, including generation of antibodies and apoptosis of infected cells within the subretinal space.[31]

1.8 – Antigen Presentation Capabilities of the RPE

Major Histocompatibility Complex (MHC) molecules have previously been observed on RPE cells.[9,39,40] MHC I is expressed on almost all human cell types and displays peptides generated within the cell to signal the cell's own physiological state to surveillant immune cells. MHC II tends to be localized to antigen-presenting cells such as macrophages and dendritic cells which are innate immune cells.[9,39] Extracellular antigens are endocytosed by antigen-presenting cells. The endocytosed antigens are degraded and processed in vesicles and mounted to MHC class II molecules. The MHC II molecules with the mounted antigen are then integrated into the cellular membrane to present the processed exogeneous antigens to other immune cells.[9,41] RPE cells presenting MHC II are able to present exogeneous antigens to adaptive immune cells and have a role in T-cell activation through their interaction with the CD4+ T-cell receptor. Activation of CD4+ T-cells allows for further inflammation, cytokine release, leukocyte recruitment as well as cell mediated immunity which are features of the adaptive immune response. [28]

1.9 – Immune Response Mitigation in the RPE

In response to damage or antigen exposure, RPE cells also release critical immune mediators which protect ocular tissues from excessive damage caused by an immune response. Upon detection of viral dsRNA by TLR-3, interferon beta (IFN- β) is released by RPE cells.[42] Previously, treatment of RPE cells with IFN- β has demonstrated a reduction in the gene expression and presence of the surface adhesion molecules sICAM, CXCL9, CXCL10 and CXCL11 from the RPE.[42] Reduction in the presentation of the adhesion molecules responsible for bringing circulating leukocytes across the blood-retinal barrier into the subretinal space indicates that IFN-

β release aims to control the immune response to the subretinal space and reduce the entrance of circulating leukocytes which can damage cells in the subretinal space. In addition to IFN-β, transforming growth factor-β (TGF- β) is a multi-functional immunosuppressive molecule primarily responsible for downregulating antigen presenting cell activity and T-cell function.[5,9] TGF-β is upregulated during numerous ocular pathologies and traumas, including retinal detachments and corneal neovascularization. IFN-β and TGF-β release during immunological events aids in mitigating the severity of the immune response. This helps to keep the immune response more targeted and localized mitigating the potential damage to post mitotic cells of the retina and the subretinal space.[9]

1.10 –Microglia in the Retina

Microglia are a type of glial cell and one of the primary innate immune cells in the retina. Microglia are important in the development of both the retina and central nervous system in addition to having robust immune and pro-inflammatory capabilities. Microglial cells infiltrate the central nervous system (CNS) and the retina during early embryonic development and have a variety of regulatory functions within their micro-environments.[43] In addition to their immune capabilities, they are essential for the maintenance of neural pathways and synapses within the eye.[43–45] Under normal conditions, the microglia can self sustain through cell division and are essential in maintaining retinal integrity through apoptosis of unwanted cells and phagocytosis of cell debris. [3,46] In addition to this, microglia are essential in proper neuronal development within the retina as they promote retinal vessel growth. In a normal retina, one which is in a healthy state and not responding to external stress or pathogenic infection, the microglia settle in the plexiform layers and have a branched morphology with long protrusions which span the nuclear layers and

origin from a small cell body.[3,47] The long protrusions are essential in surveying the retinal micro-environment sensing for PAMPS, DAMPS, as well as cytokines and chemokines.[3]

1.11 – Immune Mechanisms of the Microglia

Microglia are highly sensitive innate immune cells which have a variety of immune functions such as antigen presentation, secretion of cytokines and chemokines, and phagocytosis.[43] The microglia consistently sense PAMPs, DAMPS and other stimuli using their surface receptors which when triggered by ligand binding cause upregulation (see below) and targeted immune response. Like the RPE, the microglia have a series of TLRs which sense antigens and lead to immune response.[14,48] Upon detection of antigens, the microglia upregulate a series of pro-inflammatory cytokines, including IL-6, IL-8, IL-1β, TNF-α and CCL-2 which propagate the immune response and lead to adaptive immunity, as previously described.[45] As one of the main antigen presenting cells in the retina and the CNS, microglia have surface MHC II and recruit circulating leukocytes to the blood retinal barrier, leading to the adaptive immune response.[3] Microglia also participate in crosstalk with other immune cells such as the RPE through pro-inflammatory upregulation caused by detection of cytokines such as TNF-α, IL-6, and IFN-γ which lead to further cytokine release and the synthesis of complement fragments. These fragments are critical for innate immune system propagation through enhancing inflammation, phagocytic abilities of cells and attack on pathogen membranes.[3,47] Microglia have been observed to phagocytose a variety of things including cell debris, as well as both alive and dead cells when activated. Neurons in the CNS may present a stress signal in the form as a phosphatidylserine surface molecule which is presented during cellular stress or damage. Display of this

phosphatidylserine molecule on the surface of cells results in the rapid phagocytosis and complete removal of the cell by activated microglia.[49]

1.12 – Immune Regulation of the Microglia

Microglia are localized to areas with essential and post-mitotic tissues within the CNS and must be carefully regulated due to their potentially destructive responses that often cause unintended damage to host cells. As an example, within the retina, the microglia when activated may phagocytose viable post-mitotic cells which express stress signals (phosphatidylserine) ultimately resulting in irreversible damage.[47] RPE cells aid in the regulation of microglia through the release of TGF-β which induces IL-10 release from the microglia.[9,47,50] IL-10 then causes downregulation of antigen expression proteins including MHC II in addition to other immune surface molecules; CD80 and CD86.[47] Maintenance of TGF-β levels helps to reprogram the microglia towards an anti-inflammatory phenotype. TGF-β is secreted at high levels in both the retina and brain and depletion of TGF-β has been previously associated with increased neuronal cell death.[47,50] In addition to TGF-β, CX3CL1 (fractalkine) is consistently released from healthy retinal neurons and endothelial cells. Fractalkine binds to the fractalkine receptor on the microglia to prevent neurotoxicity which is caused through the release of free superoxide radicals.[47,50] Secretion of both fractalkine and TGF-β at high levels from healthy neuronal and endothelial cells in the retina and CNS act as the main regulatory mechanisms for microglia. However, IL-4 and IL-13 are also present at low levels in these environments and are upregulated when there is an immunological disturbance. IL-4 is a downregulatory cytokine with neuroprotective properties and IL-13 promotes microglial cell death as a method of controlling inflammatory response.[50] The upregulation of these cytokines during an immune response suggests that microglial responses in

a functional biological system are acute and targeted to eliminate the threat while mitigating any collateral damage to essential tissues caused by microglial activity.

1.13 – Crosstalk Interactions of Immune Cells

Retinal cells constantly communicate with one another using soluble factors such as cytokines and chemokines. Crosstalk refers to interactions from two or more inputs that affect one common biological output. In the case of the RPE and the microglia, the biological output affected by crosstalk is immunological regulation. Both the RPE and microglia secrete inflammatory and anti-inflammatory cytokines and chemokines which have an effect on the other cell type. Response from one cell type to the signalling of another indicates that cross talk is occurring. Through the secretion of pro-inflammatory cytokines from the RPE, the microglial cells are also put into a pro-inflammatory state due to this signalling. Similarly, the RPE are also essential in regulating the microglia through secretion of anti-inflammatory soluble factors. Understanding that there are numerous cell types participating in crosstalk, makes this interaction a process of interest as a therapeutic target. In a pro-inflammatory setting crosstalk may lead to exponential immune response escalation which in some cases may be unwanted and damaging to essential tissues within the retina.

1.14 – Inherited Retinal Dystrophies

Inherited retinal diseases (IRDs) are a group of genetic disorders which involve the degeneration of the retina and are a leading cause of reduced vision and blindness.[51,52] Currently, there are greater than 250 causative genes associated with IRDs which affect greater than 2 million people globally.[51] Early testing and diagnosis of IRDs is essential to identify treatment opportunities and improve patient outcomes. Post-mitotic cells within the retina, primarily the photoreceptors, are often affected by a series of IRDs, and damage to these cells is irreparable. Most IRDs progress over time and therefore, early diagnosis is critical. Mutations in the genes which cause IRDs can be found in all cells within the body; however, the mutation may only affect certain cells resulting in a specific phenotype. IRDs are usually distinguished through the cell types that they may affect for example, there may rod dystrophies primarily affecting the rod photoreceptors, and cone dystrophies primarily affecting the cone photoreceptors. IRDs which affect the cone photoreceptors typically cause impaired central vision and reduced color vision whereas IRDs which affect rod photoreceptors typically cause difficulties with peripheral and low light vision[52].

1.15 – Retinitis Pigmentosa

Retinitis Pigmentosa (RP; OMIM #268000) is an IRD which results in bilateral degeneration of rod photoreceptor cells. Due to the large number of genes that may be involved in its development it may present as autosomal dominant (AD), autosomal recessive (AR), X-linked, or mitochondrial disorder. Having greater than 100 genes involved in this pathology makes treatment and diagnosis very specific to each patient requiring treatment.[52] RP is considered a rod-

cone dystrophy due to the fact that rods are initially effected followed by cone photoreceptor degeneration. This results in initial night blindness and peripheral field loss, then later deterioration of central vision as cones degenerate. There are currently no treatments available for RP; however, there are a series of ongoing gene therapy clinical trials.[52]

1.16- Choroideremia

Choroideremia (CHM; OMIM #303100) is an X-linked recessive chorioretinal dystrophy which affects 1 of 50,000 patients globally who are usually male.[53–55] Although patients are born with this IRD, manifestation of the disease is often not observed until young adulthood beginning with impaired night vision and peripheral vision eventually progressing into blindness. CHM is caused by a mutation in Rab escort protein 1 (REP-1), resulting in shortening of photoreceptor segments, depigmentation of the RPE, and ultimately thinning of the retina and death of neuronal cells and photoreceptors.[54–56] REP-1 is ubiquitously expressed and responsible for intracellular trafficking, and manages prenylation and fusion of unprenylated rab proteins to associated binding partners.[54,57] Although this is a global mutation, meaning all cells in the body are REP-1 deficient, the *CHM*- phenotype manifests within the retina likely due to the elevated metabolic demands of the visual cycle. REP-2 is another protein with analogous function to REP-1 and in non-ocular tissues it is sufficient at compensating for the loss of REP-1 function which is why the *CHM*- mutation phenotype primarily is observed in the eyes. Similar to RP, CHM has no treatments available; however, there are ongoing clinical trials which use an adeno-associated viral vector delivered subretinally.[55,58]

1.17- Gene Therapy

The human genome contains approximately 25000 genes that encode a variety of proteins which are used in cellular and biological processes.[59] Mutations are the main cause of diversity among organisms and genetic mutations can be both benign and pathogenic. In some cases, inherited genetic mutations may have outcomes such as IRDs that may not have a treatment other than replacement of the affected gene. Gene therapy is reliant on masking of the mutation which causes the dystrophy or disease. In the context of choroideremia as an example, gene therapy consists of compensation for the lack of function caused by the *CHM*- gene mutation with the introduction wild type (WT) copy in order to produce a functional REP-1 protein. Gene therapy research funding has been previously reduced due to a variety of factors such as off target effects, immunological implications, and poor treatment outcomes. However, in recent years more interest has returned to the research.[59] Luxturna (voretigene neparvove-rzyl) which is a gene replacement therapy for an *RPE65*-associated retinopathy, was the first gene therapy approved by the FDA and Health Canada in 2017.[60] In recent years, interest and funding towards gene therapy has increased.[59]

1.18 – Viral Vector Based Gene Therapy Treatments

Gene therapy requires the functional compensation for dysfunction of a mutated gene with the introduction of a WT copy of the same gene in order to restore proper function. There are a variety of methods to target a mutated gene, of which the most common is through the use of a repurposed viral vector which contains the engineered WT gene copy. The viral vector serves as a delivery vehicle due to its ability to enter targeted cells efficiently.[61] Gene therapies tend to use

one of three different viral vectors, the adeno-associated virus (AAV), adenovirus, or lentivirus which each have unique viral properties affecting both immunogenicity and therapy efficacy.[61] AAV vector tends to be preferred for gene therapy treatments in spite of its limitation of low cargo-capacity due to the fact lentivirus has a risk of insertional mutagenesis and adenovirus has an elevated risk of inflammation. A variety of different diseases would benefit from viral vector-based treatments, however, the elevated cost of this procedure in addition to potential immunological implications and off-target effects are some of the primary barriers to accessibility to patients, especially those who may have underlying immunological conditions.[59,61]

1.19- Gene Therapy Treatments within the Retina

Many inherited retinal dystrophies may have their progression halted using gene therapies. Luxturna is currently the only FDA approved AAV vector-based gene replacement therapy. [60] Previously, clinical trials using viral vector-based gene therapy have been conducted on patients who have mutations in the choroideremia gene (*CHM-*) in order to treat choroideremia with varying degrees of success. Due to the structural features of the retina, there are various unique challenges that increase the complexity of viral vector gene therapy treatments. Multiple delivery methods have been previously explored which aim to get the viral vector to the target cells for *CHM-* therapy, specifically the RPE. Subretinal injection of viral vector has been the approach of vector delivery to target cells used within a clinical trial of ocular gene therapy for choroideremia in the MacDonald Lab.[58] Subretinal injection is the primary protocol used in clinical trials of gene therapies for retinopathies of the outer retina (targeting the photoreceptors and RPE) such as in choroideremia (Figure 3).[58,62] Subretinal injection is an invasive procedure that results in the detachment of the retina and formation of a bleb where the viral vector will be injected into the

retina. This approach is favourable as it delivers the vector near to the target cell site of the RPE, theoretically minimizing the amount of required vector genomes due to more direct administration and reduced off-target transduction. This may increase transduction efficiency and reduce risk of off target effects.[62] However, due to the invasive nature of this procedure involving a retinal detachment and direct injection into the subretinal space there are elevated risks and complications that may come as a result of physical damage to the retinal structures in addition to administration of the viral vector. A slightly less invasive approach that has been previously used is intravitreal injection of the viral vector. Intravitreal injection of the viral vector requires a high titer of viral vector being injected directly into the vitreous with the concept that the vector will transduce the surrounding cells including the target cells which require the therapy.[63] This approach is slightly less invasive than a subretinal injection due to the fact that disturbance and detachment of the retina is not required for administration of the viral vector. However, intravitreal injections is predicted to only be effective in ocular gene therapies targeted to the optic nerve, nerve fiber layers and the inner retina.[62] In order to successfully transduce cells in the outer retina with this method, a large viral titer is required to a point where risks may not make this therapy practical for outer retina dystrophies such as choroideremia. Although in some cases the intravitreal approach may have decreased risks, balancing the decreased risk with decreased efficacy is one of the primary difficulties when designing gene therapy treatments within the retina.

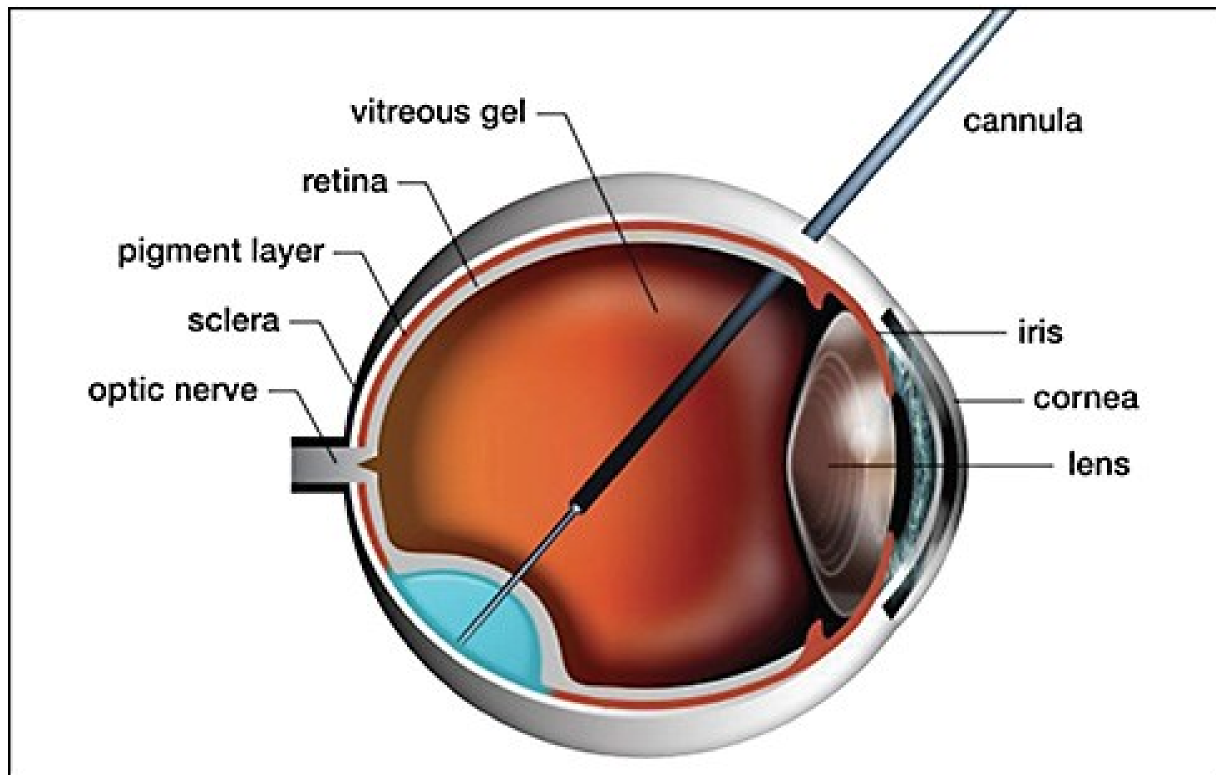

Figure 3. Depiction of a sub-retinal injection and formation of a bleb for delivery of viral vector for gene therapy. (From M. Ali Khan, MD and Allen C. Ho, MD, 2020) (https://www.retinalphysician.com/issues/2020/special-edition-2020/the-eye-as-a-biofactory)

1.20 – Immune Implications of Gene Therapy in the Retina

Beyond physical and structural damage which may occur as a result of subretinal injection or other ocular gene therapy treatment strategies, the primary risk and consideration is the immune implications of gene therapy treatments. Previously thought to be immune privileged, recent research suggests that the eye including the subretinal space has robust immune capabilities. Previous clinical trials within the MacDonald Lab and other labs involving gene therapy treatments for IRDs have recorded poor outcomes due to ocular inflammation.[58] A series of events during subretinal ocular gene therapies may cause upregulation in the immune response including retinal detachment from bleb formation and administration of the immunogenic viral vector. It has been

observed that it is important to consider and minimize the number of vector genomes (vg) being used for treatment because in both subretinal injection and intravitreal administration, an immune response occurs with inflammatory severity related to the vg number.[63] Determining an optimal titre of viral genomes that will allow successful treatment without significant inflammation is a difficulty of ocular gene therapy.

1.20.1 – Retinal Detachment

Retinal detachment is a sight threatening condition caused by the separation of the neurosensory retina (NSR) from the RPE.[64] The neurosensory retina and the RPE are not held together by any anatomical junctions which makes separation of these layers relatively easy.[64] Retinal detachment leads to separation of the photoreceptors and the RPE which may lead to upregulation of photoreceptor stress signals leading to apoptosis or phagocytosis of the photoreceptor cells.[64,65] Possible causes for retinal detachment may include various traumas. In addition, there is increasing likelihood and susceptibility of detachment with advancing age. In the case of subretinal injection, the retina is forcibly detached through an injection of a solution forming the bleb. Retinal detachments may be treated through surgical interventions and should be treated quickly to prevent irreparable damage which may come from separation of the NSR and the RPE.[64]

Retinal detachments have been observed to trigger the innate immune response within the retina which involves both the microglia and the RPE.[66] Retinal detachment has been observed to upregulate the release of pro-inflammatory cytokines and chemokines in addition to increasing TLR expression on immune cells while promoting the chemotaxis and infiltration of microglia towards the area of the retinal detachment and stressed photoreceptors.[66] Microglia migrating towards the area of retinal detachment aid in regulation of the immune response and phagocytose

stressed and dead photoreceptor cells.[65] Retinal detachments including those induced surgically for subretinal injections have immune implications and may be damaging if not properly monitored and treated.

1.20.2 – Viral Vector

Viral vectors are critical gene delivery mechanisms in genetic therapy due to the ability of the vectors to enter target cells and deliver the transgene to the target cells *in vivo.* A series of viral vectors have been used including retrovirus vectors, lentivirus vectors, adenovirus vectors and AAV.[67] AAV vector is the main vector of focus within the MacDonald lab due to widespread use and their acceptance as the least immunogenic vectors with lowest risk of vector-related toxicity.[67] AAV vectors are dependent on adenovirus to complete their replication cycle meaning that alone, AAV vectors cannot replicate due to the lack of essential genes for replication. There are thousands of variants of AAV vectors none of which are known to cause disease in humans.[67] The primary serotype used in gene therapy is AAV-2 and it is developed to target various genetic diseases in the CNS, eyes, heart, and liver.[67] Although the AAV vectors are considered to be the least immunogenic viral vectors, they are still detected and can cause an upregulation of the innate immune response.[68,69] TLR-9 is activated by the AAV vector which leads to MyD88 signalling, activation of NF-kB and upregulation of pro-inflammatory cytokines.[69] In combination with the primed innate immune response and translocation of microglia to the detached retina at the injection site, subretinal injection of AAV is likely to cause upregulation of inflammation and the immune response in the retina.

1.21 – The Aging Eye

With modern medicine, the average life expectancy of adults has increased, and patients of advanced age are the fastest growing segment of society in many countries.[70] Most major eye related diseases including the majority of IRDs have a progressive phenotype with age including choroideremia. A variety of tissues and structures within the eye and body undergo physiological changes which occur naturally as part of the aging process including changes to the vitreous which increase risk of retinal detachment, alterations to microglial regulation, and changes to the function of the RPE which may cause further progression of disease phenotypes and changes to immunological functions within the eye.[70]

1.22 – Alterations to the RPE with Age

The RPE cells are critical in homeostasis and maintenance of the visual cycle and cells within the eye. However, as RPE cells age, they undergo a series of changes which may have an immunological effect. Features of the RPE cells may change with advancing age as the cells change in shape, form multilayered cellular regions in the monolayer and lose pigmentation.[71,72] The density of melanin granules in RPE cells has been observed to decrease with age which alters the light absorption and function of the RPE. In addition, lipofuscin has been observed to accumulate with age and may affect enzyme activities and lipid peroxidation within the RPE.[71,72] Changes in densities of melanin granules and lipofuscin have an effect on the processes occurring within RPE cells which have may induce oxidative stress leading to RPE cell dysfunction. Advancing age causing increased stress and phenotypic changes to the RPE may have an effect on

the immune sensitivity of the RPE and surrounding tissues. Crosstalk and secretion of soluble factors including cytokines and chemokines are typically observed from stressed but viable cells.

1.23 – Changes to the Microglia with Age

As humans age, there are numerous physiological changes to the immune and regulatory systems in place on a cellular level. Researchers hypothesize that age related diseases in the central nervous system may be associated with changing microglial functions.[50,73] With advancing age, microglial checkpoints such as cell to cell interaction requirements, and signalling through mediators such as fractalkine appear to become dysregulated.[50] In particular, microglia may have decreased downregulatory function resulting in increased immune vigilance and activation. When combined with genetic therapy treatments, with subretinal injection and administration of viral vector, the decreased microglial immune checkpoints and downregulatory mechanisms pose increased risk of poor outcomes due to inflammation in patients particularly those of advancing age. In addition to increased immunological sensitivity, the constitutive functions of microglia have been observed to change with age.[47,50,73] Alterations to the constitutive functions of microglia may lead to damage and degeneration of essential supportive tissues within the retina.[47,50,73] Microglial cells have their regulatory checkpoints and functions altered with age which present increased risks of inflammatory upregulation and undesired interactions with host cells. When exposed to trauma through retinal detachment and antigens such as the AAV vector, there may be increased risk of poor outcomes during gene therapy treatments in patients of advancing age due to changes in microglial phenotype and functions.

1.24- Hypothesis and Experimental Design

This project planned to develop an *in vitro* co-culture model using iPSC-RPE cells and immortalized SV-40 Microglia. Through the course of these experiments, there is dissected investigation of each component of the co-culture model through testing of the immune response of the RPE alone and the microglia alone in advance of establishment of the co-culture. In addition to this, there was a series of model optimization steps which were essential in increasing the longevity of the RPE lifetime in order to successfully complete experiments. If we aim to successfully develop an immunocompetent co-culture model using iPSC-RPE cells and SV-40 microglia, then we will see a dose-dependent increase in pro-inflammatory cytokine secretion in response to treatments. This is hypothesized to occur due to the fact that this co-culture model is likely to engage in crosstalk between the two cell types and with higher initial treatment dose, it is likely to observe a more robust initial immune response to be perpetuated by RPE-microglia crosstalk. The experimental designs below were designed to build off of previous work with Geoff Casey M.Sc and, hypotheses that there are cell-cell interactions occurring which result in further pro-inflammatory perpetuation beyond RPE cells alone.

Aim 1: To investigate the response of RPE cells to pro-inflammatory cytokine treatments. The RPE cells are one of the resident immune cells in the retina and they express a variety of surface molecules involved in detecting and displaying antigens in the subretinal space. If these RPE cells are treated with a pro-inflammatory stimulus (IL-1β or TNF-α), we expect to see a dose dependent pro-inflammatory response

Aim 2: To investigate the response of microglia cells to pro-inflammatory cytokine treatments. The microglia are another immune cell which is native to the subretinal space. In addition to being effective phagocytes the microglia are known to display a variety of surface molecules involve in

detecting immune stimulus and upregulating immune response. If the microglia cells are treated with a pro-inflammatory stimulus (IL-1β or TNF-α), we expect to see a dose dependent pro-inflammatory response.

Aim 3: To improve the longevity of iPSC-RPE cell line growth on cell culture plates. In order to create a co-culture model with proper apical-basal polarization of RPE cells and associated orientation of the microglia, the microglia must be plated in the permeable supports and the RPE on the bottom of the cell culture plate. Due to previous experience with the RPE maturation process including the formation of tight junctions and apical to basal pumping of medium, the RPE cells tend to experience difficulty at sustaining long-term growth. If RPE cells are grown on cell culture plates coated in Matrigel®, there is likely to be improved longevity and mono-layer cell formation when compared to uncoated cell culture plates.

Aim 4: To investigate crosstalk between the RPE and microglia using a co-culture model. Introducing both the microglia and the RPE within the same model should facilitate crosstalk interactions which perpetuate an immune response. If the co-culture model is treated with a pro-inflammatory stimulus (IL-1β, TNF-α), we expect to see a dose dependent response to the pro-inflammatory stimulus.

Aim 5: To investigate the impact of microglial presence on the survival and growth of iPSC-RPE cells using a co-culture model. If the RPE and the microglia are introduced into a co-culture model when compared to RPE alone and the model is not treated with any pro-inflammatory stimulus, there may be improved survivability of the RPE in the presence of non-active microglia due to crosstalk between the cells that aids in maintenance of the health of RPE cells.

2. MATERIALS AND METHODS

2.1 – Retinal Pigment Epithelium Commercial Cell Line Cell Culture

Retinal pigment epithelium cells were derived from patient normal induced pluripotent stem cells (iPSC-RPE, cell line ID "VK-WT"; a gift from the lab of Dr. Vasiliki Kalatzis, Institute of Neurosciences of Montpellier, FRA). An additional cell line was also used and obtained from Axol Bioscience (discontinued cell line, Axol Bioscience, Cambridge, UK). Cells were stored in liquid nitrogen and were warmed at 37°C until completely thawed prior to plating. Plates were coated with Corning® Matrigel® (Corning. Cat. No. 354234, Corning, Corning, NY) according to the manufacturer's specifications. Thawed cells from liquid nitrogen were added dropwise to 20 mL of RPE support medium. RPE support medium is composed MEM-Alpha (Gibco, Cat. No. 12571-063, Thermo Fisher Scientific, Waltham, MA) supplemented with 250 µL gentamicin (Invitrogen, Cat. No. 15750-060, Thermo Fisher Scientific, Waltham, MA), antibiotic-antimycotic (Thermo Fisher Scientific, Cat. No. 15240062, Thermo Fisher Scientific, Rochester, NY) taurine (Sigma Life Sciences, Cat. No.T8691-25g, Sigma Aldrich, Burlington, MA), N2 (Thermo Fisher Scientific, Cat. No. 17502001, Thermo Fisher Scientific, Rochester NY), and B27(Thermo Fisher Scientific, Cat. No. 17504044, Thermo Fisher Scientific, Rochester NY),). Cells were centrifuged for 5 minutes at 300RCF and resuspended in 5 mL of fresh medium prior to counting using a hemocytometer. Cells were plated at a density of 200,000 cells/well and medium was supplemented with 10% Fetal Bovine Serum (FBS) for the first 24 hours, 5% FBS for the second 24 hours (day2) and 1% FBS for the following 48hours (day 3-4) prior to incubation in serum free medium for the remainder of the growth. Cell medium was changed every Monday, Wednesday and Friday and cells were incubated at 37°C and 5% CO_2. Cells were kept in culture for a minimum of 30 days prior to treatment.

VK-WT iPSC-RPE were characterized at various timepoints of maturation by Geoff A. Casey M.Sc. (MacDonald Lab, University of Alberta) using immunofluorescence for various targets unique to RPE cells indicating their maturity. The antibodies and targets for characterization are in Table 1. These cells were not used in co-culture and the Axol Bioscience RPE cells (discontinued cell line, Axol Bioscience, Cambridge, UK) were only for baseline measurements of RPE cytokine secretion and came with pre-validated phenotypes.

Table 1: Antibody probes and targes used to characterize VK-WT RPE. Adapted from Geoff Casey (MacDonald Lab, University of Alberta)

TARGET	**SUPPLIER**	**PART NUMBER**	**CONJUGATED FLUOROPHORE**
ZO-1	ThermoFisher Scientific, Rochester, NY	339188	Alexa Fluor 488
BEST1	Santa Cruz Biotechnology, Dallas, TX	Sc-32792 AF546	Alexa Fluor 546
CLDN-19	Santa Cruz Biotechnology, Dallas, TX	Sc-365967 AF647	Alexa Fluor 647

2.2 – iPSC Retinal Pigment Epithelium Cell Line Cell Culture

Retinal pigment epithelium cells were derived from patient normal or *CHM-* fibroblast cells (IPSC-RPE cell line ID: normal, KK-*CHM*, PS-*CHM*; derived by Manlong Xu, MacDonald Lab, University of Alberta). Cells were stored in liquid nitrogen as passage 2 (P2) after derivation from iPSC RPE cells. Prior to plating plates were coated with Corning® Matrigel® (Corning. Cat.

No. 354234, Corning, Corning, NY) according to the manufacturer's specifications and, cells were removed from liquid nitrogen and warmed at 37°C until completely thawed. iPSC RPE cells were grown in DMEM (Gibco, Cat. No. 11995-065, Thermo Fisher Scientific, Waltham, MA) supplemented with Non-Essential Amino Acids (StemCell Technologies, Cat. No. 07600, StemCell Technologies, Vancouver, BC) and Pen/Strep (Gibco, Cat. No. 15070063, Thermo Fisher Scientific, Waltham, MA). Thawed cells from liquid nitrogen were added dropwise to 20 mL of pre-warmed iPSC-RPE medium and centrifuged for 5 minutes at 300RCF. Cells were resuspended in 5 mL of fresh medium and added dropwise through a 37 µm reversible strainer (StemCell Technologies, Cat. No. 272250, StemCell Technologies, Vancouver, BC) into a second conical. Cells were counted on a hemocytometer and plated at a density of 200,000 cells per well. Medium was supplemented with 4% Knockout-Serum Replacement (KSR) (Gibco, 10828028, Thermo Fisher Scientific, Waltham, MA) for 48 hours or 80% confluency, and 2% until 100% confluent. After 100% confluency normal iPSC RPE were tapered to 0% KSR and KK and PS *CHM-* cell lines remained at 2% KSR for the duration of their growth. Medium was replaced every Monday, Wednesday, and Friday. Cells were incubated at 37°C with 5% $CO2$. Cells were kept in culture prior to treatment for 21 days. Normal iPSC-RPE and PS iPSC RPE were characterized by Manlong Xu (MacDonald Lab, University of Alberta) using PCR and immunofluorescence for RPE specific genes. The targets for PCR characterization were *BEST1, RPE-65, MITF, RLBP-1.* The antibodies and targets for immunofluorescence characterization are in Table 1.

2.3- Microglial Cell Line Cell Culture

SV-40 Microglia cells are immortalized microglia cells which are immortalized through serial passaging and transduction with recombinant lentiviruses containing the SV-40 Large T antigen (Microglia, cell line ID: SV-40 Microglia; gifted from Dr. Thomas Langmann, University

of Cologne.) Cells were cultured on plates or in T-75 flasks coated with a 1:50 dilution in DNAse RNAse free H2O of Collagen 1-Bovine (Life Technologies GmbH: 15140122). 1:50 diluted Collagen 1 was applied to culture surface and rested at room temperature for 1 hour prior to removal of the collagen solution and plating of the microglia cells. Cells were stored in liquid nitrogen and were warmed at 37°C until completely thawed prior to plating. Thawed cells were added dropwise to 20mL of Microglia medium: DMEM (High Glucose) with sodium pyruvate and L-Glutamine (Sigma-Aldrich-Chemie GmbH: D2649-500mL) supplemented with FBS and Penicillin-Streptomycin liquid solution. Cells were counted using a hemocytometer and plated at a density of 75k cells per well. Cell medium was changed every Monday, Wednesday and Friday. Cells were grown in culture for a minimum of 5 days prior to treatment and incubated at 37°C and at 5% CO2 concentration.

2.4 – Microglia and RPE Co-Culture

RPE CELLS

Retinal pigment epithelium cells were derived from patient normal or *CHM*-fibroblast cells (IPSC-RPE cell line ID: normal, KK-*CHM*, PS-*CHM*; derived by Manlong Xu, MacDonald Lab, University of Alberta). Normal iPSC RPE cells were used due to increased purity of RPE cells. Prior to plating, plates were coated with Corning Matrigel® (Corning. Cat. No. 354234, Corning, Corning, NY) according to the manufacturer's specifications and cells were removed from liquid nitrogen and warmed at 37°C until completely thawed. Cells were added dropwise to 20 mL of iPSC RPE support medium. Cells were centrifuged for 5 minutes at 300RCF and resuspended in 5 mL of fresh medium. Resuspended cells were passed through a 37 µm reversible strainer (StemCell Technologies, Cat. No. 272250, StemCell Technologies, Vancouver, BC) prior to a count on a hemocytometer and plating at a density of 200k cells per well. iPSC RPE

support medium was supplemented with 4% KSR for 48 hours or until 80% confluency was reached. KSR concentration was tapered to 2% for an additional 48 hours or until 100% confluent. Confluent iPSC RPE cells were grown in DMEM supplemented with Non-Essential Amino Acids and penicillin-streptomycin with 0% KSR. RPE cells were grown for a minimum of 23 days prior to introduction of the co-culture model and treatment. Cells were incubated at 37°C with 5% CO_2

Microglia Cells

SV-40 Microglia cells were immortalized microglia cells which were immortalized through serial passaging and transduction with recombinant lentiviruses containing the SV-40 Large T antigen (Microglia, cell line ID: SV-40 Microglia; gifted from Dr. Thomas Langmann, University of Cologne.) Prior to culture, permeable supports (EMD Millipore, Cat. No. MCHT12H48, EMD Millipore Corporation, Burlington, MA) were coated with a 1:50 dilution in DNAse RNAse free H_2O of Collagen 1-Bovine (Life Technologies GmbH: 15140122). 1:50 diluted Collagen 1 was applied to culture surface and rested at room temperature for 1 hour prior to removal of the collagen solution and plating of the microglia cells. Cells were stored in liquid nitrogen and thawed completely at 37°C prior to plating. Thawed cells were added dropwise to 20 mL of microglia support medium and centrifuged at 300RCF for 5 minutes. Cells were resuspended in fresh microglia support medium and counted using a hemocytometer prior to plating at a density of 50k cells per permeable insert. Permeable inserts were placed in unused 12 well plates with 1mL of medium added to the bottom side of the insert and 0.5mL of medium added to the top of the insert. Cells were incubated at 37°C and 5% CO_2. After 48 hours microglial support medium was replaced with iPSC support medium. Cells were grown for a minimum of 5 days prior to formation of the co-culture model for experimentation or a minimum of 80% confluency.

Co-culture

iPSC-RPE cells were grown on Matrigel® for a minimum of 23 days prior to treatment and SV-40 Microglia cells were grown for a minimum of 5 days prior to treatment. Microglia cell medium was replaced fully with iPSC-RPE support medium for one change or 48 hours prior to treatment. Treatments were applied in accordance to Table 2. to wells containing iPSC-RPE cells alone with replaced medium. After treatment, microglia on permeable supports medium was replaced and permeable supports were transferred into wells with iPSC RPE (Figure 4). Co-culture system was incubated at 37°C and 5% CO_2.

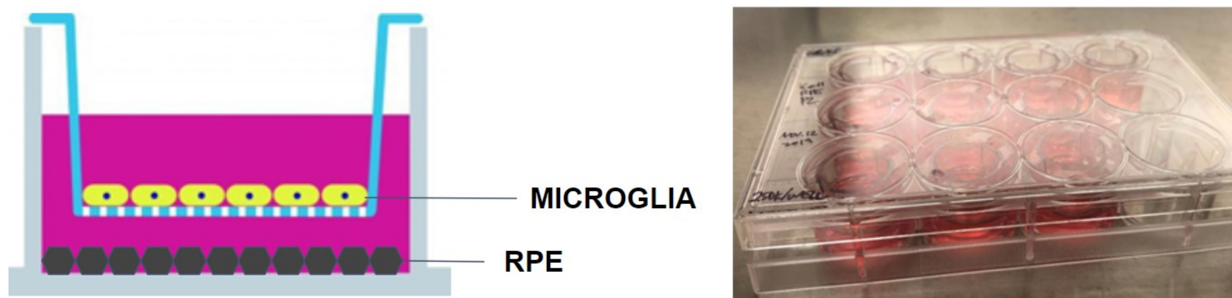

Figure 4. Summary diagram and image of the co-culture transwell system using permeable supports. RPE cells were cultured on flat cell culture plates, microglia were cultured on permeable supports which were then combined. Left shows a diagram of the set-up, and a plate with cells is shown on the right.

2.5 – ELISA analysis of Culture Medium for Cytokine and Chemokine Production/Secretion

Cytokine and chemokine secretions were measured via sandwich ELISA conducted by Eve Technologies (IL-6, IL-8, CCL-2, custom cytokine array manufactured by Millipore Sigma; Calgary, AB). 200 µL samples were collected at the desired timepoints from the center of the treatment well for single cell systems. 200µL of fresh medium was replaced after samples were collected to prevent removal of all medium and death of cell culture. Medium was sampled from

the basal side of the permeable support in the co-culture model and was replaced with 200µL of fresh medium. Media samples were stored at -80C prior to delivery to Eve Technologies for analysis.

2.6 –qPCR analysis of Cytokine and Chemokine Production/Secretion

GeneJET RNA purification kit (Thermo Scientific, Cat, No. K0732, ThermoFisher Scientific, Rochester, NY) was used for RNA extraction. RNA extraction was completed in accordance with kit protocol "B. Mammalian Cultured Cells Total RNA Purification Protocol." Medium was removed from the cell culture and wells were rinsed with DPBS prior to addition of the lysis buffer. 600 µL of lysis buffer supplemented with 20 µL of β-mercaptoethanol was added to each well and cells were lysed and removed through rinsing, pipetting, and mechanical removal using the pipet tip prior to transfer to a sterile DNAse RNAse free tube provided within the kit. Cells were vortexed to ensure complete lysis. Anhydrous ethanol (360 µL) was added to lysates and purification was performed in accordance with kit protocol B. Purified RNA was eluted using 50 µL of nuclease free water as the eluent.

2 µL of each eluted sample was loaded onto a µDrop plate (Thermo Scientific, Cat. No. N12391, ThermoFisher Scientific, Rochester, NY) in duplicate in addition to nuclease free water (control). The µDrop plate was read in a colorimetric plate reader (Thermo Scientific, Cat. No. 51119300, ThermoFisher Scientific, Rochester, NY) using SkanIt Software RE. RNA concentration was calculated using Beer's Law the A260 values determined (Equation 1). RNA purity levels were determined using the A260 A280 ratio (optimal: ~2) prior to proceeding with cDNA synthesis.

$$[DNA] = (A_{260}Sample - A_{260}Blank) * 800 \qquad \text{(Equation 1)}$$

cDNA synthesis was completed using the RevertAid Reverse Transcription kit (Thermo Scientific, Cat. No. K1691, ThermoFisher Scientific, Rochester, NY). The reverse transcription and cDNA synthesis was completed in accordance to the kit protocol. RNAse free tubes were used for this protocol. Approximately 500ng of RNA sample was added to nuclease free water to a total of 8 µL prior to the addition of 1 µL of 10x reaction buffer and 1µL of DNAse I. 1µL of 50 mM EDTA was added to the samples after a 30-minute incubation period at 37°C. The incubation was completed using a thermocycler (Bio-Rad, Cat. No. S1000, Hercules, CA). An additional incubation occurred at 65°C for 10 minutes after addition of the EDTA to the tube. In order, 1µL of random primer, 4µL of 5x reaction buffer, 1µL of RiboLock RNAse inhibitor and 2µL of RevertAid reverse transcriptase were added prior to the final incubations: 25°C for 5 minutes, 42°C for 60 minutes, and 70°C for 5 minutes.

cDNA synthesized was used in RT-qPCR which was carried out in 384 well plates (Applied Biosystems, Cat. No. 4309489, ThermoFisher Scientific, Rochester, NY). A master-mix was created with the appropriate ratios of 10 µL of Fast SYBR Green Master Mix (Applied Biosystems, Cat. No. 438616, ThermoFisher Scientific, Rochester, NY), 8 µL of nuclease free water, 0.5 µL of forward primer and 0.5 µL of reverse primer in accordance to the number of wells required. Master mix was added to each well required for the experiment and 1 µL of cDNA sample was added. 384 well plate was sealed with clear adhesive film (Applied Biosystems, Cat. No. 4306311, ThermoFisher Scientific, Rochester, NY) and centrifuged for 2 minutes at 2000RCF. The plate was run on the Real-Time PCR System (Applied Biosystems, Cat. No. 7900HT-Fast, ThermoFisher Scientific, Rochester, NY)

Real-time PCR system cycles were completed in accordance to instructions included with 10 µL of Fast SYBR Green Master Mix (Applied Biosystems, Cat. No. 438616, ThermoFisher

Scientific, Rochester, NY). Polymerase activation occurred through a 20 second incubation at 95°C in advance of 40 cycles of amplification consisting of denaturation at 95°C for 1s and annealing at 60°C for 20s. Data were collected during the 40 amplification cycles in order to determine the qPCR data.

Fold expression was calculated in accordance to equation 2 using the $\Delta\Delta C_T$ method. C_T refers to the cycle threshold which refers to the cycle number where fluorescence from the PCR product reaches a distinguishable point. ΔC_T was calculated through determining the difference between C_T from *ACTB*, the housekeeping gene, from the C_T of target gene. $\Delta\Delta C_T$ was determined by finding the difference in ΔC_T between the untreated control and the treatments.

$$Fold\ Expression = 2^{-\Delta\Delta CT} \qquad \text{(Equation 2)}$$

2.7- Treatment Summary

Stock concentrations for experimental treatments are outlined in Table 2.

Table 2: Stock concentrations of treatments applied to cell culture experiments

Treatment	**Stock Concentration**
IL-1β	10 µg/µL
TNF-α	10 µg/µL
Poly-IC	8 µg/µL

Experiments with microglia alone would have TNF-α and IL-1β stocks diluted using two sequential 1:1000 dilutions prior to treatments being added to 1mL of freshly changed media. The concentration was 1 pg/µL after these dilutions which can then be added to the wells in accordance to the treatment level. Experiments in co-culture would require treatment with 50% more volume

due to the fact that there is media both apically and basally in the transwell with a total volume of 1.5 mL. Poly-IC was diluted to a final treatment concentration of 8 pg/mL in the co-culture experiment.

2.8 – Image Processing and Statistical Analysis

Data visualization and statistical analysis were performed using GraphPad Prism (v9.3.1) and Microsoft Excel (2019). Data were received, collected and sorted in Microsoft Excel and transposed subsequently into GraphPad Prism. Specifically Fold Change using the $\Delta\Delta C_T$ method was calculated in Microsoft Excel. Figure generation and statistical analysis was carried out in Prism. Unpaired t-test comparisons of treated samples against untreated samples were carried out at each concentration and timepoint using the Mann-Whitney test as we are unable to assume a Gaussian distribution of data.

3. RESULTS

3.1 – Characterization of RPE Cell Lines

iPSC-RPE cell lines were characterized by Manlong Xu in the MacDonald Lab using both PCR characterization and immunofluorescence microscopy. The targets for PCR characterization were *BEST1, RPE-65, MITF, RLBP-1* (Figure 5.) The presence of these bands in panel A demonstrates that the WT iPSC-RPE cells express the target genes for characterization of the cells as RPE. In addition, panel C demonstrates that the *CHM-* iPSC-RPE also express the target genes and can be characterized as RPE. Panel B is the RT- control which serves to demonstrate that there is no contamination which would result in false positive results.

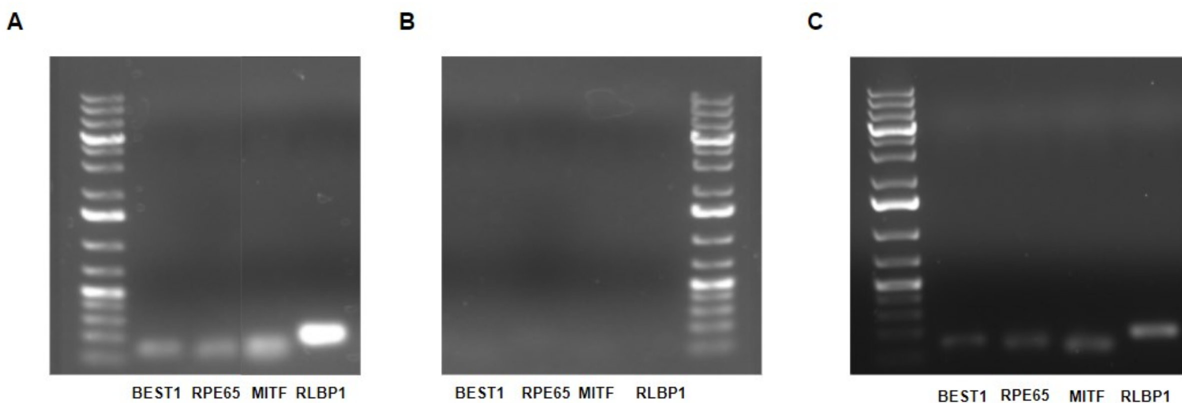

Figure 5. PCR characterization of iPSC Normal (WT) and PS (*CHM-)* iPSC RPE cell lines. BEST-1, RPE-65, MITF, RLBP1 are all RPE specific genes. A. Normal iPSC Derived RPE B. reverse transcriptase negative (RT-) to assess for genomic DNA contamination. C. PS iPSC Derived RPE

Figure 6 uses immunofluorescence to demonstrate the presence of RPE specific proteins, markers, and the demonstration of the formation of tight junctions. Both PS (*CHM-*) and normal cells demonstrate DAPI localization to the nucleus, ZO-1, BEST-1 and CLDN-19 localization to the cell boundaries which is indicative of the formation of tight junctions as well as RPE specific marker presence. Figure 5 and Figure 6 demonstrate the characterization of both normal and *CHM-* RPE completed through both immunofluorescence and PCR and aid in demonstration of the RPE morphology including visualization of tight junctions, polygonal shape, and presence of key RPE genes.

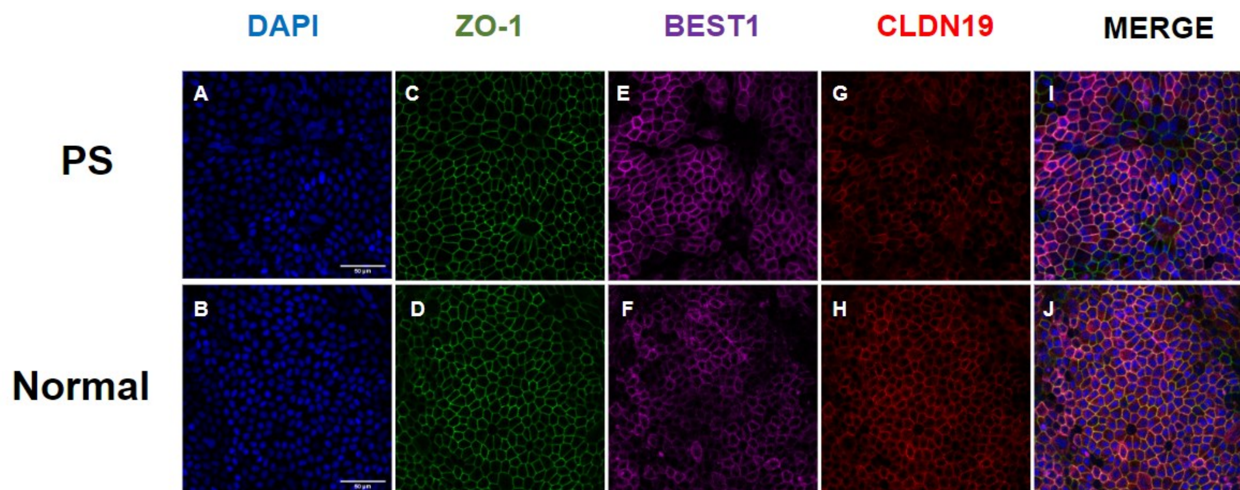

Figure 6. Immunofluorescent characterization of WT and *CHM-* iPSC RPE. Both cell types express DAPI(A-B) which localizes to the nucleus, ZO-1 (C-D) which localizes to cell boundaries, BEST-1 (E-F) which is an RPE specific marker, and CLDN-19 (G-H) which is observed in mature tight junctions.

AIM 1:

3.2 - RPE cell culture lines are capable of pro-inflammatory cytokine release in response to treatment with IL-1β and TNF-α

RPE cells were treated with 2 pg/mL and 20 pg/mL of TNF-α diluted from the stock solutions referenced in Table 2. Separate RPE cells were treated with 0.25 pg/mL and 2.5 pg/mL of IL-1β diluted from the stock solutions referenced in Table 2. Data were collected at treatment time (0hours) 6 hours, 12 hours and 24 hours post-treatment. The strongest pro-inflammatory cytokine secretion responses were observed from treatments with 20 pg/mL of TNF-α which demonstrated a potential trend towards upregulation in the concentration of IL-6, IL-8 and CCL-2 which were all measured in this experiment and shown in Figure 7. The measured concentrations of these cytokines increase across the timepoints and appear to trend towards higher pro-inflammatory cytokine secretion responses at higher initial treatment dose of TNF-α. At the 20 pg/mL treatment level IL-8 concentration was recorded at t=0 to be 0.01 pg/mL and 12.913 pg/mL at t=24. This can be compared to the 2 pg/mL treatment level with an IL-8 concentration at t=24 of 0.19 pg/mL. Similar results are observed for both IL-6 and CCL-2. This was repeated with n=3 and therefore did not reach statistical significance under non-Gaussian distribution assumption.

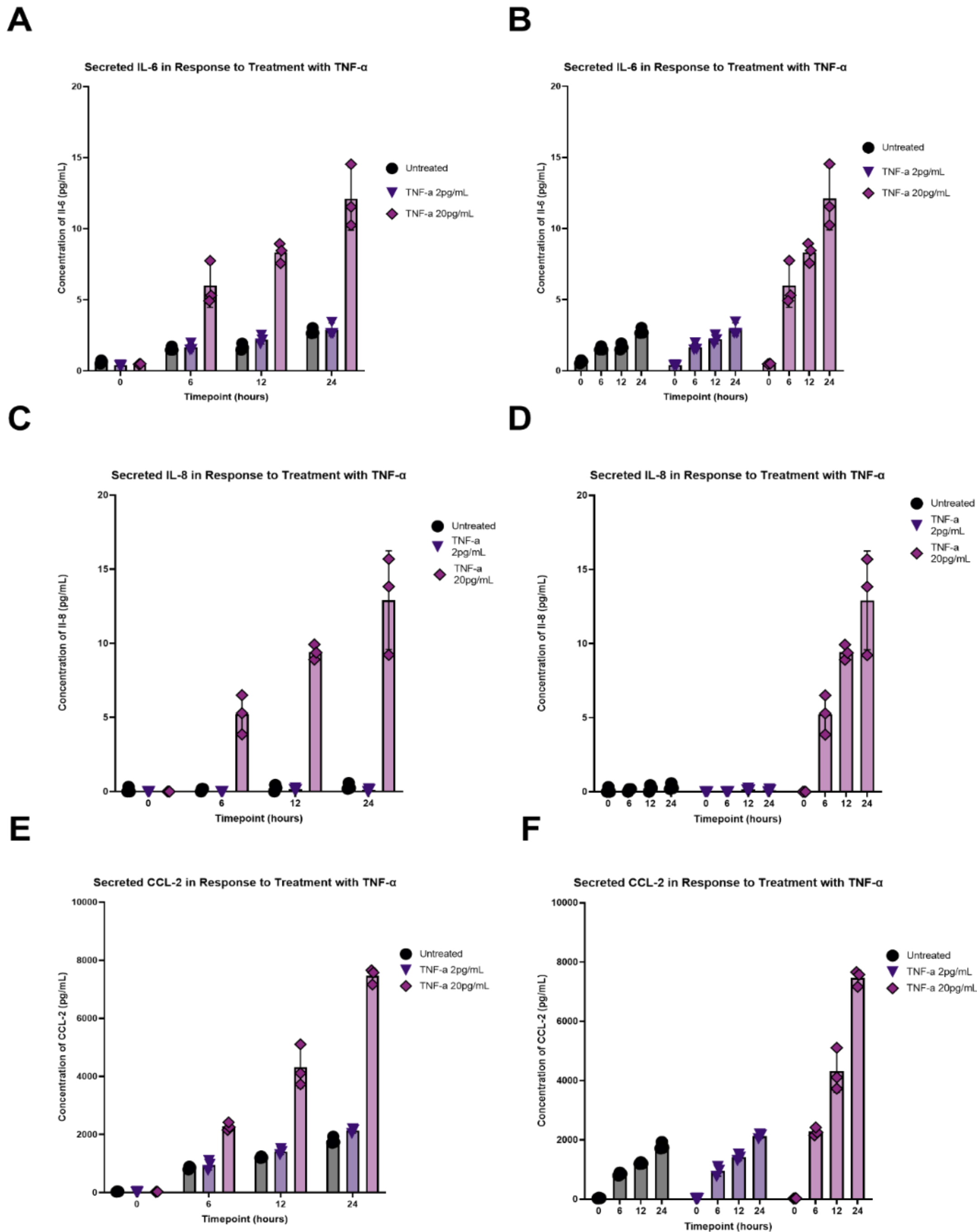

Figure 7. Cytokine secretion from commercial Axol RPE cell lines at specific time points measured using sandwich ELISA in response to treatment with TNF-α. Investigating the release of IL-6, IL-8 and CCL-2 in response to treatments with TNF-α. Graphics are grouped differently depending on time point (A,C,E) and treatment concentration (B,D,F) Increased secretion of IL-6, IL-8 and CCL-2 can be observed with increased treatment concentration although statistical significance was not reached. All p values were greater than $p < 0.05$ ($n = 3$ experimental replicates)

Response of IL-6, IL-8 and CCL-2 secretion to treatment with IL-1β are displayed in Figure 8. There appears to be some fluctuation between untreated controls and treatments with IL-1β and a clear trend does not seem evident. It can be noted that CCL-2 concentrations increase over time in all treatment levels of IL-1β. The data presented in Figure 8 did not reach statistical significance ($p>0.05$, $n=3$ experimental treatments)

After treatment of RPE cell lines with pro-inflammatory master regulators IL-1β and TNF-α a time-dependent increase in CCL-2, IL-8 and IL-6 secretion may be observed in response to treatment with 20 pg/mL of TNF-α across the timepoints of t=0, t=6, t=12 and t=24. This trend is visually depicted within the figure; however, due to experimental replicates being n=3 and constraints of the experiment, this data cannot be deemed statistically significant without the assumption of Gaussian distribution which does not apply to this case. There is evidence to suggest a dose dependency in treatments with TNF-α which may reach statistical significance with more replicates. This is not observed in the IL-1β treatments.

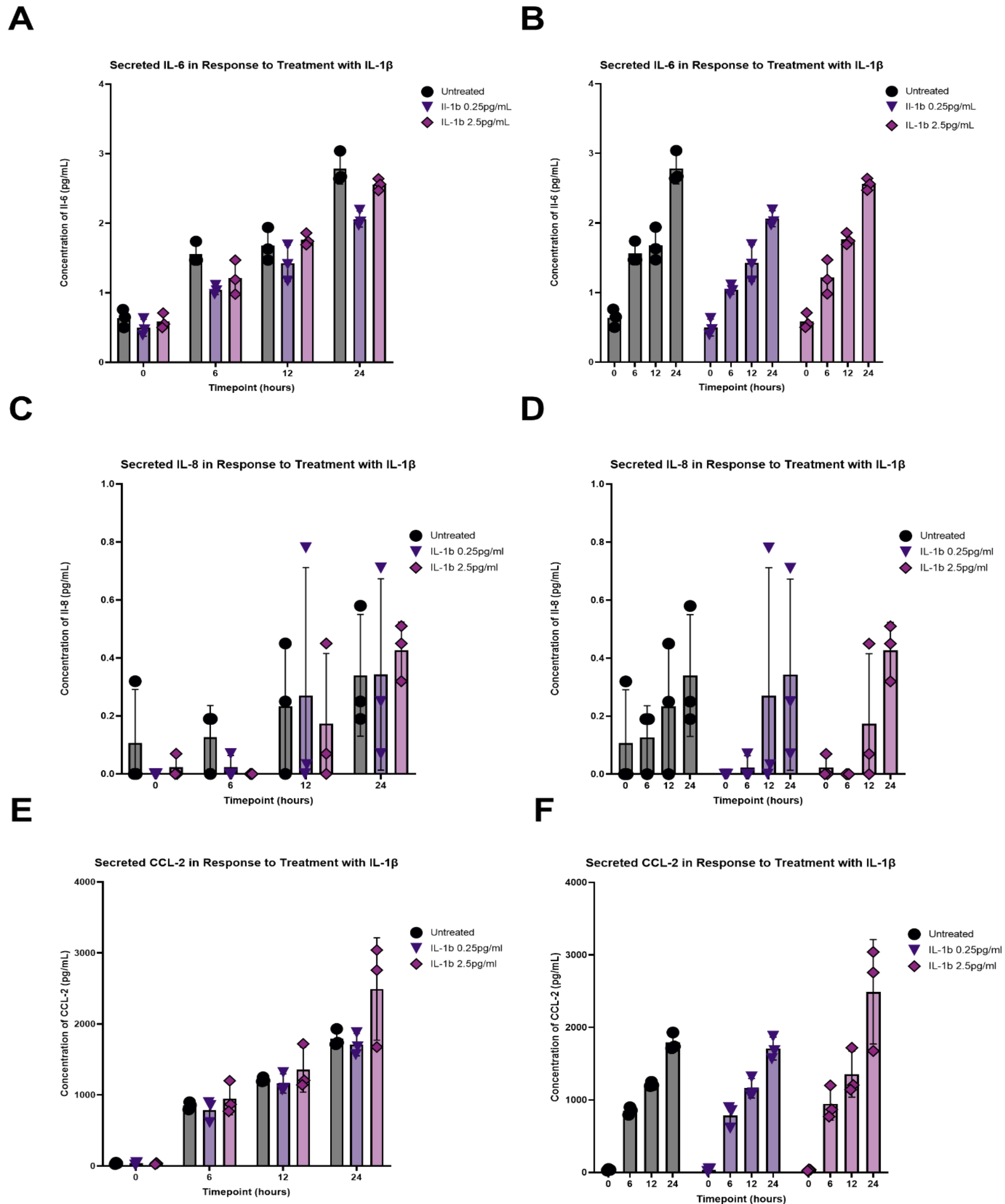

Figure 8. Cytokine secretion from commercial Axol RPE cell lines at specific time points measured using sandwich ELISA in response to treatment with IL-1β. Investigating the release of IL-6, IL-8 and CCL-2 in response to treatments with IL-1β. Graphics are grouped differently depending on time point (A,C,E) and treatment concentration (B,D,F) Statistical significance was not reached in any treatment. All p values were greater than $p < 0.05$ (n = 3 experimental replicates)

AIM 2:

3.3 - Response of SV-40 Microglial cell lines to treatment with IL-1β

3.3.1–qPCR

SV-40 microglia cells were treated with 1 pg/mL, 10 pg/mL or 100 pg/mL of IL-1β diluted from the stocks in Table 2. Data were collected at 3 hours, 24 hours, 72 hours, and 168hours post treatment for fold change in expression of CCL-2 and IL-8 in response to IL-1β treatment. Overall there appears to be minimal impact on fold change of CCL-2 and IL-8 RNA after treatment with IL-1β. A 15.5-fold increase can be observed at 3 hours post treatment with 100 pg/mL of IL-1β, however due to the low number of replicates and high variability this data did not reach statistical significance ($p>0.05$, $n=2$ experimental replicates). Fold change of both IL-8 and CCL-2 RNA concentrations in response to various concentrations of IL-1β treatments can be seen in Figure 9.

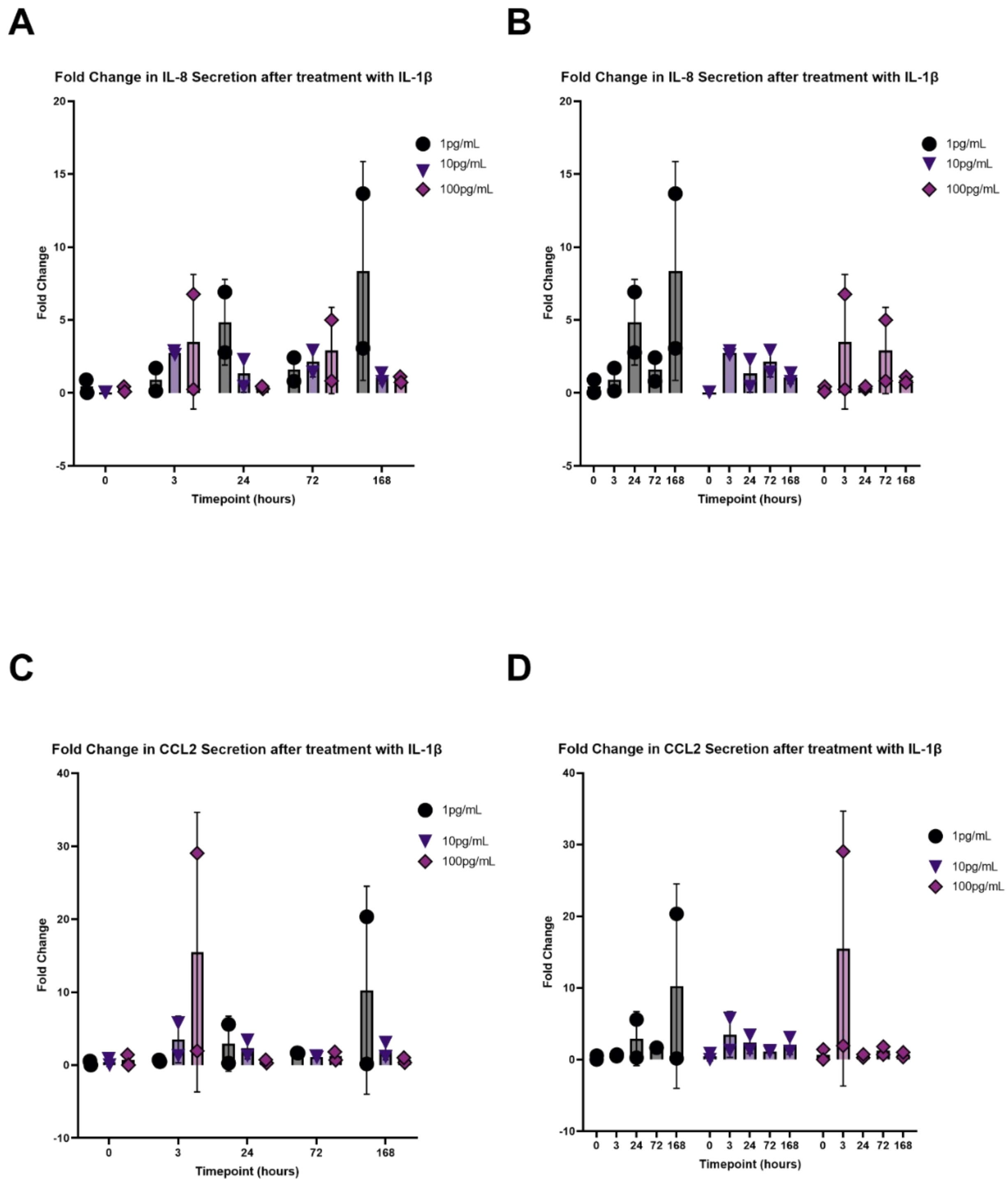

Figure 9. Fold-expression of mRNA measured using RT-qPCR for innate immune cytokine genes for IL-8 and CCL-2 in response to short and long timeframes of treatment with IL-1β in SV-40 microglia. Graphics were grouped differently based on timepoint (A,C) and treatment concentration (B,D) Statistical significance was not reached in any treatment. All p values were greater than $p < 0.05$ (n = 2 experimental replicates)

3.3.2- ELISA

The average concentrations of CCL-2, IL-8 and IL-6 secreted from SV-40 Microglia cells in response to treatment with various concentrations of IL-1β are shown in Figure 10. SV-40 microglia cells were treated with either 1 pg/mL, 10 pg/mL or 100 pg/mL of IL-1β diluted from the stocks referenced in Table 2 and data were collected at 3 hours, 24 hours, 72 hours and 168 hours. There does not appear to be a noticeable trend difference between the treatment levels however, there is a progressive increase in the concentration of CCL-2 and IL-8 measured across the timepoints which may be independent from treatment concentration. Rather than observing the hypothesized dose-dependent response, the data demonstrate a progressive increase in pro-inflammatory cytokines on a time dependent relationship which appears to be unaffected by treatment concentration. This can be observed in Figure 10 and the treatments did not reach a point of statistical significance with $p < 0.05$ (n=3).

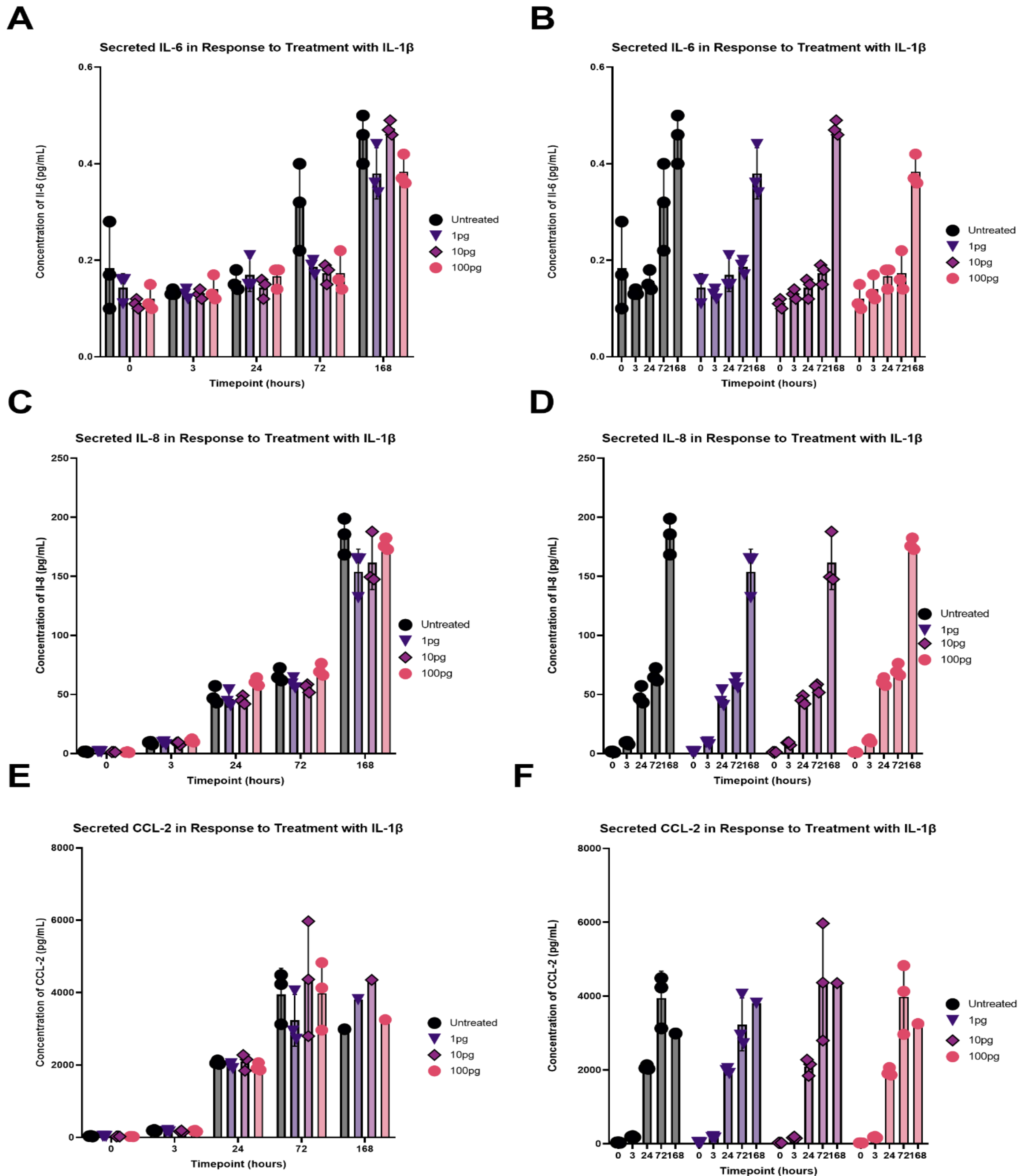

Figure 10. Cytokine secretion from SV-40 Microglia cell lines at specific time points measured using sandwich ELISA. Investigating the release of IL-6, IL-8 and CCL-2 in response to treatments with IL-1β. Graphics are grouped differently depending on timepoint (A,C,E) and treatment concentration (B,D,F) Statistical significance was not reached in any treatment. All p values were greater than $p < 0.05$ (n = 3 experimental replicates)

AIM 3:

3.4 – Improving growth of RPE cell lines on flat plates for establishment of co-culture model

The progressive growth of WT iPSC-RPE cells on cell culture plates is shown under two separate treatment conditions in Figure 11. iPSC-RPE cells in both treatments had media supplemented with 10% FBS for the first 48 hours after plating, then 5% for the next 48 hours and 1% for a final 48 hours before proceeding with growth in serum free media. The treatment difference was whether or not plates were coated in Matrigel® prior to plating of the iPSC RPE cells. Day 3 was selected as it was the first change in media serum concentration. It can be observed at this time point that on the plates coated with Matrigel® there is a higher confluence of cells. Day 7 was a selected timepoint as it was the change to serum free media. It can be observed that cells on the plate coated with Matrigel® formed more clear boundaries, are smaller in shape and appear more organized which is characteristic of the RPE maturation process. Day 19 was a selected timepoint due to the tear in the monolayer which occurred in the FBS only trial. This was an issue characteristic of RPE cell growth prior to coating the plates with Matrigel® and resulted in the discarding of the trial due to loss of integrity of the RPE monolayer. When compared to the FBS and Matrigel® treatments together, no separation of the monolayer appeared to occur. The forming of tight junctions and decreasing in cell size during maturation is what causes the issue of monolayer tearing. Day 45 was the final timepoint recorded prior to cell sacrifice. At this timepoint very small and pigmented RPE cells were observed without any loss of integrity from the monolayer in the Matrigel® coat trial. Bubbling of the monolayer was beginning to occur due to apical-basal pumping of cell culture media which caused the distortion of the focal plane.

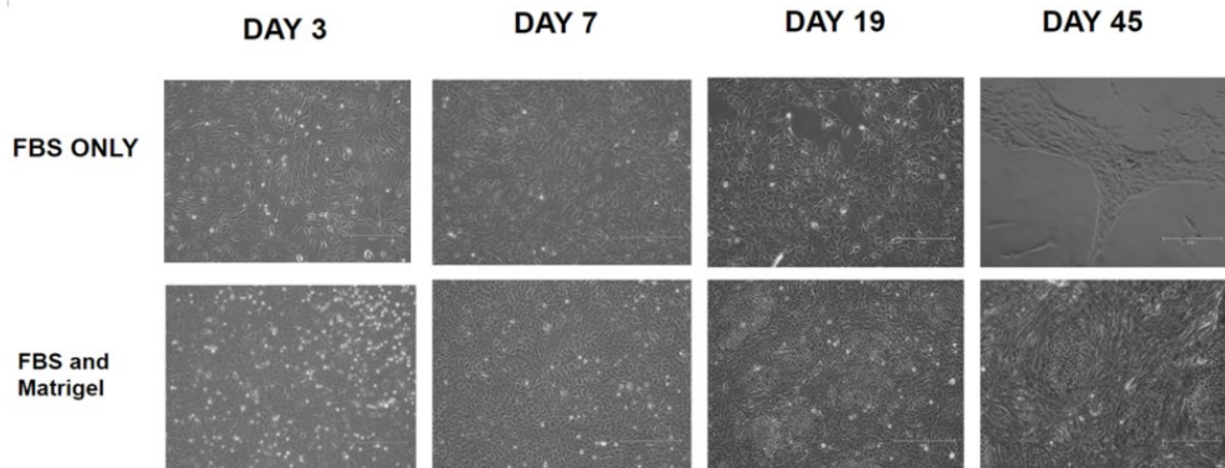

Figure 11. Progressive growth of wild type iPSC-RPE cells on cell culture plates. Plates which were coated in Matrigel® showed decrease in maturation time of the RPE demonstrated through polygonal shape and smaller cell size. Matrigel® coated plates also increased longevity of cell adhesion and survival on cell culture plates. Images were captured at 10x magnification.

AIM 4:

3.5 - Response of the Co-culture model to pro-inflammatory cytokine treatment

The average concentrations of CCL-2, IL-8 and IL-6 secreted from iPSC-RPE and SV-40 microglia in co-culture, treated with immune master regulators IL-1β and TNF-α are seen in Figures 12 and 13. Figure 12 shows the concentrations of CCL-2, IL-8 and IL-6 secreted in response to treatment with IL-1β and collected at 3 hours, 24 hours, 72 hours and 168 hours. Figure 13 shows the concentrations of the same cytokines at the same timepoints in response to treatment with TNF-α. In Figure 12 it appears as though the concentrations of cytokines released in response to IL-1β administration do not vary largely between treatments and do not reach statistical significance. However, a trend can be observed where there is an increase in cytokine concentration over time regardless of treatment level. This is particularly clear when observing IL-6 and IL-8 in response to treatments with IL-1β, observing the largest increase between 72 and 168 hours. This observed dramatic increase in cytokine concentration may be due to accumulation of cytokines in the media across the treatment timepoints.

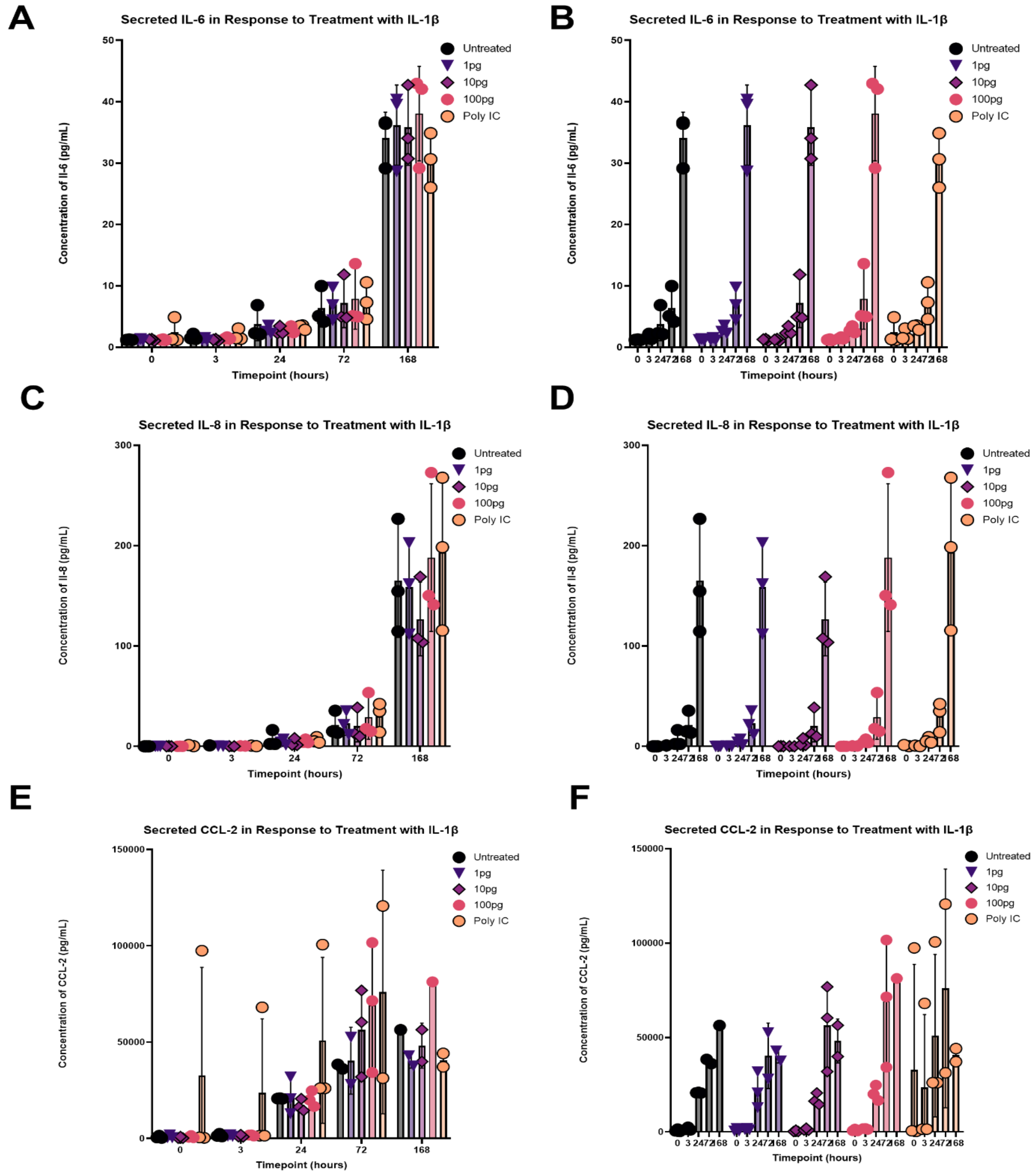

Figure 12. Cytokine secretion from co-culture of WT iPSC RPE plated on Matrigel® and SV-40 microglial cell lines treated with IL-1 β measured using sandwich ELISA at specific time points. Investigating the release of IL-6, IL-8 and CCL-2 in response to treatments with IL-1β. Graphics are grouped differently depending on timepoint (A,C,E) and treatment concentration (B,D,F) Statistical significance was not reached in any treatment. All p values were greater than $p < 0.05$ (n = 3 experimental replicates)

Figure 13 shows concentrations of cytokines released in response to TNF-α administration do not vary largely between treatments and do not reach statistical significance ($p>0.05$). Like in Figure 12, a trend can be observed where there is an increase in cytokine concentration over time regardless of treatment level. In addition, when comparing Figures 12 and 13 it is important to note that there are higher concentrations of IL-6 and IL-8 released in response to treatment with TNF-α when compared to treatment with IL-1β. Secreted CCL-2 concentrations are similar between the two treatments

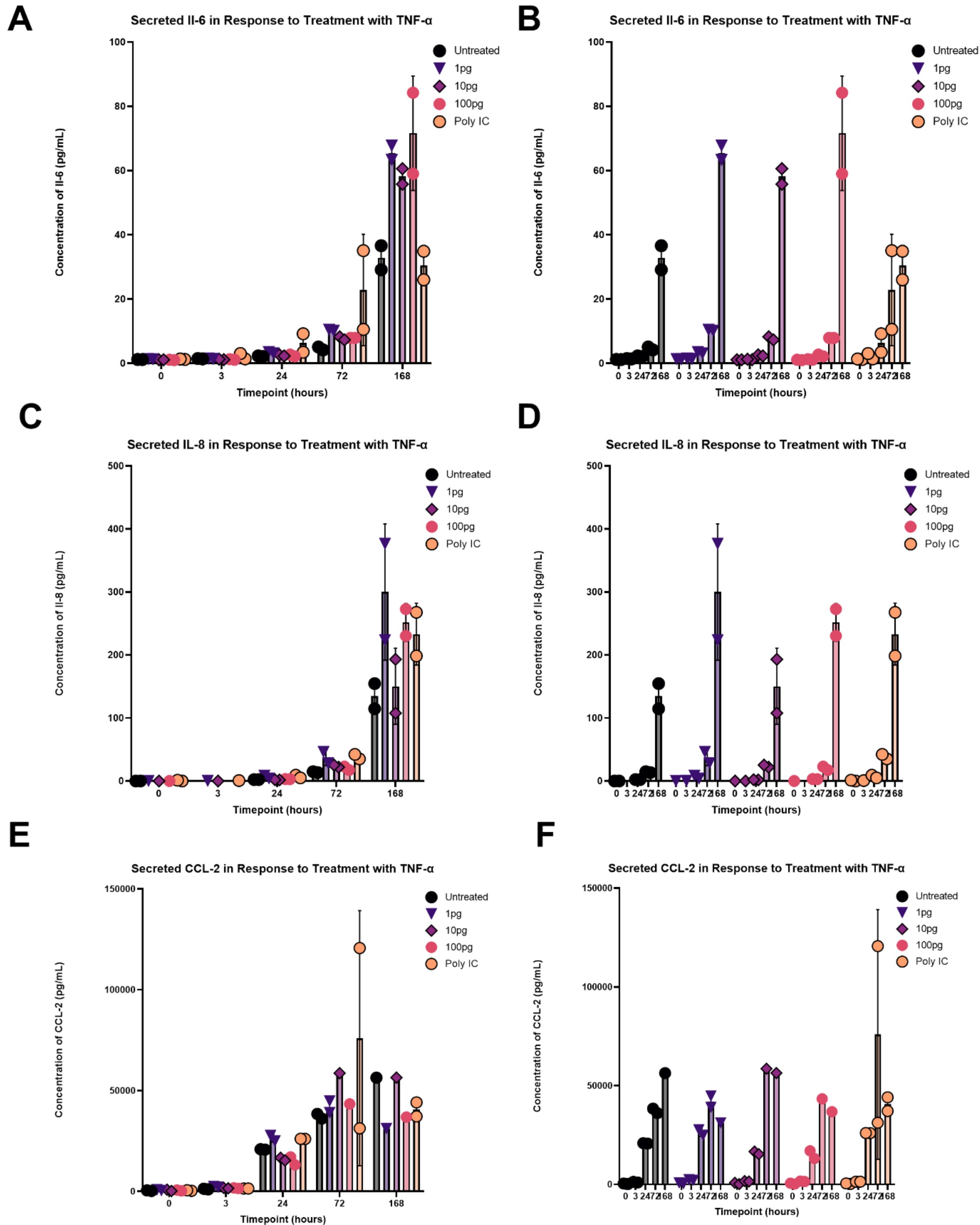

Figure 13. Cytokine secretion from co-culture of WT iPSC RPE plated on Matrigel® and SV-40 microglial cell lines treated with TNF-α measured using sandwich ELISA at specific time points. Investigating the release of IL-6, IL-8 and CCL-2 in response to treatments with TNF-α. Graphics are grouped differently depending on timepoint (A,C,E) and treatment concentration (B,D,F) Statistical significance was not reached in any treatment. All p values were greater than $p < 0.05$ (n = 3 experimental replicates)

AIM 5:

3.6 – Presence of microglia delays RPE cell death *in-vitro*

The growth and survival of RPE cells under three different conditions is demonstrated in Figure 14. RPE cells were either grown alone or including a permeable insert with or without microglial cells added to the culture system 18 days after plating of the cells. At Day 0 permeable inserts were introduced. All three conditions appear to have no distinguishing features at 3- and 7-day post-treatment. At 11 days post treatment it appears that the RPE only cells are beginning to detach from the plate with tight junctions becoming decreasingly apparent. The intervals between day 11 and day 18 could not be recorded due to the fact that I contracted COVID-19 and could not follow up with this experiment. At day 18 both the RPE only and the RPE + permeable insert treatments without microglia have had all RPE cells die and detach from the cell culture plate. When comparing these conditions to the RPE + microglia at Day 18, this treatment has the RPE monolayer remaining intact with tight junctions and maintenance of the characteristic polygonal shape. This result provides evidence to suggest that the microglia may have a role in maintaining the RPE monolayer integrity and health beyond what the RPE is capable of doing alone. It is important to note that the media color of the RPE cells and RPE + permeable insert was yellow indicating it was acidic. In contrast, the microglia + RPE medium maintained its pink-orange colour indicating that it was less acidic. This provides evidence to suggest that the microglia may also play an important regulatory metabolic role that involves pH control.

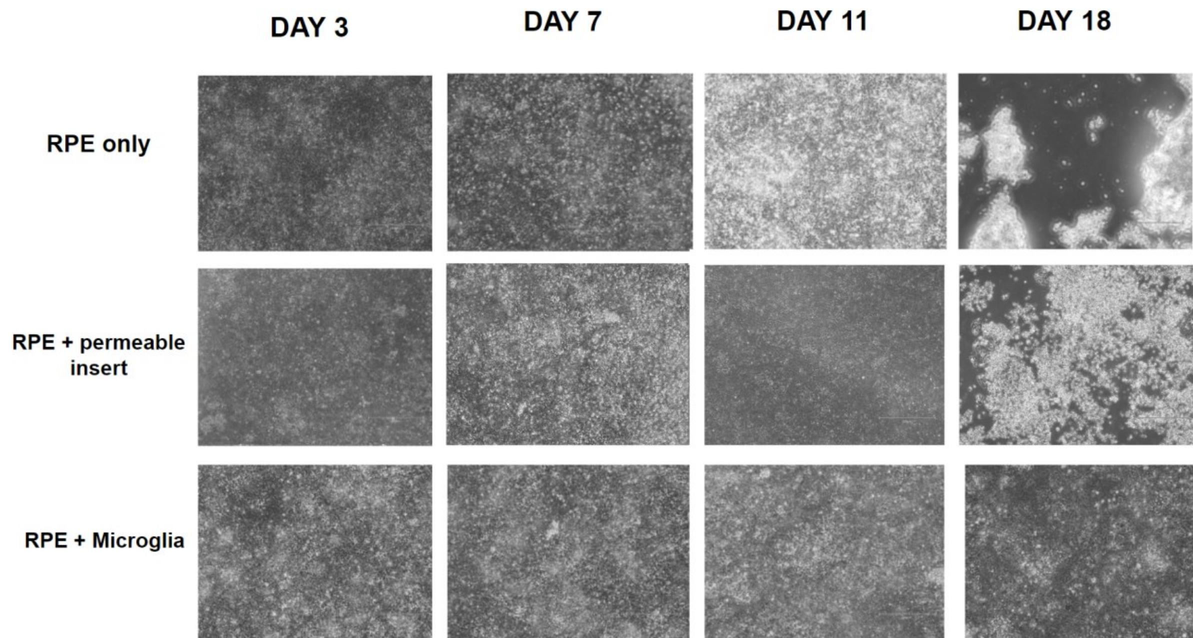

Figure 14. Growth of WT iPSC-RPE cells after addition of SV-40 microglia cells on permeable supports. RPE that were co-cultured with microglial cells outlived RPE cells with no other treatment or the addition of an empty permeable insert. Timepoints were measured starting at Day 0 being the day where treatment was introduced. Microglial cells appear to have increased the longevity of RPE cells. Images were captured at 10x magnification.

4. Discussion

4.1 – Why create an *in vitro* model of the immune mechanisms of the retina?

Ocular gene therapies are becoming a focus of research interest and therapeutics as demonstrated by the recent FDA and Health Canada approvals for Luxturna.[74] One of the primary risks of ocular gene therapy treatments is unwanted inflammation and immune response leading to poor outcomes and further damage to ocular tissues. Understanding these immune mechanisms by using a model in the lab may be helpful in identifying therapeutic targets to improve outcomes in viral vector-based gene therapy treatments. In many pre-clinical trials, an animal model is often used prior to administration of the therapy to humans; however, there are a series of shortcomings to this. It is well documented and understood that there are a variety of differences between human and animal models when comparing both ocular tissues, responses, and immune systems.[75] Previous pre-clinical testing of therapies in animal models may not display significant inflammation or raise concerns that there is a risk for potential poor outcomes. However, from experience in clinical trials using AAV-2 on human patients, it is known that these inflammatory and immunological events may occur in a dose dependent manner which narrows the therapeutic window (personal discussion, YK Chan, Wyss Institute, Harvard).[58] Although an animal model may provide insight into certain functions, structures, and responses to various gene therapies in the pre-clinical stage, it may not give an accurate representation of the full risk profile or immunological potency of various gene therapy treatments in humans. As a result of this understanding, it is important to recognize the benefit of creating a human-cell based *in-vitro* model. This model can be used in conjunction with the data collected in pre-clinical animal model studies to increase assurance of therapeutic or procedural safety to minimize risk of poor outcomes. There are benefits and limitations of both cell culture and

animal models and that collecting information and data from both will maximize safety and lead to a reduction in poor outcomes.

4.2 – Creation and assessment of the *in-vitro* co-culture model using SV-40 microglia and normal iPSC-RPE cell lines

In this study, the objective was to successfully create a co-culture model which is able to engage in crosstalk and demonstrate immunological competency when treated with pro-inflammatory stimulus. Pro-inflammatory response was measured by ELISA analysis and qPCR to measure the secretion of pro-inflammatory cytokines IL-6, IL-8 and CCL-2. In this study, I successfully formed a co-culture model through improving growth and longevity of iPSC-RPE cell lines on cell culture plates in addition to establishing a model with both microglia and RPE in a biologically relevant orientation respective to one another. When measuring the cytokine and chemokine secretion from the co-culture model, when compared with the untreated control, treatment with IL-1β or TNF-α did not yield any statistically significant differences across any of the timepoints. This makes it difficult to draw conclusions from the data presented and certainly provides grounds for further study. Through observation of results, it is clear that the cells in the co-culture system are capable of secreting cytokines as there is a noticeable increase in cytokine concentration as the timepoints progress. The lack of statistical significance between treated and untreated samples may have occurred for a variety of different reasons, which would merit future investigation into this model with appropriate modification to treatment protocols.

Microglial cells are tightly regulated due to their destructive capabilities and non-discriminatory action which may lead to host cell damage and death.[50] As a result of this,

treatment with exclusively IL-1β or TNF-α may not be sufficient to cause large microglial cell pro-inflammatory activation. IL-1β and TNF-α are both capable of activating NF-kB pathways in microglial cells, resulting in a downstream pro-inflammatory signalling cascade however; when the microglia were being tested separately, as well as in co-culture, there was no significant change in pro-inflammatory cytokine secretion between treated and untreated trials.[76,77] This may occur as a result of the tight pro-inflammatory regulation and immune checkpoints on microglia which prevent a sustained pro-inflammatory response without a continuous stimulus. In contrast, when the RPE cells were treated separately with TNF-α, I observed an increase in cytokine secretion although it did not reach statistical significance.

Although it has been noted that IL-1β and TNF-α are capable of activating NF-kB within microglia potentially leading to pro-inflammatory activation, it is likely that the downregulatory action of the microglial cells prevents this pro-inflammatory stimulus from causing a signal cascade and robust pro-inflammatory response. This is likely as a result of mechanisms in place to prevent 'false alarms' and unwanted off target damage due to acute changes in cell signalling and systems. Co-stimulation with a secondary signal required to activate immune response such as triggering a toll-like receptor or positive feedback from another cell type in addition to treatment with only TNF-α or IL-1β may be required in order to cause robust pro-inflammatory immune response from the microglia leading to the expected crosstalk response. Co-stimulatory factors such as a PAMP would act as further confirmation of immunological threat and stimulus which is in place to minimize unwanted activation and damage which occurs from the microglia. In addition to this, introducing other ocular immune cell types such as Muller cells may add necessary missing components of the proinflammatory crosstalk interaction between the RPE

and microglia preventing immunological upregulation, leading to the lack of significant differences between the untreated and treated samples.

I observed that when testing the longevity of the RPE monolayer in the presence of microglia, RPE cells had completely died in wells not containing microglia in addition to media being bright yellow indicating a low pH. In contrast, the co-cultured wells with both microglia and RPE maintained an intact monolayer and media that was an orange color suggesting that the microglia may have a role in maintaining the RPE cells and media pH. This observation helps to support the role of microglia in maintaining tissues and homeostasis in non-activated and non-immune state. It is unclear what the mechanisms underlying the pH difference between the RPE alone when compared with trials in co-culture; however, it can be assumed that the microglia contribute to pH regulation or slower metabolic rate. Previous research has demonstrated that microglia are important in development and homeostasis of central nervous system (CNS) cells and the retina through aiding in tissue development, maintaining of vasculature and secretion of factors that promote and regulate development and maintenance of cells. Further exploration into the crosstalk which promotes maintenance and survival of the RPE cells cultured in the presence of microglia should be explored in future experiments.

In this study, there was a notable improvement in the successful culturing and longevity of growth for RPE cells on coated cell culture plates which enabled the creation of a co-culture model. However, the lack of significant data occurring at all timepoints when comparing untreated to treated samples with IL-1β and TNF-α makes it difficult to draw notable conclusions about the capabilities of this model for study of immunological activity and for the testing of therapeutic targets.

The results from investigating cytokine secretion across all timepoints in the co-culture model did not demonstrate significant differences under the assumption of a non-Gaussian distribution using a Mann-Whitney test where $p<0.05$. The data presented do not support my hypothesis that the co-culture model will demonstrate a dose-dependent response to pro-inflammatory stimulation. Future study is necessary for the continued use of this model in order to gain comprehensive understanding of the immune mechanisms of the crosstalk between the RPE and the microglia in addition to the potential mechanisms which promote increased RPE cell survival in co-culture with microglia. Topics of interest for future study may include an investigation into co-stimulatory mechanisms which lead to more robust and sustained immune response. In addition to this, expanding the model to include an additional critical ocular immune cell type, notably the Muller cells, may result in observing crosstalk and the escalating robust immune response that was expected in this study.

4.3- Study Implications

Although it is difficult to draw conclusions about the immunological capabilities of the co-culture model from the presented data due to the lack of statistical significance, improvements to model design and protocols may aid with future exploration using similar techniques. Improvements and optimizations contributing to enhanced longevity and survival of cultured iPSC-RPE cells on cell culture plates that were adjustments to techniques discovered in this study, may enable future success and reduced setbacks in futures studies. The greatest point of difficulty when working with RPE cells in a culture model is ensuring that the monolayer remains intact, and the cells do not detach from the culture plate from the pumping of fluid from the apical to basal sides of he cells. The optimizations to RPE growth in cell culture in addition to the procedures for creating the co-culture model established in this study should benefit others

in future discovery. The techniques developed should mitigate difficulties that I encountered in growing the RPE cells and reduce the time used to troubleshoot these issues. This should allow for more efficient study and investigation into the immunological interactions and crosstalk which may occur in this model.

4.4 – Future Directions and Experiments

4.4.1 – Use of iPSC derived microglia and RPE cells from the same patient

In this study, the development of the co-culture model occurred with iPSC-RPE cell lines derived from patient fibroblasts and non-isogenic human SV-40 immortalized microglia (not the same genetic origin as the patient fibroblasts). It is already known that immortalization and serial passaging may cause alterations in proper cell signalling and interactions in cells undergoing the immortalization process. In order to create a further enhanced and patient specific model, it would be advisable to derive both the RPE and microglia cells for use in co-culture directly from the same patient fibroblast cells. Derivation of microglia from human pluripotent stem cells has been previously achieved per Speicher et al. but could not be completed in this study due to time restraints and inability to receive various necessary supplies due to the COVID-19 pandemic.[78] Creating a model using the same iPSC line to derive both the RPE and microglia prior to immunological testing may yield results more characteristic of *in vivo* expectations. In addition, when studying ocular genetic diseases such as choroideremia, the mutation may cause unexpected immunological changes or elicit stress signals from cells carrying the mutation which would not be observed using the SV-40 microglia cell line. Having both the microglia and RPE cells derived from the same patient iPSC cell line may provide a more accurate insight into immunological interactions in addition to any changes or unexpected occurrences which may be

caused by the mutation in the patient cells which could not be observed in the model used in this study with SV-40 microglia.

4.4.2 – Targeting the Toll-Like Receptors with a pro-inflammatory stimulus

In this study, the pro-inflammatory stimuli used were TNF-α, IL-1β and Poly(I:C). TNF-α and IL-1β are capable of activating cellular pro-inflammatory responses through the NF-kB pathway and although Poly(I:C) is capable of activating cells through triggering TLR-3, the treatment concentration used in this study was likely below the threshold amount to trigger a sustained response. In this study, TNF-α and IL-1β were selected due to their characteristics as master regulators of the pro-inflammatory response of immune cells. Using TLR-3 triggers such as Poly(I:C) at higher concentrations or an AAV viral vector at high concentrations will activate different pathways and result in the secretion of additional soluble cytokines and chemokines which may be essential for propagating the immune response and facilitating the crosstalk interaction which was hypothesized. Although treatment with TNF-α and IL-1β as master regulators is capable of triggering the release of many pro-inflammatory cytokines, using the AAV viral vector or an analogue will provide insight into the mechanism of pro-inflammatory action that would be observed in the ocular gene therapy clinical trials which use a sub-retinal injection and AAV-2 vector.

4.4.3 – Activation or priming of the co-culture model prior to treatment with the pro-inflammatory stimulus.

During the sub-retinal injection procedure, a retinal detachment is created, and a bleb is formed prior to injection of the AAV-2 viral vector for gene therapy treatment. Physical injury and damage cause the release of DAMPS which are detected by immune cells such as the RPE and microglia and cause activation and inflammatory response with the objective of healing the insult and preventing infection. The physical damage caused by the sub-retinal injection would result in activation of retinal immune cells *in-vivo*, resulting in a more robust and faster immune response when triggered with additional pro-inflammatory stimuli such as an AAV-2 vector or pro-inflammatory cytokines. In this study, prior activation of immune cells was not done because the objective was to record pro-inflammatory cytokine secretions in response to pro-inflammatory stimulation from resting state. In future studies, activation, or pre-treatment prior to experimental treatment with pro-inflammatory stimulus could yield different results which may be more characteristic of what would be expected to occur in the retina after physical damage from the sub-retinal injection for gene therapy. As suggested by Langmann et al. (personal discussion), pre-treatment with LPS under a series of protocols may activate the microglia sufficiently to their alerted state. Perhaps with prior activation of the cell-culture model the dose-dependent relationship to pro-inflammatory stimulus treatment would be observed in accordance with the hypothesis.

4.4.4- Using a mutant cell line

In the co-culture model of this study, normal (WT) iPSC-RPE cells were used. During gene therapy treatments, the target cells have a mutation which may cause stress signals or other signals to be released or not released altering the crosstalk and immune response of surrounding cells. In future studies, mutant patient iPSC cells may be used in order to explore if there is underlying immunogenicity caused by the mutation. As an example, patient fibroblasts from an affected patient (*CHM-*) could be used to derive both microglia and RPE which may be tested for baseline immunogenicity which may be caused by the *CHM-* mutation. Mutations may cause cellular changes which could lead to creation of DAMPs or display of other stress signals which lead to activation of the immune cells within the co-culture model. Prior detection of DAMPS or stress signals would serve to prime the immune cells for response increasing immunogenicity from further stimulus. Creating a co-culture model from patient cells with the mutation of interest may provide enhanced insight into immunological complications which may arise specific to the disease of study. This will allow for better anticipation of potential immunological complications which may be caused by the presence of the mutation within the cells.

4.4.5 -Investigation into RPE maintenance by the microglia

In this study, microglia in the absence of a pro-inflammatory stimulus appeared to prolong the survival of iPSC-RPE in culture. It has been previously understood that microglia are critical in neurological development and homeostasis in both the CNS and the retina. In future study, this model could be used and tested to investigate why the iPSC-RPE cells survive longer in the presence of microglia. Cytokines and signals which are important in homeostasis interactions of the microglia including IGF-1 and TGF-β could be assayed in order to use this model to investigate the role of microglia in maintenance of RPE cells and homeostasis. In addition, the

difference in color of the media being less acidic in the microglia and RPE trial provides evidence to suggest the microglia are important in control of pH. Exploring these mechanisms for control over media pH and other associated processes may provide more insight into crosstalk which occurs between RPE and microglia beyond pro-inflammatory responses.

4.5 Conclusions

In this study, I created a co-culture model using SV-40 Microglia and patient iPSC-RPE cells to investigate the immunological effects of treatment with pro-inflammatory stimuli on this model. I hypothesized that there would be a dose-dependent response to treatment measured through secretion of pro-inflammatory cytokines IL-6, IL-8 and CCL-2 in response to treatment. In this investigation, it is difficult to draw conclusions from the data investigating cytokine secretion from this model due to the lack of statistically significant data. In this study, I successfully optimized the growth of RPE cells on cell-culture plates which maintained the RPE monolayer over extended periods of time. The model allows for the microglia and RPE to exist in a biologically relevant orientation with respect to one another and allows for structural success of the model. Using this model optimization in future studies will reduce the difficulties of growing the RPE cells. Future models may also want to expand on the work completed here by integrating additional cell lines into the model which may result in additional intercellular interactions and crosstalk providing further insight into the immune mechanisms of this retina. Future optimization and study using this model will provide insight into the immune mechanisms of the retina and may be tested using therapeutics such as glucocorticoids or receptor antagonists to reduce pro-inflammatory crosstalk interactions. In addition to this, downregulatory signals for microglial activation may also be explored such as signalling with downregulatory interferons

and fractalkine in order to prevent microglia from propagating their immune response. Refining and testing of this model optimized in this study may lead to future identification of therapeutic targets and agents which can reduce pro-inflammatory action caused by subretinal injection of AAV vectors for gene therapy. This will lead to the discovery of strategies and therapies for mitigation of poor outcomes during gene therapy treatments of ocular disease in the retina.

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