Flinders University

Effects of Inflammatory Cytokines on Human Retinal Endothelial Cells

By

Lisia Barros Ferreira

MD

Thesis
Submitted to Flinders University
for the degree of

Doctor of Philosophy

College of Medicine and Public Health
July 2023

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ABSTRACT

Uveitis is among the leading causes of blindness worldwide. The treatment of non-infectious uveitis is not ideal, especially for those forms of the disease that involve the posterior segment of the eye. In this setting, retinal endothelial dysfunction results in vascular leakage and macular oedema, consequently leading to visual loss.

Cytokines are major mediators of uveitis, and some are also known to alter endothelial cell function, potentially contributing to loss of the inner blood-retinal barrier which is mainly composed of tightly interconnected retinal endothelial cells. In research documented in this thesis, the core mechanisms involved in cytokine-induced human retinal endothelial cell dysfunction, the presence of a key cytokine receptor in these cells, and the expression of long non-coding RNAs (lncRNAs) potentially involved in the cell activation were examined.

The effects of selected cytokines on the electrical impedance of human retinal endothelial cell monolayers were tested with a real-time biosensor, demonstrating that tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 induced the breakdown of the retinal endothelial barrier, while IL-8, IL-17 and C-C motif chemokine ligand 2 had no significant effect on barrier function. Moreover, IL-6 did not enhance the impairment of the endothelial barrier when applied in combination with TNF-α or IL-1β.

The decline of the cellular electrical impedance provoked by IL-6 suggested that the IL-6 receptor (IL-6R) was expressed by human retinal endothelial cells, a surprising finding considering the available literature. Reverse transcription-polymerase chain reaction (RT-PCR) and flow cytometric studies were employed to examine IL-6R expression in human retinal endothelial cells, and *in silico* methodology combined with small interfering RNA experiments were employed to assess IL-6R regulation by transcription factors. Expression of IL-6R was confirmed in human retinal endothelial cells, and TNF-α, IL-1β and lipopolysaccharide were shown to downregulate IL-6R in these cells. Also, the transcription factor, ETS proto-oncogene 1, was revealed to play a role in IL-6R regulation.

To address the underlying mechanisms of the observed cytokine-induced barrier changes in the retinal endothelial cell monolayers, RT-PCR and immunolabeling studies, as well as a cell survival assay and flow cytometric analyses of apoptosis and necrosis, were performed. Both TNF-α and IL-1β disturbed the intercellular junctional components and induced cell death, in the case of TNF-α by necrosis, while IL-6 mildly affected the integrity of the junctional complexes, without being cytotoxic.

Long non-coding RNAs influence endothelial cell function, but the literature on retinal endothelial lncRNAs is limited, and lncRNAs have not been investigated in the context of uveitis. A number of lncRNAs putatively expressed in human retinal endothelial cells were identified. Due to the documented pronounced effects of TNF-α and IL-1β on the retinal endothelial cell monolayer, the impact of these cytokines on retinal endothelial lncRNA expression was assessed by RT-PCR, and differential expression of a cluster of lncRNA, especially MIR155HG, was identified in activated human retinal endothelial cells.

The work presented in this thesis progresses knowledge of uveitis mechanisms in unveiling the central cytokines that mediate retinal endothelial dysfunction during inflammation as TNF-α, IL-1β and IL-6, demonstrating the expression and regulation of IL-6R in human retinal endothelial cells, and linking specific lncRNAs to retinal endothelial cell activation. The uncovering of essential targets in retinal endothelial cell dysfunction may translate clinically to improved management of vascular leakage in uveitis, thereby reducing the visual loss ensuing from this condition.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Lisia Barros Ferreira

PREAMBLE:
EFFECT OF COVID-19 PANDEMIC ON CANDIDATURE

The candidate arrived in Adelaide, Australia in August 2019 and commenced her candidature as a research higher degree student under the supervision of Professor Justine Smith in September of the same year. She was based in her supervisor's research laboratories that are situated within the Flinders Medical Centre, a public hospital affiliated with Flinders University. Five months later, Australia closed its international borders to try to contain, or at least delay, the spread of SARS-CoV-2. State-based restrictions including rolling lockdowns, delays in elective surgery, work-from-home requirements, social distancing and mandatory use of personal protective equipment followed. Restrictions were particularly stringent in patient-facing institutions, including the Flinders Medical Centre.

The candidate’s research was thus impacted in a number of ways. She nonetheless managed to complete the experimental work described within this thesis over the two years from September 2019 to November 2021. At that time, international borders re-opened. A constellation of local, national and international issues related to the pandemic then necessitated relocation of the candidate, together with her immediate family, back to her home country of Brazil in early December 2021. Her thesis was written entirely in Brazil, with weekly phone calls and virtual meetings held with her supervisor in support. No further experimental work was able to be performed during this period.

ACKNOWLEDGMENTS

I had a dream of pursuing a Doctor of Philosophy degree even before I started medical school. My studies began in Brazil, with my medical degree, Ophthalmology residency and clinical fellowship in uveitis, which then led me to Canada for a clinical fellowship in medical retina. It was the work of the clinician-scientist Professor Justine Smith which inspired me to contact her while I was in Canada, and that led us to meet during the American Academy of Ophthalmology Congress in 2018, where it all started. Firstly, I would like to thank Professor Justine Smith for her support and trust in getting me to the other side of the world for a journey of personal and career growth, and for providing me with a privately funded scholarship. Professor Justine Smith is a unique person with an unprecedented passion for research and whose teachings I will carry for life. Having Emeritus Professor Keryn Williams as my co-supervisor was an honour and of great support for my candidature, much encouraging and inspirational. I am also grateful for having Dr. Giles Best as my co-supervisor, whose teachings and assistance were essential for the completion of this thesis.

To all the Eye and Vision Health personnel, I praise their support: Liam Ashander, for his great knowledge of molecular biology and patience; Dr. Yuefang Ma, for her expertise and teachings of cell culture; Dr. Alix Farrall, for her great feedbacks during laboratory meetings and presentations; Dr. Binoy Appukuttan for his molecular biology and genetics knowledge; Dr. Amanda Lumsden, for her scientific advice while we shared the same office; my colleague Alwin Tan Chun Rong for his companionship, and Ms. Janet Matthews, for all her superb administrative work, always with a lovely smile on her face. I also want to thank Dr. Nicholas Eyre for his lessons on confocal microscopy.

This degree would not have been possible to undertake without the tuition fee waiver and institutional support I generously received from Professors Briony Forbes, Tara Brabazon, Paul Ward, Jonathan Craig, Peter Eastwood, and Robert Saint. Lastly, I would like to acknowledge all the encouragement of my family, especially of my beloved husband Alexandre for having accompanied me throughout the world while I followed my career dreams, always supportive and taking care of me.

My gratitude to you all.

MANUSCRIPTS PUBLISHED DURING THE CANDIDATURE

Publications arising directly from this thesis

Barros Ferreira L, Ashander LM, Appukuttan B, Ma Y, Williams KA, Smith JR. Expression of long non-coding RNAs in activated human retinal vascular endothelial cells. Ocul Immunol Inflamm. 2022;1-6.

Ferreira LB, Ashander LM, Appukuttan B, Ma Y, Williams KA, Best G, Smith JR. Human retinal endothelial cells express functional interleukin-6 receptor. J Ophthalmic Inflamm Infect. 2023;13(1):21

Ferreira LB, Ashander LM, Ma Y, Appukuttan B, Best G, Williams KA, Smith JR. Effects of tumour necrosis factor-α and interleukin-1β on human retinal endothelial cells. Cytokine. *Under review.*

Additional publications related to this thesis

Ferreira LB, Smith AJ, Smith JR. Biologic drugs for the treatment of noninfectious uveitis. Asia Pac J Ophthalm (Phila). 2021;10(1):63-73.

Ferreira LB, Farrall AL, Furtado JM, Smith JR. Treatment of noninfectious uveitis. Arq Bras Oftalmol. 2021;84(6):610-621.

Additional publications during the PhD candidature

Ashander LM, Lumsden AL, Dawson AC, et al. Infection of human retinal pigment epithelial cells with dengue virus strains isolated during outbreaks in Singapore. Microorganisms. 2022;10(2):310.

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Haydinger CD, Ferreira LB, Williams KA, Smith JR. Mechanisms of macular edema. Front Med. 2023;10:1128811.

PREFACE

The work of this thesis was carried out by the author, except for:

- Isolation of primary human retinal endothelial cells, which was performed by Dr Yuefang Ma (Chapter 4 and Chapter 6).

- Generation of human retinal endothelial transcriptome dataset used for *in silico* prediction of transcription factors regulating interleukin-6 receptor expression (Chapter 4) and long non-coding RNAs involved in endothelial activation (Chapter 6), which was the work of Professor David Lynn and Professor Justine Smith and colleagues at the South Australian Health and Medical Research Institute and Flinders University.

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LIST OF ABBREVIATIONS

2-ME	2-mercaptoethanol
ACKR1	atypical chemokine receptor 1
ADAM	a desintegrin metalloproteinase
ALAS1	5'-aminolevulinate synthase 1
ANOVA	analysis of variance
APC	allophycocyanin
APC-A	allophycocyanin area
AQP	aquaporin
ATP	adenosine triphosphate
B2M	beta-2-microglobulin
BLAST	basic local alignment search tool
bp	base pairs
C/EBPs	CCAAT-enhancer-binding proteins
CAR	coxsackie virus and adenovirus receptor
cat.	catalogue number
CCL	C-C motif ligand
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
ceRNA	competitive endogenous RNA
ChIP-seq	chromatin immunoprecipitation with subsequent sequencing
cIAPs	cellular inhibitor of apoptosis proteins

Cq	quantification cycle
CTNBB1	β-catenin
CV	coefficient of variance
CXCL	C-X-C motif ligand
DAPI	4’, 6-diamidino-2-phenylindole
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide-triphosphate mix
E-cadherin	epithelial cadherin
E-plate	electronic plate
EAU	experimental autoimmune uveitis
EDTA	ethylenediaminetetraacetic acid
EGM2	endothelial cell growth medium 2
EIU	endotoxin-induced uveitis
ERK	extracellular signal-regulated kinase
ESAM	endothelial cell-specific adhesion molecule
ETS1	ETS proto-oncogene 1, transcription factor
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
FITC-A	fluorescein isothiocyanate area
FSC-A	forward scatter area
FSC-H	forward scatter height

g	gravitational force equivalent
GAS5	growth arrest specific 5
gp80	glycoprotein 80
gp130	glycoprotein 130
GTP	guanosine-5'-triphosphate
HLA	human leukocyte antigen
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
IκB	I kappa B
IL	interleukin
IL17-RA	interleukin-17 receptor A
IL1RA	interleukin-1 receptor antagonist
IL1RAcP	interleukin-1 receptor accessory protein
IL1R	interleukin-1 receptor
IL-6R	interleukin-6 receptor, interleukin-6 receptor α, CD126, glycoprotein 80
Inc.	Incorporation
JAK	janus kinase
JAM	junctional adhesion molecules
JNK	c-Jun N-terminal kinase
KCNQ1OT1	potassium voltage-gated channel subfamily Q member opposite strand/antisense transcript 1
LDL	low density lipoprotein
LFA	lymphocyte function-associated antigen

LINC00294	long intergenic non-protein coding RNA 00294
lncRNA	long non-coding RNA
LPS	lipopolysaccharide
M	stability measurement
MAC	macrophage antigen
MAL	myelin and lymphocyte protein
MALAT1	metastasis associated lung adenocarcinoma transcript 1
MAPK	p38 mitogen-activated protein kinase
MARVEL	myelin and lymphocyte protein and related proteins for vesicle trafficking and membrane link
MCP-1	monocyte chemoattractant protein-1
MEG3	maternally expressed 3
MEM	modified Eagle's medium
mIL-6R	transmembrane interleukin-6 receptor
MIR155HG	microRNA 155 host gene
miRNA	microRNA
MITOMI	mechanically induced trapping of molecular interactions
MYD88	myeloid differentiation primary response gene 88
NCBI	National Center for Biotechnology Information
NEAT1	nuclear paraspeckle assembly transcript 1
NF-κB	nuclear factor kappa B
NK	natural killer
ncRNA	non-coding RNA

NORAD	non-coding RNA activated by DNA damage
OIP5-AS1	opa interacting protein 5 antisense RNA 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PECAM	platelet endothelial cell adhesion molecule
PFA	paraformaldehyde
PI3K	phosphoinositol-3 kinase
PKB or AKT	protein kinase B
PKC	protein kinase C
PLVAP	plasmalemma vesicle-associated protein
PPIA	peptidylprolyl isomerase A
PSGL-1	P selectin glycoprotein ligand-1
r	Pearson correlation coefficient
RFU	relative fluorescence unit
RNA-seq	RNA sequencing
RPLP0	ribosomal protein lateral stalk subunit P0
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription-polymerase chain reaction
RT-qPCR	quantitative reverse transcription polymerase chain reaction
SELEX	systematic evolution of ligands by exponential enrichment
SENCR	smooth muscle and endothelial cell enriched migration/differentiation-associated lncRNA
sgp130	soluble glycoprotein 130

sIL-6R	soluble interleukin-6 receptor
siRNA	small interfering RNA
SN	serial number
SNX32	sortin nexin 32
SSC-A	side scatter area
STAT	signal transducer and activator of transcription
TACE	tumour necrosis factor-α-converting enzyme
TAE	tris base, acetic acid and ethylenediaminetetraacetic acid
TGF-β	transforming growth factor-β
Th	T-helper
TIFF	tagged file image format
TNF-α	tumour necrosis factor-α
TNFR	tumour necrosis factor-α receptor
TRADD	tumour necrosis factor receptor 1 associated protein with death domain
TRAF2	tumour necrosis factor receptor-associated factor 2
Triton X-100	p-tert-octylphenol ethoxylate
TUG1	taurine up-regulated 1
VCAM-1	vascular adhesion molecule-1
VE-cadherin	vascular endothelial cadherin
VE-PTP	vascular endothelial protein tyrosine phosphatase
VEGF-A	vascular endothelial growth factor-A
VLA	very late antigen

vol/vol	concentration by volume/volume
w/v	concentration by weight/volume
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
ZO	zona occludens

Statistical significance

*	$p < 0.05$
**	$p < 0.01$
***	$p < 0.001$
****	$p < 0.0001$

Standard international units of measure

ºC	degree Celsius
cm	centimetres
g	gram
h	hour
Hz	hertz
L	litre
M	molarity, moles per litre
mol	mole
V	volt

CHAPTER 1: INTRODUCTION

1.1 Introduction

Uveitis, an umbrella term for intraocular inflammation, is a common and potentially blinding disease that mostly affects the working-age population.[1, 2] Due to secondary complications, a great burden is associated with uveitis that affects the posterior segment of the eye, involving the retina and the choroid.[2-6] The retina is vascularized by non-fenestrated blood vessels lined by a monolayer of endothelial cells tightly interconnected by junctional complexes, forming a barrier against infectious agents, cells, proteins, and fluid, hence protecting the retina and visual function. Under the influence of several inflammatory mediators, including cytokines, the endothelial barrier may become dysfunctional, resulting in leukocyte transendothelial migration, vascular leakage, and accumulation of fluid in the centre of the retina – termed macular oedema – which is the main cause of visual loss in uveitis.[7]

Cytokines are key molecules in the intricate regulatory process of inflammation. The study of cytokines has led ultimately to the discovery of new drug targets concurrently with the development of biologic immunomodulatory therapy. The invention of biologic agents has revolutionized the treatment of several autoimmune diseases. Even though biologic drugs targeting different cytokines have been used in clinical practice for the treatment of non-infectious uveitis, none have complete efficacy and an ideal safety profile. The mechanisms by which cytokines affect the human retinal endothelial barrier have not been fully established; therefore, a thorough investigation of the roles of cytokines in human retinal endothelial dysfunction would potentially aid in the development of new treatments to combat non-infectious uveitis.

1.2 The eye

1.2.1 Structure of the eye

The eye is the visual organ of the body, containing three structural layers: the outermost layer, a fibrous layer composed of the sclera and the cornea; a middle vascular layer named the uvea, consisting of the iris, ciliary body and the choroid; and the innermost layer, represented by the retina, the neural tissue of the eye.[8] The eye is divided into the anterior and posterior segments. The anterior segment, limited anteriorly by the cornea and posteriorly by the lens, encompasses the iris and the

ciliary body.[8] The aqueous humour, mainly composed of water, ions and glucose, is produced by the ciliary body and fills the anterior segment, nourishing its structures and maintaining intraocular pressure.[9, 10] The posterior segment of the eye comprises the retina, the choroid, and the optic nerve. The retina is the neuronal tissue of the eye, while the choroid is composed of melanocytes and fibroblasts, and is rich in blood vessels.[10] The optic nerve begins anatomically at the optic nerve head and is formed by the convergence of axons of the retinal ganglion cells.[10] Filling the posterior segment, the vitreous humour, composed mostly of water, collagen and hyaluronic acid, maintains ocular shape and acts as a diffusion barrier between the two segments of the eye. Due to its proximity to the ciliary body and the retina, the vitreous reflects the metabolic changes of these tissues.[8, 11]

1.2.2 The retina

The retina, a sensory layer that lies on the internal surface of the posterior segment, is responsible for the perception of light.[10] Light is converted into electrochemical impulses that are transmitted to the visual cortex, where visual interpretation occurs. The retina is composed of 10 layers: the inner limiting membrane, corresponding to the basement membrane of the Müller cells; the nerve fibre layer, formed by the axons of the ganglion cells; the ganglion cell layer, formed by the nuclei of ganglion cells; the inner plexiform layer, created by the axons of the bipolar and amacrine cells and the dendrites of the ganglion cells; the inner nuclear layer, corresponding to the bipolar cells nuclei, Müller, horizontal and amacrine cells; the outer plexiform layer, made by the connections among the axons and synaptic bodies of the photoreceptors, horizontal and bipolar cells; the external limiting membrane, originated from the connections of the photoreceptors and Müller cells; the photoreceptors, produced by the inner and outer segments of cones and rods; and the retinal pigment epithelium, a cell monolayer lying on the Bruch's membrane.[10] The macula is anatomically defined as the region situated between the temporal vascular arcades.[12] Located in the centre of the macula, the fovea is responsible for the sharpest visual acuity.[10, 12]

The retinal and choroidal circulatory systems nurture the posterior segment of the eye. The retinal vasculature originates from the central retinal artery, which in turn arises from the ophthalmic artery or one of the posterior ciliary arteries.[13] Entering the eye at the optic disc, the central retinal artery

divides into two main arterioles, which branch to supply the retinal quadrants. These branches form interconnected capillary plexuses: the superficial and the deep vascular complex, the former nurturing the nerve fibre, ganglion cell and part of the inner plexiform layers, and the latter nourishing the inner nuclear and the outer plexiform layer.[14] A dense capillary network connects the precapillary arterioles with the postcapillary venules, sparing the extreme periphery, the periarteriolar region and usually the fovea. The venules drain to the central retinal vein, and from there to the cavernous sinus. The choroid is supplied by the posterior and anterior branches of the ciliary arteries. The posterior ciliary arterioles form the non-fenestrated outward vessels layer (Haller's layer), the medium vessel layer (Sattler's layer) and the fenestrated innermost layer (choriocapillaris), nurturing the retinal pigment epithelium, the photoreceptors, and the outer nuclear layers.[15] Most of the choroidal venous drainage flows into the vortex veins, orbital veins, and subsequently to the cavernous sinus.[16]

1.3 The blood-retinal barrier

Two blood-ocular barriers operate to protect the eye: the blood-aqueous barrier and the blood-retinal barrier, which act to control the entrance of toxins, microorganisms and inflammatory cells into the anterior and posterior segments, respectively.[8] These barriers provide immune privilege, an evolutionary adaptation to protect vulnerable and highly specialized tissues from inflammation-induced damage.[17] The blood-ocular barriers allow adequate oxygen and nutrient supplies to the anterior and posterior segment tissues, whilst restraining the free intercellular influx of potentially deleterious cells and substances, ultimately protecting vision, a critical survival sense.[18, 19]

The blood-aqueous barrier is formed by the tight junctions of the non-pigmented epithelium of the ciliary body, the non-fenestrated vascular endothelial cells of the iris, the posterior iris epithelium and the inner wall endothelium of the Schlemm canal.[20-22] The blood-aqueous barrier keeps the aqueous humour essentially devoid of proteins to allow for a transparent medium for the transmission of light and regulates active transport and selective cell trafficking.[23] The breakdown of this barrier leads to protein and cellular accumulation in the aqueous humour, clinically seen as flare and an anterior chamber reaction, respectively. The blood-retinal barrier has two components: the inner component,

which is mainly provided by the intercellular tight junctions of the retinal endothelium, and the outer component, which consists chiefly of the interepithelial tight junctions of the retinal pigment epithelium.[19, 24-26] The blood-retinal barriers protect the retinal tissue from the accumulation of fluid and influx of cells and harmful substances that may lead to oedema and tissue destruction, endangering visual function.

1.3.1 The inner blood-retinal barrier

The inner blood-retinal barrier is composed of the retinal endothelial cells, pericytes and glial cells (Müller cells and astrocytes).[24, 25] The retinal endothelium is the main component of the inner blood-retinal barrier, being firmly interconnected by tight junctions, which restrict paracellular transport. Besides functioning as a barrier and regulating the movement of water and substances into and out of the retina, the retinal endothelium also secretes cytokines and growth factors.[25] Pericytes are separated from the retinal endothelial cells by a basal lamina, making contact with the endothelium by intercellular junctional proteins.[27] Pericytes are essential for blood-retinal barrier development, acting in capillary contraction, structural support, phagocytosis, production of growth factors, modulation of the junctional molecule expression, survival and proliferation of endothelial cells.[28-31] Pericytes also communicate with other cell types, and the functional interconnection among them, endothelial cells, astrocytes, microglia and neurons constitute the neurovascular unit.[25] Müller cells aid in barrier function by regulating ionic and water transport, secretion of cytokines and growth factors, and in forming the inner limiting membrane with their footplates.[8, 25, 32] Astrocytes modulate junctional molecule expression, secretion of growth factors, cytokines and antioxidants.[33, 34]

1.3.2 The outer blood-retinal barrier

The outer blood-retinal barrier comprises the retinal pigment epithelium, Bruch's membrane, the external limiting membrane and the choroid.[34] The retinal pigment epithelium is a monolayer of hexagonal cells with multiple microvilli on their apical surfaces and is situated between the neuroretina and the choriocapillaris. The presence of tight junctions interconnecting the apical surface of the retinal pigment epithelial cells, as well as polarization provided by the asymmetrical allocation of membrane proteins, are the basis for the barrier function, preventing the free influx of

water and substances from the choriocapillaris.[35] Underlying the retinal pigment epithelium is Bruch's membrane, formed by the basement membranes of the retinal pigment epithelial cells and choriocapillaris, interposed by collagenous and elastic fibres. Bruch's membrane is essentially impervious to large molecules.[34] The external limiting membrane is formed by junctional complexes located at the border of Müller cells and inner segments of the photoreceptors, limiting the transit of large molecules throughout the retina.[26, 36] The choriocapillaris is fenestrated, nourishing the retinal pigment epithelium and the outer retina while removing metabolic waste from the region. Choroidal endothelial cells are also involved in the control of the retinal pigment epithelial barrier function.[37]

1.4 The retinal vascular endothelium

1.4.1 Overview

Retinal endothelial cells are generally long spindle-shaped cells with a compressed cytoplasm, rich in mitochondria and ribosomes.[38] Unlike other endothelial cells, the cells of the retinal endothelium are non-fenestrated, have scarce pinocytic vesicles and caveolae (microinvaginations in the plasma membrane), and are packed with specialized junctional complexes.[25, 38] All these characteristics confer the barrier function on the retinal endothelium. Some aspects differ among arteriolar, venular and capillary endothelial cells, possibly due to differences in their response to shear stress.[38] In the arterioles, endothelial cells are spindle-shaped with stretched nuclei, while venular endothelial cells assume a polygonal form, with oval nuclei. Capillary endothelial cells are thin and spindle-shaped.[39, 40] Endothelial characteristics also change depending on the vessel order, correlating with cellular and nuclear length in arterioles and width in venules.[40] Smaller vessels are wrapped by more astrocytic extensions than larger vessels. A single layer of smooth muscle cells is seen in smaller arterioles, while larger arterioles appear to have more than one layer.[39, 40] The extracellular matrix of the endothelial basement membrane supports the endothelium and is necessary for endothelial cell proliferation and survival.[41]

1.4.2 The junctional complexes

The junctional complexes are dynamic structures essential for barrier function and integrity maintenance, as well as for intercellular communication.[42] The intercellular anchorage is provided

by junctional complexes composed of transmembrane proteins, which in turn are connected to intracellular molecules that regulate the fixation of the junctional complex to the cytoskeleton actin microfilaments. There are three main types of junctional complexes: tight junctions, adherens junctions and gap junctions. Tight junctions are distributed along the lateral membrane and adherens junctions are intermixed with the tight junctions.[19, 25] Adherens junctions are also found between photoreceptors and Müller cells in the outer limiting membrane.[43] Gap junctions are usually found intermingled with the tight and adherens junctions at the cell membranes, frequently in proximity to the apical surface of endothelial cells. The endothelial-astrocyte and endothelial-pericyte contacts also contain gap junctions.[25, 44] Desmosomes are adhesive intercellular structures common in epithelium, but absent in the endothelium, although vimentin filaments appear to be connected to endothelial adherens junctions in desmosome-like structures, named complexus adherens.[42, 44, 45]

1.4.2.1 Tight junctions

Tight junctions comprise transmembrane proteins (claudins, occludins and junctional adhesion molecules – JAMs), and cytoplasmic proteins (zona occludens – ZO).[19, 46] Claudins are a large family of tetraspanin proteins which form ion-selective channels involved in paracellular transport and which interact with ZO-1.[19, 44, 46] Claudin-5 is highly expressed by endothelial cells, playing an essential role in the retinal endothelial barrier permeability.[19] Occludins are members of the myelin and lymphocyte protein (MAL) and related proteins for vesicle trafficking and membrane link (MARVEL) family, possessing four transmembrane domains, which interact with claudins and ZO-1. A regulatory function of the barrier is attributed to occludin, despite being dispensable for tight junction formation.[19, 46-48] The JAMs are an immunoglobulin-like family, constituted of JAM-A, -B, -C and the related proteins JAM-4, JAM-L, endothelial cell-selective adhesion molecule (ESAM) and coxsackie virus and adenovirus receptor (CAR). Junctional adhesion molecules are involved in barrier formation, function and polarity, as well as leukocyte trafficking.[19, 25, 46] The ZO family comprises the members ZO-1, -2, and -3, scaffolding proteins which interact with claudins, occludin and JAMs. Zona occludens-1 assembles transmembrane proteins via cingulin to the actin cytoskeleton, being crucial for endothelial barrier formation.

1.4.2.2 Adherens junctions

Adherens junctions are required for tight junction formation.[49] The adherens junction family is composed of cadherins, catenins and nectin proteins. The cadherin family is divided into type I, including the epithelial (E)-cadherin and type II, which includes the vascular endothelial (VE)-cadherin.[50] The main component of the endothelial adherens junction is VE-cadherin, a transmembrane protein with single transmembrane and cytoplasmic domains.[51] Vascular endothelial cadherin has binding sites to the cytoplasmic proteins known as armadillo plaque proteins (β-catenin, p120, p0071, plakoglobin), density-enhanced phosphatase and vascular endothelial protein tyrosine phosphatase (VE-PTP), associated with structural support, and regulation of permeability and morphogenesis.[25, 51] Catenins are intracellular proteins, named β- and α-catenin. β-Catenin is linked to cadherins and regulates the Wnt growth-factor signalling pathway, controlling angiogenesis.[46, 52] α-Catenin interacts with β-catenin and cytoskeleton proteins, not being directly bound to cadherins.[44] Nectins belong to the immunoglobulin superfamily, with nectin-2, -3 and -5 being present in endothelial cells, aiding in junction complex integrity and polarity.[44, 53] Platelet endothelial cell adhesion molecule (PECAM)-1 or cluster of differentiation (CD)31 is a cell membrane protein associated with adherens junction, interacting with β-catenin.[54]

1.4.2.3 Gap junctions

Gap junctions are composed of hemichannels (connexons) of two adjacent cells, each hemichannel being built by six proteins (connexins), forming homomeric and heteromeric structures.[55] More than 21 connexin genes have been described in humans. The gap junctions allow the direct passage of small molecules, ions, and metabolites between neighbouring cells. In addition to the endothelium, which expresses connexin 30.2, 37, 40 and in great abundance, connexin 43, several types of connexins are present in pericytes, photoreceptors, and horizontal, bipolar, amacrine and ganglion cells. Besides acting in intercellular communication, gap junctions aid in the assembly of tight and adherens junctions, influence cell differentiation, paracrine signalling, and embryogenesis.[25, 55] Specifically, connexin 43 influences occludin and ZO-1 expression, and regulates monocyte-endothelial adhesion.[55, 56] Reduction of connexin 43 is associated with vascular cell apoptosis in retinal capillaries[57] and disturbance of the endothelial barrier homeostasis due to pericyte-endothelial

cell interaction loss.[58]

1.4.3 The molecular phenotype of the human retinal vascular endothelium

Both retinal and choroidal vascular endothelial cells exhibit a characteristic cobblestone morphology, form capillary-like tubes in a basement membrane matrix, and express CD31 and von Willebrand factor.[59] However, transcriptomics and proteomics studies have revealed a unique molecular profile of human retinal endothelial cells.[38, 60, 61] Although the retinal and choroidal vascular beds both originate from the ophthalmic artery, the difference in their transcriptomic profiles reaches around 9%.[38, 60] At a proteomics level, retinal and choroidal endothelial cells differ in approximately 25% of proteins.[61] The transcripts expressed by the retinal endothelium are associated with the immune response, encompassing cell adhesion molecules – e.g. intercellular adhesion molecule (ICAM)-1; vascular adhesion molecule (VCAM)-1 – molecular messengers such as cytokines – e.g. interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α – and chemokines – e.g. C-C motif ligand (CCL) 2, C-X-C motif ligand (CXCL) 8 – and angiogenesis regulators – e.g. supervilin, cathepsin B.[60] Thus, despite forming a barrier against fluid and cellular trafficking under physiological conditions, the retinal endothelium is equipped with the genetic apparatus to allow for leukocyte adhesion and migration.

1.4.4 Fluid & solutes transport across the retinal vascular endothelium

The retinal endothelium is a highly selective fence, where the transit of fluid and substances is tightly regulated via the transcellular and paracellular pathways. Small lipophilic molecules passively cross the endothelium via the paracellular pathway; however, larger and hydrophilic molecules require adenosine triphosphate (ATP)-dependent transport.[62] The retinal endothelium has limited vesicular and transporter-mediated conveyance systems, but abundant efflux pumps.[34] Transcellular transport systems include caveolar transport, ion transport, receptor-mediated transport, carrier-mediated transport, and efflux pumps.[25, 34] Transcellular movement is provided mainly by caveolar transport.[19, 34] Caveolae are plasma membrane invaginations composed of caveolin-1 and lipids, involved in endocytosis, signal transduction, and possibly in tight junction regulation.[19, 34] Albumin-binding proteins, such as gp60, also aid in transcytosis by forming a complex with caveolae, contributing to

the creation of an osmotic pressure gradient throughout the barrier.[19, 34] Transport across the endothelium occurs mainly via the transcellular route, since the paracellular way is restricted by the junctional complexes.[24, 25] However, a low rate of paracellular transit is established by the claudins. Claudins form ion-specific channels, with their first extracellular loop determining ion-selectivity.[19, 34]

1.4.5 Leucocyte trafficking across the retinal vascular endothelium

Leukocytes are recruited from the circulation to the tissue to perform immunosurveillance under physiological and pathological states.[63] Leukocyte adhesion to the retinal endothelium is enabled by ICAM-1 and VCAM-1 interactions with leukocyte αLβ2/αMβ2 integrins and α4β1 integrin, respectively.[64, 65] Frequently, leukocyte adhesion triggers the phosphorylation of cortactin, an actin component, creating a "docking site" whereby the endothelial cell projections "embrace" the leukocyte, before transcellular or paracellular migration occurs. Other cell surface receptors such as JAMs, PECAM-1, ESAM, CD99, and E- and P-selectins interact with leukocyte ligands to mediate adhesion, rolling, crawling and leukocyte migration.[64-69] The most common route of leukocyte migration in the retinal endothelium is paracellularly, through the opening of junctional complexes. After leukocyte adhesion, free calcium ions and reactive oxygen species are released into the cytoplasm, resulting in action-myosin fibre contraction and loosening of intercellular adhesions.[65] Phosphorylation of VE-cadherin is also triggered, dissociating it from VE-protein tyrosine phosphatase (PTP), hence destabilizing the adherens junction and allowing leukocyte trafficking.[64, 65] Transcellular migration occurs to a lesser extent, involving ICAM-1, caveolin-1, vimentin, and other molecules which also participate in paracellular leukocyte migration.

1.5 Uveitis

1.5.1 Definition

Uveitis is a broad term that characterizes intraocular inflammation affecting the uveal tract and adjacent structures.[1, 2] Depending on the primary site of inflammation, uveitis is categorized as: anterior, if affecting primarily the iris (iritis), anterior ciliary body (anterior cyclitis) or both (iridocyclitis); intermediate, if the vitreous humour is the main area of involvement (pars planitis); posterior, if the retina (retinitis), choroid (choroiditis) or both (retinochoroiditis) are most affected; and as panuveitis,

if all tissues are equally involved.[70] Uveitis is deemed acute if lasting less than three months; recurrent if there are repeated occurrences followed by periods of inactivity of three months or more without treatment; or chronic if inflammation persists, with reactivation in less than three months after treatment discontinuation. Uveitis may be infectious or non-infectious. Common causes of infectious uveitis include toxoplasmosis, tuberculosis, syphilis, and herpes virus infection.[2] Non-infectious uveitis may be idiopathic or represent a myriad of ocular syndromes such as birdshot retinochoroidopathy, serpiginous choroiditis, and multifocal choroiditis, or be linked to systemic inflammatory diseases such as human leukocyte antigen (HLA)-B27-associated spondyloarthropathy, sarcoidosis, Vogt-Koyanagi-Harada syndrome, and Behçet's disease.[2]

1.5.2 Epidemiology & clinical burden

Uveitis is a common disease, with an incidence that ranges from 17 to 52 new cases per 100,000 individuals per year, and a prevalence of 38 to 714 cases per 100,000 people.[3-6, 71] Incidence and prevalence differ in different populations, depending on age, ethnicity, sex, environment, genetic factors and socio-economic development status.[3, 72] Anterior uveitis is the most common form of uveitis, followed by posterior or panuveitis, depending on the studied population, with intermediate uveitis being the least frequently reported.[2, 3, 73, 74] Uveitis is one of the leading causes of visual impairment, being accountable for 5-20% of legal blindness in the Western world and approximately 25% in developing regions, mainly when chronic and affecting the posterior segment of the eye.[2-6, 75] In developing countries, infectious uveitis is more frequently observed than in industrialized nations, mainly attributed to toxoplasmosis and tuberculosis.[75-77] As uveitis onset tends to occur during the working years, the burden on quality of life is substantial, not only because of the risk of vision loss accumulating over many years but also because of onerous treatment regimens and the possible co-existence of systemic disease.[78-80]

1.5.3 Clinical manifestations and complications

Clinically, uveitis may manifest with a variety of symptoms or even be asymptomatic. Ocular pain and photophobia are more likely associated with anterior uveitis, while posterior and intermediate uveitis may cause visual floaters, distorted vision (metamorphopsia), flashes of light in the visual

field (photopsias) and decreased visual acuity. Clinical signs of posterior uveitis include vitreous cellularity and haze, retinal or choroidal infiltrates, vascular sheathing and retinal haemorrhages, potentially resulting in vision-threatening complications such as retinal vascular occlusions, retinal detachment, optic neuropathy, hypotony, choroidal neovascularization, and macular oedema.[5, 81-83] Macular oedema, manifesting as swelling of the macular region, occurs in 20-33% of patients with uveitis and is a major cause of long-term vision loss due to macular scarring.[4, 5, 7, 81, 84] Macular oedema is caused by vascular leakage, which may occur in any part of the retina and is seen clinically as sheathing or cuffing of exudates and/or blood surrounding retinal vessels. It is detected in the retinal angiogram by contrast extravasation.[85]

1.5.4 Treatment

Historically, glucocorticosteroids have been the cornerstone of treatment of non-infectious uveitis affecting the posterior segment, and may be administered systemically or by intraocular or periocular injection.[86] However, corticosteroids may only be administered for a limited time due to the long list of adverse reactions caused by prolonged use.[86] Immunomodulatory drugs are the most modern agents used in the treatment of non-infectious uveitis, allowing for a corticosteroid-sparing effect.[87] Conventional immunomodulatory drugs such as antimetabolites and T-cell signalling inhibitors have been widely used in the past decades to treat non-infectious uveitis.[88] However, since the publication of the VISUAL I and II clinical trials showing the efficacy of a monoclonal antibody against TNF-α (adalimumab) in delaying time to treatment failure in patients with active and non-active non-infectious uveitis, respectively, a number of biological drugs targeting different cytokines and other inflammatory mediators have been developed.[89, 90] Despite these recent advances, treatment of non-infectious uveitis is suboptimal due to adverse drug events and the refractoriness of a considerable proportion of cases.[88, 91]

1.6 Mechanisms of uveitis

1.6.1 Overview

Immune-mediated non-infectious uveitis involves both innate and adaptative immune pathophysiologic mechanisms.[92] Cells of the adaptative response include CD4-positive T-cells, T-

helper cells (particularly Th1, Th2, Th9 and Th17 lineages, named according to the chief cytokines they produce),[92-96] tissue-resident cells (e.g. microglia, Müller cells and astrocytes), monocytes, neutrophils, natural killer (NK) cells, and γδ T-cells.[92-95, 97] Data from animal and human studies suggest that following the production of inflammatory cytokines and metalloproteinases, which activate the endothelium and disrupt the intercellular tight junctions, activated antigen-specific Th cells adhere to the retinal endothelium and migrate via paracellular and/or transcellular routes into the retina.[92, 98] Local resting antigen-presenting cells are activated, and other leukocytes, such as neutrophils and monocytes are recruited, enhancing the inflammatory reaction. The migration of cells into the retina is associated with increased endothelial permeability, allowing fluid and protein extravasation and subsequent tissue damage. The escape of retinal self-antigens to the systemic circulation primes, expands and recruits more autoreactive cells, perpetuating inflammation.

1.6.2 Mechanisms of blood-retinal barrier breakdown

One of the key events in the pathogenesis of uveitis is the breakdown of the blood-retinal barrier. Many mediators are implicated in this process, including cytokines, chemokines, growth factors, hormones and other inflammatory peptides.[25, 26, 35, 99] These molecules are produced by the activated endothelium, activated Th cells, monocytes, neutrophils, NK cells, γδ T-cells, microglia, Müller cells, astrocytes, and pericytes. The mechanisms underlying the blood-retinal barrier breakdown include increased paracellular permeability through the impairment of junctional complexes; increased paracellular transport; and direct or indirect damage to endothelial cells and other blood-retinal barrier-supportive cells, such as pericytes and glial cells.[25, 35, 69] After infiltrating the retina, activated T-cells, macrophages and neutrophils release cytokines, chemokines, reactive oxygen species, and nitric oxide, also triggering complement activation, and thus leading to tissue destruction and amplification of the inflammatory response.[95, 100-102] The blood-retinal barrier breakdown results in the infiltration of leukocytes, fluid and protein into the retina, disrupting the retinal architecture, causing macular oedema, tissue necrosis and ultimately vision loss.

1.6.3 Leukocyte infiltration

The blood-retinal barrier breakdown is triggered by leukocyte migration across the retinal

endothelium.[69, 92] The sequence of events for leukocyte trafficking initiates with leukocyte adhesion, which is mediated by the interaction of endothelial adhesion molecules and the leukocyte integrins, cell adhesion receptors that facilitate cell adhesion to the extracellular matrix and to other cells.[103] During the early stages of uveitis, the retinal endothelium is activated by cytokines, such as TNF-α and IL-1β, upregulating adhesion molecules (E- and P-selectins, ICAM-1, VCAM-1, PECAM and CD44), while chemokines direct leukocytes to the inflamed sites.[69, 92, 104-107] E- and P-selectins mediate tethering and rolling of leukocytes via interactions with leukocyte L-selectin, and P-selectin [69]glycoprotein ligand (PSGL)-1. Leukocyte adhesion and rolling also involve ICAM-1-lymphocyte function-associated antigen (LFA)-1 (αLβ2 integrin) and VCAM-1-very late antigen (VLA)-4 (α4β1 integrin) interactions.

Chemokines increase integrin affinity via chemokine-receptor G-protein-dependent activation, allowing firm leukocyte adhesion.[69] Different leukocyte subtypes appear to require specific ligands during migration.[67, 108] Th1 and Th2 migration rely on CD44-CD44R and ICAM-1-LFA-1 interactions, selective Th1 recruitment requires P/E-selectin-PSGL-1 ligation, monocyte rolling is dependent on L-selectin, while Th17 infiltration is mediated by VCAM-1-VLA-4 interaction. After leukocyte adhesion and rolling, firm attachment is secured by conformational changes in adhesion molecule-integrin ligation driven by chemokines. Leukocyte transmigration occurs via paracellular and transcellular routes.[64, 65, 69] The transendothelial migration of leukocytes elicits loss of astrocytes processes surrounding the endothelium and disruption of tight junctions at the site of infiltration.[104] The main route of leukocyte migration occurs paracellularly by damaging tight junctions, resulting in leukocyte extravasation into the retinal parenchyma.[69, 104-106]

1.6.4 Molecular mechanisms of blood-retinal barrier breakdown

The main molecular mechanism involved in blood-retinal barrier breakdown is the disruption of endothelial cell tight junctions, permitting increased endothelial permeability.[109] Cytokines, vascular endothelial growth factor (VEGF)-A, prostaglandins, histamine, and cell adhesion molecules are all implicated in this process. Changes in integrity, phosphorylation and localization of tight and adherens junction components are associated with increased barrier permeability.[25, 99, 110-126]

Phosphorylation of junctional proteins via kinases and phosphatases precedes their internalization and degradation, often being transitory.[25, 127] As junctional complexes are damaged, the retina suffers from increased capillary pressure, driving more fluid into the retina.[36] Besides the intercellular adhesion damage, impairment of endothelial cell ion channels and aquaporin (AQP)-4 and potassium (Kir4.1) channels expressed by glial cells lead to accumulation of ions within the cells and osmotic influx of water, causing intracellular swelling.[25, 128-130] The VEGF-A-induced expression of plasmalemma vesicle-associated protein (PLVAP), a component of endothelial fenestrae, increases transcytosis and endothelial permeability.[19, 131] Loss of the endothelial glycocalyx is also associated with increased endothelial permeability.[25, 34, 132]

1.6.5 Cellular mechanisms of blood-retinal barrier breakdown

Cellular mechanisms of blood-retinal barrier breakdown involve enhanced vesicular transport across endothelial cells and degenerative cellular changes.[99, 133] Vesicular transport is increased after cytokine and VEGF-A stimulation.[99, 133-136] Vesiculo-vacuolar structures and caveolae have been observed in the retinal endothelium of rabbits after IL-1β, TNF-α and VEGF-A intravitreal injection,[99, 133] with VEGF-A increasing the number of caveolae observed at the luminal side of the retinal endothelium.[136] Vascular endothelial growth factor-A also induces the creation of vesiculo-vacuolar organelles involved in the extravasation of macromolecules, and caveolar transport in a nitric oxide synthase-dependent way.[134, 135] Increased transendothelial transport was reported in an animal model of posterior uveitis (experimental autoimmune uveitis, EAU), predominantly at the abluminal side.[137] Blood-retinal barrier disruption is also caused by the loss of endothelial cells, pericytes and glial cells. Endothelial cell death is triggered by different mechanisms, including cytokine release, oxidative stress and leukostasis.[25, 109] In a co-culture of retinal endothelial cells and pericytes, endothelial barrier disruption occurred following IL-1β-induced pericyte apoptosis.[138] Astrocyte apoptosis induced by TNF-α and IL-1β increased retinal vascular barrier permeability, and loss of retinal venular encapsulation by astrocytic processes colocalized with decreased junctional molecule expression.[104, 139]

1.7 Inflammatory cytokines

1.7.1 Definition

Cytokines are small molecules essential for cell-cell communication and regulation, acting via autocrine, paracrine, or even endocrine pathways.[140-143] As a wide-ranging term, cytokines include interleukins (produced by leukocytes generally) and chemokines (involved in chemotaxis), lymphokines (produced by lymphocytes) and monokines (produced by monocytes). However, a single cytokine may be secreted by or act on diverse cell types and be redundant, agonistic or synergistic in its roles. One cytokine can stimulate the production of several others, resulting in pleiotropic effects. Cytokines are also secreted by endothelial cells, mediating interactions with leukocytes, and modulating inflammation and angiogenesis.[142] A cytokine is considered inflammatory when its action promotes inflammatory responses. Tumour necrosis factor-α, IL-1β and IL-6 are considered master inflammatory cytokines due to their pleiotropic roles in inflammation.[144-146] The inflammatory potential of a cytokine depends on the target cells and their responsiveness.

1.7.2 Inflammatory cytokines in uveitis

As has already been documented, cytokines enact a central role in uveitis pathogenesis, involving the activation of the retinal endothelium, induction of chemokine production, migration of leukocytes and activation of Th cells, neutrophils and macrophages, with most of the knowledge deriving from the study of animal models of uveitis.[96, 143, 147, 148] Cytokine assessment in intraocular humours (aqueous and vitreous) is of particular interest since their cytokine load is a proxy for the disease process affecting the ocular tissues. In multiple studies, specific cytokines have been found in increased concentrations in intraocular fluids during uveitis.[149-184] In some cases, different studies have found a specific cytokine to be increased, decreased or unchanged in comparison with controls. However, the profiling of intraocular cytokines may be a promising investigative tool to determine uveitis aetiology.[160, 185] The direct comparison of intraocular cytokine levels among non-infectious anterior, intermediate, and posterior uveitis has not been extensively researched. Intraocular cytokine profiles appear to vary with disease activity,[178, 179, 186] treatment status,[160] extraocular manifestations,[176] and uveitic complications.[155, 156, 171, 181, 186-189] Thus far among the published

literature, a range of cytokines stand out as prominent agents of intraocular inflammation.

As key protagonists of inflammation, TNF-α, IL-1β and IL-6 all play a role in the pathogenesis of uveitis. Tumour necrosis factor-α is a key cytokine of Th1 cells, a chief lineage for uveitis development.[190] In different models of experimental uveitis, TNF-α is increased in the inflammatory cell infiltrate, and TNF-α blockade alleviates the severity of inflammation. The differentiation of Th17 cells relies on IL-1β expression, so the latter is a crucial cytokine for uveitis initiation,[191] while uveitis is greatly mitigated in IL-6-deficient mice and during IL-6 antagonism.[192, 193] Interleukin-8, IL-17 and CCL2 also play major roles in uveitis. Intravitreal injection of IL-8 induces uveitis in animals, whereas IL-8 blockade reduces disease severity.[194-197] Interleukin-17 is necessary for the expansion and pathogenicity of Th17 cells and the development of experimental autoimmune uveitis.[198, 199] In addition to their role in experimental models of uveitis, IL-1β,[151, 169] IL-6,[157, 166, 169, 172, 200] IL-8,[151, 157, 160, 164, 166, 177, 182] IL-17,[166, 169] TNF-α,[151, 157, 163, 166, 169] and CCL2[160, 166] are upregulated in intraocular fluids during posterior uveitis, while IL-6, IL-8 and CCL2 are also increased intraocularly in patients with uveitic macular oedema.[171] Tumour necrosis factor-α, IL-6, IL-8, and CCL2 have been shown to distinguish idiopathic uveitis from non-inflammatory controls,[160] and IL-6[186] and IL-17[159] are linked to uveitis activity.[201]

1.7.2.1 Tumour necrosis factor-α

Tumour necrosis factor-α is a transmembrane protein that can be cleaved by matrix metalloproteases such as TNF-α-converting enzyme (TACE) – also known as a desintegrin metalloproteinase (ADAM) 17 – into soluble TNF homotrimers.[202] Tumour necrosis factor-α is secreted by activated macrophages, microglia, astrocytes, T-cells and mast cells.[188] Tumour necrosis factor-α binds to two TNF-α receptors (TNFR): TNFR1 (p55), which is widely expressed throughout the body, and TNFR2 (p75), which is more restricted to the different cells of the immune system, being also expressed in vascular, muscle and brain cells.[202, 203] Following TNF-α binding to the TNFR1, the TNF receptor 1-associated protein with death domain (TRADD), receptor-interacting protein kinase 1, TNF receptor-associated factor 2 (TRAF2), and cellular inhibitor of apoptosis proteins (cIAPs) 1 and 2 are conscripted to the receptor, resulting in the activation of the nuclear

factor kappa B (NF-κB), p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways.[202] The activation of TNFR2 results in the clustering of TRAF2, cIAP1/cIAP2 and HOIP (a component of the linear ubiquitin assembly complex), which form a signalling complex that activates and induces the non-canonical NF-κB pathway and protein kinase B (PKB) phosphorylation. Tumour necrosis factor-α actions include cytotoxicity, host defence against intracellular microorganisms, activation of endothelial cells and T-cells, stimulation of NK cells, and modulation of leukocyte function.[204]

Levels of TNF-α were demonstrated to be increased in the aqueous[149, 151, 153, 155, 157, 159, 163, 168, 169] and vitreous humours[166, 171] of humans with uveitis, when compared with controls. Tumour necrosis factor-α is increased in the aqueous humour and/or vitreous of patients with uveitic aetiologies mainly affecting the posterior segment of the eye, such as Behçet's disease,[151, 157, 163] sarcoidosis,[151, 166] Vogt-Koyanagi-Harada syndrome,[151, 163] and birdshot chorioretinopathy.[169] Elevated intraocular levels of TNF-α correlate with disease activity,[163, 175] and with the development of glaucoma secondary to uveitis.[187] Overall, TNF-α is a major cytokine involved in intraocular inflammation, being present at elevated concentrations in the aqueous and vitreous fluids of non-infectious uveitis patients, especially in those with concurrent systemic diseases and posterior uveitis.

The effect of TNF-α on the human retinal endothelium has been evaluated in a number of studies.[117-119, 122, 124, 205-208] Tumour necrosis factor-α has been demonstrated to decrease cell proliferation,[206] induce apoptosis,[208] and prompt the production of IL-6[206] and matrix metalloproteinase 3[207] in these cells. Decreased transcellular electrical resistance[117, 122 124, 205] and disruption of junctional molecules have also been reported.[117, 118, 122, 205] However, others have noted no impact on the endothelial barrier function, enhancement of adherens junction component, or change in junctional molecule expression.[119, 205] In studies using human brain vascular endothelial cells, downregulation of junctional molecules was reported, but contradictory findings persisted regarding the effects of TNF-α on the endothelial barrier function.[209-211] In bovine retinal endothelial cells, TNF-α induced endothelial barrier breakdown, downregulation of junctional complexes and apoptosis,[115, 120, 121, 126, 212] whereas in rodents and rabbits, TNF-α was associated with increased transendothelial vesicular

transport, leukocyte adhesion, and disruption of tight junctions.[116, 133, 213-215]

There is convincing evidence for the clinical efficacy of TNF-α blockers, such as infliximab[216-218] (chimeric monoclonal anti-TNF-α antibody), and adalimumab[89, 219-223] and golimumab[224] (human monoclonal anti-TNF-α antibodies) in non-infectious uveitis. Two large clinical trials showed a delayed time to treatment failure in patients with active and inactive uveitis, with the use of adalimumab,[89, 90] with a 50% and 43% reduction in the risk of treatment failure in the adalimumab group compared with placebo at 18 months. In a study randomizing patients with juvenile idiopathic arthritis with active uveitis to methotrexate only or combined with adalimumab, treatment failure occurred in 60% in the methotrexate-only group and 27% in the adalimumab group at 18 months.[223] The development of anti-drug antibodies may occur during treatment with adalimumab or infliximab therapy, decreasing their efficacy, and anti-TNF-α drugs have been associated with paradoxical intraocular inflammation.[225-228] Some studies have focused on anti-TNF-α efficacy for the treatment of persistent uveitic macular oedema, with an overall response rate ranging from 22% to 85% after 6-12 months of follow-up.[216, 218-220, 229, 230]

The intravitreal use of anti-TNF-α drugs has been explored for the treatment of recalcitrant non-infectious uveitis.[231-237] Temporary improvement of inflammation has been reported with the use of intravitreal infliximab; however, monthly treatment over a 9-month period was largely unsuccessful, and 20% of patients had worsening of intraocular inflammation.[232, 236, 237] Despite improving uveitis in patients with Behçet's disease and uveitis in some studies, macular oedema resolved in fewer than 20% of patients.[231, 233] Furthermore, intravitreal infliximab has been reported to be unsuccessful for the treatment of diabetic macular oedema and choroidal neovascularization, and concerns were raised over retinotoxicity and new onset of intraocular inflammation.[238-240] Intravitreal adalimumab controlled uveitis in 75% of patients, and resolution of macular oedema was achieved in around 60% of cases, although a small case series showed that intraocular adalimumab was ineffective in non-infectious uveitis.[234, 235] Data from existing studies of local TNF-α blockade are insufficient to determine efficacy for the management of uveitis and uveitic macular oedema, and reports of toxicity demand careful consideration of the clinical risk-benefit ratio involved.

In summary, TNF-α plays a major role in uveitis pathogenesis.[241] Tumour necrosis factor-α is upregulated intraocularly in several aetiologies of posterior uveitis,[151, 157, 163, 166, 169] and its blockade has been proved successful in many instances in clinical practice,[89, 90, 223] including cases of macular oedema and vascular leakage,[216, 218-220, 229, 230] although the use of intravitreal TNF-α antagonists was only partially successful for uveitis control.[231-237] The role of TNF-α in retinal endothelial dysfunction seems to involve increased permeability, secondary disruption of junctional complexes, increased leukocyte adhesion and trafficking, upregulation of vesicular transport and possibly cytotoxicity.[115-122, 124, 126, 133, 205-210, 212-215] However, much of the knowledge about the role of TNF-α in blood-retina barrier impairment has stemmed from *in vivo* animal studies and *in vitro* research using animal and non-ocular human endothelial cells. Most of the publications that evaluated the impact of TNF-α on the human retinal endothelium used commercial cells, and focused on diabetic retinopathy and other vitreoretinal diseases.[117-119, 122, 124, 205-208] A debate lingers on the effect of TNF-α on retinal endothelial permeability and damage of junctional complexes.[117-119, 122, 124, 205] Furthermore, the effects of TNF-α on retinal endothelial cell viability have not been extensively researched.

1.7.2.2 Interleukin-1β

Interleukin-1β is produced by dendritic cells, epithelial cells, endothelial cells, monocytes, NK cells, B-cells, and T-cells.[147, 242] Interleukin-1β acts through the IL-1 receptor (IL1R) type 1, which is blocked by the naturally-occurring IL-1 receptor antagonist (IL1RA).[243] Interleukin-1 receptor type 1 is widely expressed in immune cells, such as macrophages, neutrophils, eosinophils, T-cells, and endothelial cells.[244-246] The production of IL-1β is elicited by cytokines or activation of toll-like receptors via pathogen-associated molecular patterns. An inactive precursor of IL-1β is cleaved by caspase-1 following activation by danger-associated molecular patterns.[247] After binding to its receptor, a co-receptor, IL-1 receptor accessory protein (IL1RAcP), is engaged to the IL-1β/IL1R complex, forming a ternary structure, which attaches to the Toll/IL1R intracellular domains, recruiting the myeloid differentiation primary response gene 88 (MYD88), toll-interacting protein, and IL1R-associated kinase 4.[248] Down-stream events activate the NF-κB or MAPK/JNK/ extracellular signal-regulated kinase (ERK) pathways, the former being related to immune responses and regulation of cell survival and proliferation, and the latter with tumour suppression, apoptosis and cell

growth.[247-249] Interleukin-1β has pleiotropic actions in autoinflammatory diseases, including the activation of various cells involved in immune responses, and induction of endothelial adhesion molecules.[243, 248]

Many studies have reported higher concentrations of IL-1β in the aqueous humour of uveitic patients when compared with controls.[149, 151, 152, 159, 169, 178, 184] Zhao et al.[184] and Abu El-Asrar et al.[151] described raised levels of IL-1β in the aqueous humour of patients with HLA-B27-associated uveitis. Elevated concentrations of IL-1β were also found in the aqueous humour of patients with uveitis affecting the posterior segment, such as Behçet's disease,[151] birdshot retinochoroidopathy,[169] sarcoid uveitis,[151] and Vogt-Koyanagi-Harada syndrome.[151] While IL-1β upregulation in the aqueous humour during non-infectious uveitis has been reported by many authors, none has reported it to be reduced in comparison with controls, suggesting an important role of IL-1β in the mediation of uveitis.

In the human retinal endothelium, IL-1β has been demonstrated to trigger oxidative stress, mitochondrial dysfunction, and production of TNF-α, IL-6, CCL2, ICAM-1, VCAM-1, VEGF-A, matrix metalloproteinases-2 and -9.[206, 250-252] Some reports have shown that IL-1β can induce the breakdown of the human retinal endothelial barrier,[117, 124, 205, 253] and downregulate junctional molecules;[117] however, another study reported induction of retinal endothelial hyperpermeability by IL-1β under hyperglycaemia only when human retinal endothelial cells were in co-culture with pericytes, via induction of pericyte apoptosis and impairment of tight junctions.[138] Conversely, progressive strengthening was observed in human brain vascular endothelial cells following an acute reduction in transcellular electrical resistance after IL-1β administration.[211] No changes in the apoptosis rate and cell viability have been reported after IL-1β treatment of human retinal endothelial cells,[138] but disparate results have been reported for other species, including bovine[126, 254] and rodent retinal endothelial cells.[116, 255] Interleukin-1β promoted endothelial tight junction opening, leukocyte migration, increased vesicular transport and endothelial permeability when injected into the eyes of rodents and rabbits.[99, 133, 256, 257] Downregulation of junctional molecules, and upregulation of genes related to apoptosis and leukocyte migration, have been observed in human umbilical vein endothelial cells exposed to IL-1β.[258]

Interleukin-1β blockers, such as anakinra (recombinant human IL1RA) and canakinumab (a human anti-IL-1β monoclonal antibody), were shown to be effective in treating non-infectious uveitis.[259-264] Seven of 9 patients with refractory Behçet's uveitis responded to treatment with anakinra in a case series.[261] Nineteen patients with Behçet's disease experienced a reduction in the annual number of flares of uveitis from 200 to 49 per 100 patients per year after the introduction of IL-1β antagonists as monotherapy or given with another immunomodulatory drug.[259] The effectiveness of anakinra and canakinumab has been correlated with a shorter duration of Behçet's disease and ocular involvement, with the group that presented a sustained response over a year achieving disease control by 3 months of therapy.[260] However, another initially-promising anti-IL1β drug, gevokizumab (humanized anti-IL-1β monoclonal antibody), did not decrease the risk of uveitis flares in patients with Behçet's disease, although the vision was maintained, and macular oedema was reduced in the first 6 months of treatment.[265, 266] Intravitreal blockade of IL-1β has only been tried in animal models, reducing neutrophilic infiltration of the retina, but not preventing mononuclear cell trafficking and protein leakage.[267]

As a chief cytokine associated with autoinflammatory conditions, IL-1β is implicated in the mechanisms driving uveitis,[99, 117, 124, 133, 205, 256, 257] being found in elevated intraocular concentrations in different posterior uveitis conditions.[151, 169] Clinically, the antagonism of IL-1β has not been specifically assessed in the treatment of uveitic macular oedema and vascular leakage, although IL-1β blockade has achieved uveitis control and macular oedema resolution in some instances.[259-263] The disruption of the inner blood-retinal barrier triggered by IL-1β appears to be secondary to tight and adherens junction damage, enhanced transendothelial vesicular transport, promotion of leukocyte migration, mitochondrial dysfunction and oxidative damage.[99, 116, 117, 124, 126, 133, 205, 206, 211, 250-252, 254-258] As in the case of TNF-α, most publications have reported work using non-human and extra-ocular cell lines, and data on human retinal endothelial cells have mostly been examined in the context of diabetic retinopathy and other retinal diseases.[117, 124, 138, 205, 206, 250-252] The influence of IL-1β on junction molecule expression has not been extensively assessed, and controversies remain on the effect of IL-1β on barrier permeability and cell viability in human retinal endothelial cells.[117, 124, 138, 205]

1.7.2.3 Interleukin-6

Secreted by multiple cell types including monocytes, T-cells, B-cells, endothelial cells, and fibroblasts, IL-6 is a pleiotropic cytokine that signals through the IL-6 receptor (IL-6R), also known as IL-6Rα, CD126, and glycoprotein 80 (gp80).[144, 268-278] The IL-6R exists in two forms: a membrane-bound IL-6R (mIL-6R), which is limited to a few cell types, and a soluble IL-6R (sIL-6R), which is mainly generated via proteolytical cleavage of the mIL-6R by proteases, or by alternative splicing, extending IL-6 actions throughout the body. The sIL-6R lacks the transmembrane domain of the receptor.[268, 270, 274-276] When IL-6 binds to mIL-6R, a signalling pathway termed classical signalling is elicited, while IL-6/sIL-6R ligation triggers the trans-signalling pathway.[277-279] Both pathways require the association of the IL-6/IL-6R complex with the widely expressed signal-transducing glycoprotein 130 (gp130), activating tyrosine kinase Janus kinases (JAK) 1 and 2, and tyrosine-protein kinase 2. Janus kinase 1 phosphorylates tyrosine residues in the intracellular portion of gp130, activating the MAPK, phosphoinositol-3 kinase (PI3K)/AKT, and the signal transducer and activator of transcription 3 (STAT3) pathways. Downstream effects of this signalling include cell proliferation, differentiation and survival, immune regulation stress responses, and angiogenesis.[280-282]

Increased levels of IL-6 have been found in the aqueous[152, 154, 157, 159, 160, 167, 169, 170, 172, 173, 178, 180] and vitreous humours[166, 171, 174] of patients with non-infectious uveitis, compared with controls. The levels of IL-6 in the aqueous humour of uveitic patients were 137 times higher than controls, corresponding to the cytokine that was most increased in a study by Chen et al.[159] Raised intraocular IL-6 levels have been measured in several uveitides, including those affecting the posterior segment, such as Behçet's disease, Vogt-Koyanagi-Harada syndrome, sarcoidosis, and birdshot chorioretinopathy.[157, 160, 166, 167, 169, 170, 172] Intraocular concentration of IL-6 correlated with the number of cells and neutrophils in the anterior chamber of patients with idiopathic anterior uveitis, Behçet's disease and Vogt-Koyanagi-Harada syndrome.[157, 160] Uveitis activity and the grade of secondary cataracts have been associated with the level of IL-6 in the aqueous humour.[152, 181] Overall, IL-6 plays a major role in non-infectious uveitis, with several studies reporting increased levels in aqueous and vitreous humours, and no reports of IL-6 downregulation, suggesting this cytokine is involved in both anterior and posterior segment inflammation.

Interleukin-6 enhances cell proliferation and viability in human retinal endothelial cells.[206, 283] A couple of studies have reported that IL-6 decreases the transcellular electrical resistance and increases the permeability of human retinal endothelial cells,[125, 205] diminishing the expression of some tight junctional components.[125] Others have reported no change in human retinal endothelial barrier function or tight junctional molecule expression,[284] with one study noting the absence of effect without showing the data.[117] The co-administration of sIL-6R and IL-6 phosphorylates STAT3, prompting ICAM-1 expression, mitochondrial dysfunction, and oxidative stress in human retinal endothelial cells, impairing the barrier function.[285] Endothelial barrier breakdown and disruption of junctional complexes are also seen after simultaneous administration of IL-6 and sIL-6R in human vein umbilical endothelial cells, but not after sole administration of IL-6.[286] However, Desai et al.[287] described decreased transcellular electrical resistance using the same endothelial cell type after exclusively administrating IL-6 alone in a dose-dependent manner. Downregulation of junctional molecules and enhanced permeability were reported in human brain microvascular endothelial cells.[209, 210] In mice, uveitis was attenuated in IL-6-deficient animals or with intravitreal anti-IL-6 treatment.[192, 193] Interleukin-6 is crucial for Th17 differentiation, an important lineage in the pathogenesis of uveitis.[192]

Retinal endothelial cells are believed to lack the transmembrane IL-6R[284, 285, 288] while expressing the glycoprotein gp130,[285, 288] which could allow for IL-6 trans-signalling. Although the IL-6R transcript expression was reported in human retinal endothelial cells,[289] a few reports failed to demonstrate the presence of IL-6R protein in the retinal endothelium.[284, 285, 288] Low levels of sIL-6R were identified in the medium of cultured human retinal endothelial cells, but were insufficient to allow for endothelial barrier breakdown after exogenous IL-6 administration.[284] During uveitis, in addition to increased IL-6 intraocular level, raised sIL-6R levels were also observed.[290] Therefore, it has been suggested that during inflammatory states, sIL-6R is generated by limited proteolysis or alternative splicing from mIL-6R expressed by infiltrating leukocytes, enabling IL-6 to activate the retinal endothelium through the IL-6/sIL-6R/gp130 complex.[285, 291, 292]

Clinical experience shows that broad IL-6R blockade improves non-infectious uveitis, particularly in refractory cases of macular oedema and vasculitis, both associated with the impairment of the retinal endothelial barrier.[293-296] Tocilizumab is a humanized monoclonal antibody against sIL-6R and mIL-6R. Patients with a range of non-infectious uveitides and macular oedema refractory to conventional immunomodulatory therapy, anti-TNF-α drugs and interferons experienced control of the intraocular inflammation with the use of tocilizumab.[294, 296-299] In contrast, a study in patients with juvenile idiopathic arthritis-associated refractory uveitis – a condition mostly featuring anterior uveitis – demonstrated tocilizumab efficacy in only around one-third of patients after 12 weeks.[300] At the same time, coexistent macular oedema resolved in three of four patients. Treatment for 16 weeks with sarilumab, a human anti-IL-6 receptor monoclonal antibody, achieved a 2-step or greater reduction in vitreous haze and/or a decrease in corticosteroid dose in 64% of patients compared with 35% in the placebo group.[301] In mice, intravitreal IL-6 blockade reduced inflammation and vascular leakage in 75% of the treated eyes.[193]

As a chief mediator of inflammation, the role of IL-6 in uveitis and other retinal diseases has been the subject of growing interest.[279, 302, 303] Elevated intraocular levels of IL-6 have been reported in cases of posterior uveitis, and IL-6 blockade has been particularly effective in treating uveitic vascular leakage and macular oedema.[294, 296-299] The effects of IL-6 on the inner blood-retinal barrier are variable and poorly understood, with existing data mainly derived from animal and *in vitro* studies of extra-ocular endothelial cells and only a few studies using human retinal endothelial cells. There is an ongoing debate on the permeability effects of IL-6 on the retinal endothelium, since two articles observed decreased barrier impedance,[125, 205] while a couple of publications showed no effect.[117, 284] Some investigators have suggested that endothelial permeability is induced only by trans-signalling since the addition of sIL-6R in conjunction with IL-6 elicited hyperpermeability.[285, 286] A central controversy that needs to be addressed is the presence of the IL-6R in the retinal endothelium since disagreement exists about transcript and protein expression,[284, 285, 288, 289] added to the incongruities around the IL-6 impact on endothelial barrier permeability.[117, 125, 205, 284] Moreover, IL-6 effects on cell viability and junctional complexes have not been broadly explored in human retinal endothelium.

1.7.2.4 Interleukin-17

Interleukin-17, or IL-17A, is one of the six members of the IL-17 family and the hallmark of Th17 cells.[304-306] Besides being produced by Th17 cells, IL-17 is secreted by γδ T-cells, cytotoxic CD8+ T-cells, NKT cells, neutrophils, and microglia. Interleukin-17 receptors are heterodimeric structures comprised of the IL-17 receptor A (IL-17RA) plus a second chain, IL17-RC, which provides ligand or signalling specificity.[307] Interleukin-17RA is widely expressed, while IL17-RC is expressed by non-immune cells in the retina, including Müller cells, retinal endothelial cells, and photoreceptors.[308, 309] Interleukin-17 binds to the IL-17RA/IL17-RC receptor complex, initiating an interaction between the cytoplasmic SEFIR domains of the receptor and the signalling adaptor protein Act1, recruiting TRAF6, and activating the NF-κB, MAPK, ERK, JAK/PI3K, JAK/STAT, p38, JNK, and CCAAT-enhancer-binding proteins (C/EBPs) pathways.[304, 307] Downstream events include the transcription of inflammatory cytokines, chemokines, and antimicrobial peptides. In addition, a noncanonical pathway, dependent on I kappa B (IκB) kinase and phosphorylation of Act1, can be induced via the recruitment of TRAF2 and TRAF5 to stabilize mRNAs, especially those involved in the transcription of cytokines and chemokines.[307]

Interleukin-17 is reported to be increased in the aqueous humour[159, 163, 169, 310] and vitreous humour[166] of patients with non-infectious uveitis, in comparison with healthy controls. Raised intraocular levels of IL-17 have been observed in Behçet's disease,[163] birdshot retinochoroidopathy,[169] sarcoidosis,[166] and Vogt-Koyanagi-Harada syndrome.[163] Several studies have demonstrated a correlation between aqueous levels of IL-17 and the clinical severity of uveitis.[159, 163] Kuiper et al.[169] evaluated both intraocular and serum levels of IL-17 in patients with birdshot retinochoroidopathy, and described higher levels in the aqueous humour than in serum.

A few studies have demonstrated that, in human retinal endothelial cells, IL-17 is associated with monolayer hyperpermeability, downregulation of junctional components, induction of IL-6, IL-8, ICAM-1 and VEGF-A, secretion of neutrophil elastase, promotion of cell migration, angiogenesis, increased apoptosis, and reduced cell proliferation.[308, 311-314] Conversely, IL-17 has been associated with enhancement of cell proliferation and no effect on human retinal endothelial barrier function in

other studies.[117, 312] Interleukin-17 is pro-angiogenic in human choroidal endothelial cells, enhancing cell migration and tube formation via the production of chemokines by the retinal pigment epithelium.[315, 316] In human extra-ocular endothelial cells, IL-17 upregulates IL-6, IL-8 and other chemokines and fosters neutrophilic recruitment and migration, inducing endothelial apoptosis.[306, 317-319] The barrier function of human retinal pigment epithelial cells and mouse brain endothelial cells is decreased by IL-17 via junctional proteins disruption.[320, 321] Blockade of IL-17 was shown to ameliorate uveitis in some animal models, although causing photoreceptor toxicity;[199, 322, 323] however, injection of IL-17 also reduced the severity of experimental autoimmune uveitis.[324]

Systemic IL-17 blockade has been used therapeutically in the spondyloarthropathies.[325-327] In one study, the use of subcutaneous secukinumab – a human monoclonal anti-IL-17 antibody – for the treatment of non-infectious uveitis showed no reduction in uveitic recurrences during withdrawal of immunomodulatory therapy, compared with placebo.[328] However, others showed that intravenous secukinumab controlled intraocular inflammation in over two-thirds of patients with non-infectious uveitis.[329] Miserocchi et al.[330] reported that secukinumab controlled intraocular inflammation in a case series of four patients with HLA-B27-associated uveitis.[330] Some authors have reported the onset of Behçet's-like and Behçet's disease, and uveitis following the introduction of anti-IL-17 drugs.[331-334] However, pooled data from three trials does not suggest an elevated risk of incidence of uveitis in patients with active ankylosing spondylitis treated with secukinumab.[335]

Most of what is known so far about IL-17 effects on the inner blood-retinal barrier has been obtained through research using commercial human retinal endothelial cells and non-ocular endothelial cells largely focusing on diabetic retinopathy, as well as from experimental animal models.[117, 199, 306, 308, 311-324, 336-340] Interleukin-17 is an important cytokine in the pathogenesis of uveitis,[336-338] but only a few studies have evaluated the activity of IL-17 in the human retinal endothelium and did so by using commercial cell lines not phenotypically characterized by the authors.[117, 308, 311, 312, 314] Data suggests that IL-17 would promote the production of other inflammatory cytokines and cytotoxic mechanisms;[306, 308, 311-314, 317-319] however, it is unclear if IL-17 affects the retinal endothelial impedance and cell proliferation due to contradictory findings. Moreover, the direct effect of IL-17 on

the junctional complexes of the retinal endothelium has not been assessed.

1.7.2.5 Interleukin-8 (C-X-C motif chemokine ligand 8)

Interleukin-8 – or CXCL8 – is member of the CXC chemokine family, categorised by an amino acid between the two cysteines. Interleukin-8 is produced by macrophages, lymphocytes, neutrophils, γδ T-cells, mast cells, endothelial cells, and epithelial cells.[341-343] Two receptors from the guanosine-5'-triphosphate (GTP) binding protein (G-protein) coupled rhodopsin-like receptor family mediate IL-8 activity. C-X-C motif chemokine receptor 1 (CXCR1 or IL-8RA) and CXCR2 (IL-8RB), both linked to heterotrimeric G proteins, are composed of α, β, and γ subunits. CXCR1 interacts only with IL-8 and CXCL-6, whereas CXCR2 responds more broadly to CXC chemokines.[344] Different cell types express CXCR1 and CXCR2, including T-cells, neutrophils, fibroblasts, epithelial cells, and vascular endothelial cells.[345, 346] Following the binding of IL-8 to its receptor, several signalling pathways can be activated, such as PI3Kγ, phospholipase C, and tyrosine-protein kinase HCK, mediated by small GTPases Ras/Rac/Rho/cdc42/Rap1, protein kinase C (PKC) and AKT. Additionally, IL-8 can promote cell adhesion, cytoskeletal changes, membrane protrusion, cell migration, exocytosis of lysosomal enzymes, and endothelial permeability.[344, 347] Alternatively, ERK1/2, MAPK and JAK/STAT pathways can be activated, regulating cell proliferation, growth, differentiation, and survival.[342, 343, 348, 349] Interleukin-8 is chemotactic for granulocytes and induces phagocytosis, oxidative stress and angiogenesis.

Several studies have reported raised levels of IL-8 in the aqueous[155-157, 167, 170, 173, 177-179, 182, 310] and vitreous[162, 171] humours of patients with non-infectious uveitis. Raised aqueous[178] and vitreous humour[171] levels of IL-8 were associated with uveitic macular oedema, and aqueous humour concentrations correlated with disease activity.[178] Intraocular levels of IL-8 may also correlate with the total number of cells and neutrophils in the anterior chamber during uveitis.[157, 160] High IL-8 levels have been detected in the eyes of patients with juvenile idiopathic arthritis-associated uveitis,[167] acute anterior uveitis,[182] idiopathic uveitis,[160] HLA-B27-associated uveitis,[164] and Behçet's disease,[157, 160, 164, 177, 182] Vogt-Koyanagi-Harada syndrome,[164, 182] idiopathic posterior, intermediate or panuveitis,[310] and sarcoidosis.[164, 166] The biological activity of intravitreal IL-8 was confirmed by

the assessment of the neutrophilic chemotactic activity and the use of a monoclonal anti-IL-8 antibody, which decreased chemotaxis by around 60%.[162] Additionally, clones of T-cells from intraocular fluids of patients with Behçet's uveitis spontaneously produced higher amounts of IL-8 than controls.[350]

In human retinal endothelial cells, IL-8 is induced by lipopolysaccharide (LPS), TNF-α, hyperglycaemia, and low shear stress.[351-354] Addition of IL-8 to human non-ocular endothelial cells increases endothelial permeability via phosphorylation and downregulation of junctional molecules, and also by transactivation of the VEGF receptor.[355-358] Intravitreal injection of IL-8 in rabbits induced uveitis transiently,[196] whereas blockade of IL-8 reduced the severity of endotoxin-induced uveitis (EIU), suppressing leukocyte infiltration, but not reducing protein levels in the aqueous.[197] Intravitreal blockade of IL-8 decreased neutrophilic infiltration by 66% in EIU in rabbits.[194]

The use of interleukin-8 blockers has not been implemented for the treatment of inflammatory diseases in humans. In animal models, an anti-IL-8 antibody reduced the severity of EIU.[197] HuMax-IL8, a human monoclonal antibody directed against IL-8, has been investigated in different clinical trials as an adjuvant or single therapy for metastatic solid tumours,[359] lung and liver cancer,[360] pancreas,[361] and prostate[362] cancer due to its anti-neoplastic and anti-inflammatory potential. A CXCR2 blocker was evaluated for the treatment of chronic obstructive pulmonary disease and influenza infection, with a good safety profile, whereas CXCR1/CXCR2 antagonism improved the response of NK and T-cells in immunotherapy for cancer treatment.[363-367]

The investigation of IL-8 activity on the human retinal endothelial barrier has not yet been published, and a few studies have reported the induction of IL-8 in the human retinal endothelium by different conditions.[351-354] From what is gathered from the limited publications using retinal endothelial cells, extra-ocular endothelial cells and animal studies, IL-8 seems to promote neutrophilic infiltration and alter junctional complex constituents;[194, 196, 197, 355-358] however, it is uncertain whether IL-8 per se impairs the endothelial barrier or does so as a result of fostering leukocyte migration. Interleukin-8 is an important mediator of intraocular inflammation, and raised levels have been found intraocularly during posterior uveitis[157, 160, 164, 166, 177, 182, 310] and macular oedema.[178] Increased serum IL-8 levels

have been associated with disease activity and occurrence of vitreous exudates, periphlebitis and papillitis in patients with intermediate uveitis.[368] Despite improving intraocular inflammation in *in vivo* animal studies,[197] IL-8 inhibition has not been attempted for the treatment of non-infectious uveitis in human clinical trials.

1.7.2.6 C-C motif chemokine ligand 2

The C-C motif chemokine ligand 2, also known as monocyte chemoattractant protein (MCP)-1, is a member of the C-C chemokine superfamily which possesses two adjacent cysteine residues next to the N-terminus.[369] C-C motif chemokine ligand 2 is synthesised by monocytes, macrophages, mast cells, fibroblasts, astrocytes, microglia, epithelial cells, endothelial cells, and smooth muscle cells.[369-372] Several receptors recognize CCL2 – i.e. C-C motif chemokine receptor (CCR) 5, CCR10, CCR11, atypical chemokine receptor 1 (ACKR1). However, CCR2 is the main receptor through which CCL2 exerts its actions.[369, 373] C-C motif chemokine receptor-2 is expressed by monocytes, macrophages, neutrophils, activated T-cells and NK cells, and endothelial cells.[374-377] Upon ligation to CCR2, CCL2 activates signalling pathways including PI3K/AKT, NF-κB, MAPK/p38, ERK, JAK/STAT3, which eventually enhance cell survival, migration, angiogenesis, and tumour growth, among other functions.[369, 374, 378] C-C motif chemokine ligand 2 is a crucial mediator of inflammation, Th1, Th2 and Th17 responses, recruitment of effector cells to the site of inflammation, regulation of cell adhesion via expression of integrins, modulation of macrophage polarization, activation of monocytes, and maturation of dendritic cells.[369, 373, 374, 378-381] Many disorders implicate CCL2 in their pathogenesis, including cancer, cardiovascular diseases, neuroinflammatory disorders, and autoimmune diseases.[378]

Various studies have reported raised levels of CCL2 in the aqueous humour[151, 160, 170, 178, 179, 183, 310] and the vitreous humour[166] of patients with non-infectious uveitis, as well as in the vitreous of patients with uveitic macular oedema.[171] Increased concentrations of this chemokine have been found in the aqueous humour of patients with Behçet's disease and uveitis,[160] idiopathic uveitis,[160, 310] Vogt-Koyanagi-Harada syndrome,[151, 157] and in the vitreous of patients with sarcoid uveitis,[166] all disorders associated with posterior segment intraocular inflammation. Raised aqueous humour levels of CCL2

were higher in active non-infectious uveitis patients when compared with quiescent cases.[178, 179] In one study in which patients with non-infectious uveitis and rubella virus-associated uveitis were grouped, CCL2 was associated with the development of secondary glaucoma.[382]

The expression of CCL2 may be induced by IL-1β, LPS, VEGF-A, and hyperglycaemia in the human retinal endothelium,[107, 250, 383, 384] and an angiogenic effect of CCL2 has been observed in non-ocular human endothelial cells linked to the upregulation of VEGF-A.[385] Rangasamy et al.[107] reported a lack of effect of CCL2 on the impedance of a commercial human retinal endothelial cell line, but when cells were exposed to conditioned medium from activated human macrophages, the impedance decreased. In rat retinal endothelial cells, CCL2 upregulated TNF-α, IL-1β and interferon (IFN)-γ,[386] but neither impacted cell viability and migration, nor disrupted the adherens junctions in murine retinal endothelial cells.[116] C-C motif chemokine ligand 2-induced endothelial hyperpermeability has been observed in brain endothelial cells from mice, via an alteration of tight junctional complexes.[371, 387, 388] During the course of experimental autoimmune uveitis, CCL2 was detected during peak disease, being evident in retinal and choroidal vessels, and in infiltrating cells.[389] Intraocular injection of CCL2 in mice elicited monocyte infiltration and microglial activation in the retina,[371] while intravitreal antagonism of CCL2 decreased leukocytic infiltration and protein leakage in EIU.[194]

Clinical implementation of CCL2 blockade for uveitis treatment in humans has not been attempted. A CCR2/CCR5 antagonist had been proposed to reduce diabetic macular oedema and vascular leakage, but the efficacy was non-inferior to standard treatment with the anti-VEGF-A drug, ranibizumab in a clinical trial.[390] C-C motif chemokine ligand 2 blockers, such as carlumab and ABN912, have been studied in humans in rheumatoid arthritis, cancer, and idiopathic pulmonary fibrosis, with no apparent benefit.[391-394] A randomized clinical trial of an anti-CCL2 antibody for the treatment of rheumatoid arthritis did not show favourable clinical or immunohistological outcomes, despite being well tolerated.[392]

Only a couple of studies have assessed the biological functions of CCL2 in the human retinal endothelium, using a non-characterized cell line and focusing on diabetic retinopathy.[107, 384] Data from studies using animal models, non-human retinal endothelial cells and non-ocular endothelial

cells suggest that CCL2 promotes angiogenesis, leukocyte transendothelial infiltration, and impairment of junctional complexes.[194, 371, 385, 387, 388] C-C motif chemokine ligand 2 is an important mediator in uveitis development,[194, 371, 387-389] and high levels of CCL2 have been found intraocularly in patients with non-infectious uveitis affecting the posterior segment,[151, 157, 160, 166, 310] being associated with active ocular disease.[178, 179] In spite of attenuating intraocular inflammation in uveitic animal models,[194] CCL2 inhibitors have not been studied in humans with non-infectious uveitis.

1.7.2.7 Other inflammatory cytokines

Other cytokines, such as IFN-γ, IL-2, IL-12, and IL-23, are also implicated in the pathogenesis of non-infectious uveitis. Interferon-γ is produced mainly by activated Th1 and NK cells, and is essential in the defence against viral infections.[395, 396] Interferon-γ was shown to be active in human non-ocular endothelial cells,[397-399] aggravated inflammation in some cases,[338, 400] but also attenuated it.[401] However, there is no published evidence that it affects the retinal endothelial barrier, and it has been associated with intraocular viral infection,[402] which is not the scope of this thesis. Th1 cells are the main source of IL-2, a cytokine that fosters the expansion of Th17 cells.[403, 404] Raised intraocular levels of IL-2 have been found in patients with uveitis,[153, 157, 160, 166, 168, 169, 174] and IL-2 blockade improved cases of refractory non-infectious uveitis;[405-407] however, the drug was discontinued due to its association with meningitis and encephalitis.[408] There is some evidence of IL-2 activity in non-ocular endothelial cells,[409-411] but it has not been studied in relation to the retinal endothelial barrier.

Interleukin-12 is produced by dendritic cells and macrophages and promotes the expansion of Th1 cells.[412] Interleukin-12 levels are raised in the intraocular humour of uveitic patients,[150, 153, 160, 165, 171, 178, 310] but IL-12 appears to have opposing effects depending on the phase of experimental autoimmune uveitis development.[413, 414] Interleukin-23 is part of the IL-12 superfamily and is mainly produced by macrophages and dendritic cells, being essential for the differentiation of pathogenic Th17 cells.[152, 159, 169, 310, 415-417] An imbalance in the IL-23/IL-17 axis has been associated with autoimmune diseases, such as Vogt-Koyanagi-Harada syndrome, Behçet's disease and sarcoidosis.[198] Although IL-23 was required for EAU development in mice, its antagonism during the course of the disease did not decrease uveitis severity,[95] and intraocular levels of IL-23 were not

detected or increased in patients with non-infectious uveitis. Transforming growth factor (TGF)-β and IL-10 are also important mediators of uveitis, but these cytokines are mostly associated with the induction of regulatory T-cells and suppression of inflammatory responses.[147, 418-423] Although IL-12, IL-23, TGF-β and IL-10 appear to be important in uveitis, there is no published evidence to suggest that they would be implicated in retinal endothelial dysfunction.

1.7.3 Summary of inflammatory cytokines in uveitis

Cytokines act in a complex way and exert several biologic functions during inflammation, inducing or acting synergistically with other cytokines, and having autoregulatory mechanisms.[424-429] At the outset of this candidature, the bulk of the *in vitro* research conducted assessing the impact of these cytokines on the inner blood-retinal barrier had relied on the use of non-human retinal endothelial cells or extra-ocular endothelial cells and, even when human retinal endothelial cells were employed, studies mostly used commercial cell lines, focused on diseases other than uveitis, and the research was limited to a few cytokines in each publication. Therefore, a thorough examination of the effect of inflammatory cytokines in the human retinal endothelium had yet to be performed, and there were deficiencies and controversies regarding the impact of cytokines on the inner blood-retinal barrier in uveitis. Tumour necrosis factor-α, IL-1β, IL-6, IL-8, IL-17, and CCL2 were identified as priority candidates for investigation in relation to the human retinal endothelial cell barrier for multiple reasons: (1) they are upregulated inside the human eye during non-infectious uveitis; (2) they have previously documented roles in endothelial dysfunction *in vitro* and *in vivo* during inflammation; (3) biologic drugs targeting these cytokines are available; and (4) they are generally well-characterized cytokines, even though not fully studied in uveitis.

1.8 Molecular mediators regulated by inflammatory cytokines

1.8.1 Overview

The inflammatory cascade which leads to endothelial dysfunction in posterior uveitis involves numerous molecules and an intricate network. Besides cytokines, important mediators of inflammation include VEGF-A, eicosanoids, matrix metalloproteinases, the complement system, reactive oxygen species and non-coding RNAs (ncRNAs). These elements may be induced by

inflammatory cytokines and/or stimulate cytokine production, propagating inflammation and endothelial dysfunction. For example, VEGF-A promotes angiogenesis, chemotaxis,[136, 430-434] retinal endothelial permeability and tissue remodelling,[112, 124, 435-439] while complement factors foster cytokine production and intercellular gap formation.[102, 440-443] Stimulated by TNF-α and IL-1β, matrix metalloproteinases degrade extracellular matrix, contributing to endothelial dysfunction.[207, 251, 252] Release of reactive oxygen species by the retinal endothelium is triggered by TNF-α,[116, 125, 209] IL-1β[116] and IL-6,[209, 285] leading to oxidative stress.[444] Cyclooxygenase 2 and lipoxygenase pathways are upregulated by TNF-α and IL-1β,[116, 445, 446] eliciting the production of eicosanoids (e.g. prostaglandins and leukotrienes), enhancing endothelial permeability, leukostasis and cell death.[116, 447-452] Lastly, non-coding RNAs were shown to regulate several biologic processes, including cytokine production, cell survival, and vascular permeability.[453-455]

1.8.2 Long non-coding RNAs

1.8.2.1 Definition

Non-coding RNAs are transcribed genomic sequences that do not encode proteins.[456, 457] They include microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), the latter arbitrarily defined as having over 200 nucleotides of length. Long non-coding RNAs interact with DNA and RNA, and may fold into secondary structures, which frequently are determinants of their functions.[457, 458] Among diverse classifications of lncRNAs, they may be classified based on their genomic location as intergenic or intronic lncRNAs, and sense or anti-sense lncRNAs, while according to the effects on DNA sequences, lncRNAs are termed cis-lncRNAs (*cis*-regulation of nearby genes) or trans-lncRNAs (*trans*-regulation of distant genes).[459, 460] Long non-coding RNAs are implicated in a myriad of molecular regulatory processes, such as transcriptional and post-transcription gene regulation.[457] Transcriptional regulation occurs either via transcriptional interference or chromatin remodelling, whereas post-transcriptional regulation ensues by splicing or translation regulation. Additionally, lncRNA may act through fostering degradation of mRNA, replicating telomeres, interfering with RNA and protein localization, and interacting with miRNAs by mimicking their targets, hence acting as competitive endogenous RNAs (ceRNAs).[460]

1.8.2.2 Role of long non-coding RNAs in endothelial dysfunction

Several lncRNAs have been reported to be associated with endothelial dysfunction. Only a small fraction have been functionally characterized, and their actions frequently involve sponging and negative regulation of miRNAs.[461, 462] Examples of lncRNA associated with endothelial dysfunction include MALAT1, H19, TUG1, and NEAT1. MALAT1 is one of the most studied lncRNAs and has been associated with cardiovascular, neurovascular diseases, and cancer.[455, 463-466] MALAT1 was linked to the promotion of inflammation and apoptosis induced by hyperglycaemia,[467, 468] hypoxia,[469] oxidized-low density lipoprotein (LDL),[470, 471] and LPS exposure[472] in human endothelial cells. However, in mouse brain endothelial cells, MALAT1 was anti-inflammatory and anti-apoptotic during oxygen and glucose deprivation.[466] Silencing of H19 protected oxidized-LDL-treated and high glucose-treated endothelial cells from inflammation, apoptosis and oxidative stress.[473, 474] Overexpression of TUG1 alleviated inflammation and apoptosis in LPS-treated human umbilical endothelial cells and murine pulmonary endothelial cells.[475, 476] Inhibition of NEAT1 decreased inflammation and apoptosis induced by oxidized-LDL in human umbilical vein endothelial cells,[477] but worsened inflammation and apoptosis under cyclic stress.[478]

1.8.2.3 Expression of long non-coding RNAs in retinal endothelial cells

Among high-throughput RNA-sequencing (RNA-seq) studies of the human retinal endothelium[292, 479-483] performed under diverse conditions, Shao et al.[482] specifically described around 12,400 lncRNA transcripts in the retinal endothelium in a study focusing on diabetic retinopathy. Apart from publications on the retinal endothelial transcriptome, various studies focusing on ischaemic vasculopathies, such as diabetic retinopathy and retinopathy of prematurity, have validated 23 lncRNAs by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and/or protein studies in human retinal endothelial cells or in an endothelial cell line derived from the retina of the rhesus monkey. The lncRNAs expressed by the retinal endothelium were ANRIL,[484, 485] Arid2-IR,[486] FENDRR,[487] HOTAIR,[484, 488] HOTTIP,[489] HULC,[484] H19,[484, 485] ICR,[490, 491] KCNQ1OT1,[492] LNC01136,[493] MALAT1,[484, 491, 494-498] MIAT,[496, 499] MEG3,[491, 500, 501] MIR497HG,[502] NEAT1,[503] RNCR3,[504] SNHG16,[505] TUG1,[506-508] UCA1,[509] ZFAS1,[484] SNHG5,[510] SNHG7,[511, 512] and SNGH16.[505, 513] The full names of these lncRNAs are listed in Table 1.1.

Table 1.1. Long non-coding RNAs expressed in the retinal endothelium

Abbreviation	Description
ANRIL[484, 485]	Antisense non-coding RNA in the INK4 locus
Arid2-IR[486]	AT-rich interaction domain 2-IR
FENDRR[487]	FOXF1 adjacent non-coding developmental regulatory RNA
HOTAIR[484, 488]	HOX antisense intergenic RNA
HOTTIP[489]	HOXA distal transcript antisense RNA
HULC[484]	Highly upregulated in liver cancer
H19[484, 485]	Long non-coding RNA H19
ICR[490, 491]	Intercellular adhesion molecule 1 related long non-coding RNA
KCNQ1OT1[492]	KCNQ1 opposite strand/antisense transcript 1
LNC01136[493]	long intergenic non-protein coding RNA 1136
MALAT1[484, 491, 494-498]	Metastasis-related lung adenocarcinoma transcript 1
MIAT[496, 499]	Myocardial infarction associated transcript
MEG3[491, 500, 501]	Maternally expressed 3
MIR497HG[502]	miR-497 host gene
NEAT1[503]	Nuclear enriched abundant transcript
RNCR3[504]	Retinal non-coding RNA3
TUG1[506-508]	Taurine-upregulated gene 1
UCA1[509]	Urothelial carcinoma-associated 1
ZFAS1[484]	ZNFX1 antisense RNA 1
SNHG5[510]	Small nucleolar RNA host gene 5
SNHG7[511, 512]	Small nucleolar RNA host gene 7
SNGH16[505, 513]	Small nucleolar RNA host gene 16

1.8.2.4 Long non-coding RNAs and retinal disease

Retinal disease progression or protection has been associated with several lncRNA through different mechanisms in ischaemic vasculopathies, such as promoting or hindering the production of inflammatory cytokines[503, 514] and VEGF-A,[484, 487-489, 498, 503, 507-510, 512, 515-518] promoting vascular permeability[488, 499, 516, 518] and oxidative stress.[484, 497, 501, 503, 513, 519] MALAT1 and NEAT1 were upregulated in the mitochondria of retinal endothelial cells exposed to high glucose, triggering oxidative stress and mitochondrial damage.[496, 497] MALAT1 silencing reduced the production of inflammatory cytokines,[494] cell proliferation,[498] migration,[495, 498] and angiogenesis[495, 498] induced by high glucose in retinal endothelial cells, but worsened cell viability and migration.[495] Decreased levels of MEG3 in hyperglycaemic conditions were associated with increased inflammation, angiogenesis, and apoptosis.[500, 501] Due to the implications in retinal endothelial dysfunction, lncRNAs are of interest in the pathogenesis of uveitis. However, there are only a few publications of lncRNAs in the context of non-infectious uveitis, and these have focused on associations between lncRNA single-nucleotide polymorphisms and specific forms of uveitis.[520-523] A transcriptome from murine irises with EAU suggested signalling pathways in the disease pathogenesis.[524]

Publications investigating the role of lncRNA in the eye have concentrated on diabetic retinopathy,[482, 484, 485, 487-489, 492, 494-499, 501, 503-506, 509, 511-519, 525-554] glaucoma,[555-563] retinoblastoma[564-580] and other retinal diseases,[507, 508, 581-589] and retinal development.[590-594] However, the role of lncRNAs during uveitis is unknown. The involvement of lncRNAs in inflammation has to be inferred by studies focused on ischaemic vasculopathies using retinal endothelial cells and other studies using non-ocular endothelial cells focused on atherosclerosis and other vascular diseases, which is not ideal given the particularities of each condition.[455, 456, 461, 462] The association between lncRNA and cytokines in retinal endothelial cells may be postulated on the basis of studies reporting that the expression of cytokines induced by other conditions, such as hyperglycaemia, was altered by the silencing or expression of a specific lncRNA.[494, 500, 503] The study of the expression of lncRNAs in the retinal endothelium under inflammatory conditions that mimic uveitis is lacking, and it is still to be determined if inflammatory cytokines can regulate lncRNA expression in retinal endothelial cells.

1.9 Research resources for studies of human retinal endothelial cells

Refined technical resources are essential to study the effects of inflammatory cytokines on the retinal endothelium. Flinders University facilities provide state-of-the-art molecular and cell biology platforms, including a biosensor for real-time impedance assessments, RT-qPCR thermal cyclers, dedicated tissue culture facilities and a specialized flow cytometry facility. The Eye Bank of South Australia, which is onsite, retrieves human eyes for the purposes of clinical transplantation. With consent from the next of kin, human posterior eyecups may be used for research after removal of corneas for use in transplantation surgery. The provision of such material is regulated by approval from the local Human Ethics Research Committee. The home laboratory has long-standing expertise in the isolation, characterization, and culture of a wide variety of cells from human eyes, including retinal endothelial cells.

Research on the human retinal endothelium forms the core of the work presented in this thesis. The gold standard *in vitro* approach is the use of primary human retinal endothelial cells, given that endothelial pathophysiology differs between human and animal cells, and between ocular and extra-ocular cells.[595-597] The acquisition of primary human retinal endothelial cells is time-consuming and demands high standards of technical expertise. For optimal cell yields, donors should preferably be young and should not have a clinical history of vascular disease. Isolation of retinal endothelial cells should begin as early as possible, ideally less within 24 hours post-mortem.[38] Following cell isolation, technical steps need to be performed to achieve an endothelial cell culture of at least 99% purity. Since the number of primary cells obtained is limited, the cells can be transduced with viral construct encoding human papilloma virus E6 and E7 genes to expand the population.[38] These 'immortalized retinal endothelial cells' are characterized phenotypically by their typical morphological cobblestone pattern, and the expression of CD31 and von Willebrand factor.

1.9.1 Assessment of transcellular electrical impedance

The barrier function of cell monolayers is commonly assessed *in vitro* by macromolecular permeability assays, which measure the flux of macromolecules via fluorescently-labelled tracers, and by transcellular electrical resistance assays, including electrical impedance analyses, which are

measured by applying an alternating electric current to the cell monolayer.[598, 599] The macromolecular permeability is an inexpensive and simple method that uses a semipermeable membrane on cell culture inserts (transwells). However, it is an end-point assay and the molecular tracers may interfere with the cellular barrier function. More recently, impedance has been assessed by non-invasive and label-free real-time biosensor devices, which are equipped with an alternate current system, such as the iCELLigence instrument, manufactured by ACEA Biosciences. The continuous measurement of impedance can extend over cell attachment, spreading, proliferation and interventions performed, allowing for kinetic studies in real-time.[600] Results of macromolecular permeability and real-time impedance assays have been shown to be comparable, but the former is unable to gauge fast and transient changes in barrier function.[599] Conventional transwell assays and biosensor devices display good comparability and correlation.[601] The home laboratory operates an iCELLigence.

1.9.2 Cellular and molecular analyses techniques

The culture of human retinal endothelial cells laid the groundwork for subsequent in-depth cellular and molecular biology studies. Standard reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-qPCR can be employed to verify the expression and to compare the differential expression of molecules in human retinal endothelial cells. Cell growth and viability can be assessed in fluorescence-based DNA quantification assays and by flow cytometry. Access to infrastructure and molecular consumables for transfecting retinal endothelial cells with siRNA supports studies to assess the function of individual molecules.

1.9.3 Bioinformatics tools

High-throughput sequencing methods permit assessment of whole genomes or transcriptomes when combined with analysis with bioinformatic tools.[602] Novel high-throughput platforms include RNA-seq, which enables thorough transcriptome sequencing.[603, 604] The RNA-seq methodology has advanced the research of non-coding RNAs and transcription factor binding sites.[602] A number of bioinformatic algorithms have been created to predict the interactions of binding sites with transcription factors.[605-607] The JASPAR database is a catalogue of transcription factor binding profiles manually curated and continually updated from the literature, allowing for predictions of

binding sites.[604, 607] A comprehensive human retinal endothelial transcriptome, generated by RNA-seq[483] technology at the home laboratory, was used to investigate lncRNAs in the human retinal endothelium and, in conjunction with the JASPAR database, to predict transcription factors involved in the transcription of specific genes.

1.10 Synopsis

Non-infectious uveitis is a group of inflammatory eye diseases that pose a huge medical burden, not only due to vision loss, but also because of the frequent need for aggressive immunosuppression and the common co-existence of systemic diseases. When inflammation involves the retina, the risk of vision loss is greatest. Historically, corticosteroids and conventional immunomodulatory drugs have been standard treatments for non-infectious uveitis. However, there is an increasing use of biologic drugs, particularly those that target inflammatory cytokines.

The retinal vascular endothelium plays a key role in the development of forms of non-infectious uveitis based in the retina. As the main component of the inner blood-retinal barrier, this endothelium impedes the traffic of molecules and cells to maintain local homeostasis. During uveitis, dysfunction of the barrier is associated with migration of leukocytes and protein extravasation into the retina. Inflammatory cytokines constitute a major family of molecular mediators, released by resident ocular cells and infiltrating leucocytes, that act on the retinal endothelium to promote barrier dysfunction.

A detailed understanding of how inflammatory cytokines disrupt the inner blood-retinal barrier in a person with non-infectious uveitis is important to the development of biologic drugs. To date, most research on the involvement of the retinal endothelium in this condition has relied on animal models or commercial cell lines. A comprehensive investigation of how diverse inflammatory cytokines affect the human retinal endothelium is lacking, and the expression of IL-6R by human retinal endothelial cells is one particular controversy that needs to be addressed. Another group of molecules implicated in cytokine responses and regulation of endothelial function, the lncRNAs, has not been studied in the context of uveitis.

1.11 Aims of the thesis

This experimental work described in this thesis encompasses a detailed examination of the effects of inflammatory cytokines on cells of the human inner blood-retinal barrier and specifically, on human retinal endothelial cells. Both primary human retinal endothelial cell isolates and a well-characterized human retinal endothelial cell line were studied. Inflammatory cytokines were selected based on their upregulation in the eyes of patients during uveitis. Elucidation of mechanisms contributing to retinal endothelial cell dysfunction and inner blood-retinal barrier breakdown may provide insights into the therapeutic application of cytokine blockade for non-infectious uveitis involving the retina.

The aims of the thesis were:

1. To assess changes in the electrical resistance of human retinal endothelial cell monolayers in response to inflammatory cytokines, and to investigate the impact of selected cytokines on the viability and junctional complexes in those cells (Chapters 3 and 5).
2. To explore the expression of the IL-6R in human retinal endothelial cells, and to define transcription factors that may regulate IL-6R gene (Chapter 4).
3. To examine the expression of lncRNAs that may be involved in the activation of human retinal endothelial cells following exposure to inflammatory cytokines (Chapter 6).

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Consumables

- Assay plate, 96-well black with clear flat bottom (Merck-Sigma Aldrich St. Louis, MO, USA; cat. CLS3340)
- Cell scrapers (Merck-Sigma Aldrich St. Louis, MO, USA; cat. CLS3010)
- Corning® centrifuge tubes (Merck-Sigma Aldrich St. Louis, MO, USA) 15 mL (cat. CLS430791) and 50 mL (cat. CLS430829)
- Corning® Deckworks low binding barrier tip sterile pipette tips (Merck-Sigma Aldrich St. Louis, MO, USA) 10 µL (cat. CLS4135), 20 µL (cat. CLS4136), 200 µL (cat. CLS4138), 100-1000 µL (cat. CLS4140-4X768EA)
- Corning® sterile cell culture dish 10 cm (Merck-Sigma Aldrich St. Louis, MO, USA; cat. CLS430167)
- Corning® sterile cell culture dish 6 cm (Merck-Sigma Aldrich St. Louis, MO, USA; cat. CLS430166)
- Corning® Thermowell® Gold 0.2 mL PCR tubes (Merck-Sigma Aldrich St. Louis, MO, USA; cat. CLS3745)
- Corning® tissue culture flasks 75 cm^2 (Merck-Sigma Aldrich St. Louis, MO, USA; cat. CLS430720U)
- Cryogenic freezing vials 1.2 mL internal thread (Merck-Sigma Aldrich St. Louis, MO, USA; cat. CLS430487)
- Disposable glass 230 mm Pasteur pipettes (Bacto, Mt Pritchard, NSW, Australia; cat. D812)
- Flow cytometry tube caps (Falcon, Corning, NY, USA; cat. FAL352032)

- Hard-shell® PCR plates 96-well low-profile, thin wall, skirted (Bio-Rad Laboratories Hercules, CA, USA; cat. HSP9601B)
- Microcentrifuge tubes 0.65 mL (Merck-Sigma Aldrich St. Louis, MO, USA; cat. CLS3208)
- Microcentrifuge tubes 1.7 mL (Merck-Sigma Aldrich St. Louis, MO, USA; cat. CLS3621)
- Microseal® B for 96-well plates (Bio-Rad Laboratories Hercules, CA, USA; cat. MSB-1001)
- Nitrile gloves (SA Health stores, Adelaide, SA, Australia; cat. 20020206)
- Pipetman® pipettes (Gilson Inc., Middleton, WI, USA) P2G (cat. F144054M), P10G (cat. F144055M), P20G (cat. F144056M), P100G (cat. F144057M), P200G (cat. F144058M), P1000G (cat. F144059M)
- Razor blade – single use (SA Health stores, Adelaide, SA, Australia; cat. 85300027)
- Round bottom polystyrene tubes (Falcon, Corning, NY, USA; cat. FAL352058)
- RTCA E-plate L8 (ACEA Biosciences Inc., San Diego, CA, USA; cat. 300600850)
- RTCA resistor plate (ACEA Biosciences Inc., San Diego, CA, USA; cat. 380600980)
- Serological glass pipettes (Merck-Sigma Aldrich St. Louis, MO, USA) 5 mL (cat. CLS4487), 10 mL (cat. CLS4488), 25 mL (cat. CLS4489)
- SSI 0.2 mL strip tubes with flat cap (Scientific Specialties Inc., Lodi, CA, USA; cat. SSIB3135-00S)
- SSI Vertex® filter tips (Scientific Specialties Inc., Lodi, CA, USA) 10 µL XL (cat. SSIB4137NSFS), 20 µL (cat. SSIB4237NAFS), 200 µL (cat. SSIB4237NSFS), 1000 µL (cat. SSIB4337NSFS)
- Sterile cell culture well plates (Merck-Sigma Aldrich St. Louis, MO, USA) 12-well plates (cat.

CLS3513), 24-well plates (cat. CLS3524), 96-well plates (CLS3795)

- TC20™ counting slides (Bio-Rad Laboratories Hercules, CA, USA; cat. 1450011)
- White strip tubes 0.2 mL (Bio-Rad Laboratories Hercules, CA, USA; cat. TLS-0851)

2.1.2 Equipment

- Allegra X-12R centrifuge (Beckman Coulter, Brea, CA, USA; SN. ALX13E03 and ALX13E05)
- Bio-Rad CFX connect™ real-time PCR detection system thermocycler (Bio-Rad Laboratories Hercules, CA, USA; SN. BR001934)
- BioAir AURA PCR cabinet (EuroClone, Milan, Italy; SN. N01PC1N6836)
- Biological safety cabinet (Thermo Fisher Scientific, Carlsbad, CA, USA; SN. 42098915)
- Canon DS126181 (Canon, Tokyo, Japan; SN. 1970526974)
- Cell counter Bio-Rad TC20™ (Bio-Rad Laboratories Hercules, CA, USA; SN. 508BR04141)
- Corning® LSE™ vortex mixer (Merck-Sigma Aldrich St. Louis, MO, USA; SN. S2091206 and S2091209)
- Corning LSE® digital dry bath (Merck-Sigma Aldrich St. Louis, MO, USA; SN. 3033012)
- CytoFLEX S Flow cytometer V4-B4-R3-12 (Beckman Coulter, Brea, CA, USA)
- Dry bead bath (Thermo Fisher Scientific, Carlsbad, CA, USA; SN. 3033012)
- Electrophoresis power supply (Thermo Fisher Scientific, Waltham, MA, USA; cat. EC300XL)
- Gilson™ Macroman™ pipette controller (Gilson Inc., Middleton, WI, USA; cat. F110756)
- Invitrogen™ Safe imager™ 2.0 blue-light transilluminator (Thermo Fisher Scientific, Waltham, MA, USA; SN. 13049426)

- MCO-19AIC CO_2 Incubator (Panasonic, Kadoma, Japan; SN. 13050133 and 13050134)
- Microcentrifuge 22R (Beckman Coulter, Brea, CA, USA; SN. MHD12B001 and MHD12B003)
- Nalgene® Mr Frosty® Cryo 1 °C freezing container (Nalge Nunc International, Rochester, NY, USA; cat. 5100-0001)
- Olympus IX53 Inverted Microscope (Olympus Corporation, Tokyo, Japan; SN. 3E06836)
- RTCA iCELLigence® instrument (ACEA Biosciences Inc., San Diego, CA, USA; SN. 431151214079; model 2XL8)
- Smoothflow Fume Cupboard (E & I Lewis Plastics, Addison, IL, USA; SN. FC5740)
- Spinfuge microcentrifuge (Bioline Global, Narellan, NSW, Australia; SN. LG3001286)
- T100™ Touch Thermocycler (Bio-Rad Laboratories Hercules, CA, USA; SN. 621BR12612 and 621BR12620)
- VICTOR™ X3, multilabel plate reader (PerkinElmer, Waltham, MA, USA; SN. 20301733)
- Wide Mini-Sub Cell GT Cell (Bio-Rad Laboratories Hercules, CA, USA; cat. 68963-3)

2.1.3 Software

- Adobe Photoshop version 20.0.6 (Adobe Creative Cloud, San Jose, CA, USA)
- Canon Digital Photo Professional version 3.14 (Canon, Tokyo, Japan)
- Canon EOS Utility version 2.14 (Canon, Tokyo, Japan)
- CellSens Imaging Software version 1.11 (Olympus Corporation, Tokyo, Japan)
- CFX Manager™ version 3.1 (Bio-Rad Laboratories Hercules, CA, USA)
- CytExpert software for CytoFLEX platform (Beckman Coulter, Brea, CA, USA)

- FlowJo™ Software version 10.7.1 (BD Biosciences, Franklin Lakes, NJ, USA)
- GraphPad Prism version 6.04 (GraphPad Software, La Jolla, CA, USA)
- RTCA Data Analysis Software version 1.0 (ACEA Biosciences Inc., San Diego, CA, USA)

2.1.4 Chemicals

- 2-mercaptoethanol (2-ME) 14.3 M for molecular biology (Sigma-Aldrich, St. Louis, MO, USA; cat. M3148)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA; cat. 67-68-5)
- Glacial 100% acetic acid (Sigma-Aldrich, St. Louis, MO, USA; cat. 320099)
- Isopropanol (Chem Supply, Gillman, SA, Australia; cat. PA013)
- Methanol (Sigma-Aldrich, St. Louis, MO, USA; cat. 494437)
- Molecular biology ethanol (Chem Supply, Gillman, SA, Australia; cat. ET00110500)
- p-tert-octylphenol ethoxylate (Triton X-100) (LABCHEM, Zelienople, PA, USA; cat. AJA1552)
- Paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO, USA; cat. P6148)
- Tris(hydroxymethyl)aminomethane (tris base) (VWR, Radnor, PA, USA; cat. 28808.294)
- TopVision Agarose (Thermo Fisher Scientific, Vilnius, Lithuania; cat. R0492)

2.1.5 Commercial cell lines

- THP-1 – human origin, monocyte-derived (American Type Culture Collection, Manassas, VA, USA; cat. TIB-202)

2.1.6 Cell culture medium and other reagents

- Collagenase type II (Thermo Fisher Scientific-Gibco, Grand Island, NY, USA; cat. 17101015)

- Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific-Gibco, Grand Island, NY; cat. 11965-092)

- Endothelial cell growth medium 2 (EGM2) Endothelial SingleQuots™ kit (Lonza-Clonetics Walkersville, MD, USA; cat. CC4176)

- F12 nutrient mixture (Thermo Fisher Scientific -Life Technologies, Carlsbad, CA, USA; cat. 11765-054)

- Foetal bovine serum (FBS) Thermo Fisher Scientific-Gibco, Foster City, CA, USA; cat. 10099-141)

- MCDB-131 (Sigma-Aldrich, St. Louis, MO, USA; cat. M8537)

- Minimum essential medium Eagle (MEM) (Sigma-Aldrich, St. Louis, MO, USA; cat. M4526)

- Opti-MEM™ I reduced serum medium (Thermo Fisher Scientific-Gibco, Grand Island, NY, USA; cat. 31985070)

- RPMI (Roswell Park Memorial Institute) 1640 medium (Thermo Fisher Scientific-Gibco, Grand Island, NY, USA; cat. A1049101)

- Trypan blue 0.4% (Thermo Fisher Scientific-Gibco, Grand Island, NY, USA; cat. 15250061)

- Trypsin-ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific-Gibco, Foster City, CA, USA), 0.05% (cat. 25300054), 0.25% (cat. 25200056)

2.1.7 Antibodies and fluorescent reagents

- 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA; cat. D9542; working concentration 300 nM)

- Allophycocyanin (APC)-anti-human CD126 (IL-6R subunit α) antibody (BioLegend, San Diego, CA, USA; cat. 352805)

- Fluorescein isothiocyanate (FITC)-Annexin V (BD Pharmingen™, San Jose, CA, USA; cat. 51-65874x)
- FITC-anti-human CD31 antibody (BioLegend, San Diego, CA, USA; cat. 303103)
- Fluoromount aqueous mounting medium (Sigma-Aldrich, St. Louis, MO, USA; cat. F4680)
- Goat anti-rabbit IgG Alexa Fluor 488® (Thermo Fisher Scientific – Molecular Probes, Eugene, OR, USA; cat. A11008)
- Goat serum (Vector Laboratories, Burlingame, CA, USA; cat. S-1000)
- Propidium iodide (Merck-Sigma Aldrich St. Louis, MO, USA; cat. P4170)
- Rabbit anti-human ZO-1 (Thermo Fisher Scientific-Gibco, Grand Island, NY, USA; cat. 402200)
- Rabbit IgG (Vector laboratories, Burlingame, CA, USA; cat. I-1000-5)

2.1.8 Enzymes

- HotStarTaq™ Plus DNA polymerase kit (QIAGEN, Hilden, Germany; cat. 203607)
- iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Hercules, CA, USA; cat. 1708841)
- SsoAdvanced™ universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA; cat. 172-5274)
- DNAse I digestion set (Merck-Sigma Aldrich St. Louis, MO, USA; cat. DNASE70-1SET)

2.1.9 Polymerase chain reaction reagents

- Gel loading dye 6X (New England Biolabs, Ipswich, MA, USA; cat. B7021S)
- GelPilot DNA 100 bp plus ladder (Qiagen, Hilden, Germany; cat. 239045)

- SYBR® Safe DNA Gel Stain 10,000X concentrate (Thermo Fisher Scientific-Invitrogen, Carlsbad, CA, USA; cat. S33102)

- Deoxynucleotide-triphosphate mix (dNTP) (QIAGEN, Hilden, Germany; cat. 201901)

2.1.10 Buffers and solutions

- Distilled H_2O for cell culture (Thermo Fisher Scientific-Gibco, Grand Island, NY, USA; cat.15230001)

- Dulbecco sterile phosphate buffered saline (PBS) calcium and magnesium-free (Thermo Fisher Scientific, Carlsbad, CA, USA; cat. 14190-250) – used in cell culture

- Non-sterile 1X PBS was employed for immunolabelling, made following the recipe described in Table 2.1.

- Nuclease-free H_2O (Sigma-Aldrich, St. Louis, MO, USA; cat. W4502)

- 10X Annexin V binding buffer (BD Pharmingen™, San Jose, CA, USA; cat. 51-66121E)

- Tris base, acetic acid and EDTA (TAE) buffer 50X was made in-house and used for agarose gel electrophoresis. After mixing the contents below, the volume was adjusted to 1 L with distilled H_2O. To obtain a 1X TAE buffer, a 1:49 dilution was achieved in distilled H_2O. Table 2.2 shows the constituents of 50X TAE buffer.

2.1.11 Reaction systems

- CyQUANT® NF Cell Proliferation Assay Kit (Thermo Fisher Scientific-Life Technologies, Carlsbad, CA, USA; cat. C35006)

- GenElute™ Gel Extraction Kit (Sigma Aldrich St. Louis, MO, USA; cat. NA1111)

- GenElute™ Mammalian Total RNA Extraction Miniprep Kit (Sigma Aldrich St. Louis, MO, USA; cat. RTN350)

Table 2.1. Components of 1X phosphate buffered saline

Components	Concentration (mg/L)	mM
Potassium phosphate monobasic (KH_2PO_4)	144.0	1
Sodium chloride (NaCl)	9000.0	155
Sodium phosphate dibasic (Na_2HPO_4-$7H_2O$)	795.0	3

Table 2.2. Components of the 50X tris base, acetic acid and ethylenediaminetetraacetic acid buffer

Components	Quantity (mass or volume)
Tris base	242 g
Glacial 100% acetic acid	57 mL
Ethylenediaminetetraacetic acid	18.5 g
Distilled H_2O	900 mL

- PureLink™ Genomic DNA Mini Kit Genomic DNA Mini Kit (Thermo Fisher Scientific- Invitrogen, Vilnius, Lithuania; cat. K1820-01)

2.1.12 Small interfering RNA and transfection reagents

- Negative control small interfering RNA (siRNA) (Thermo Fisher Scientific, Grand Island, NY, USA; cat. 4390843)
- Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific- Invitrogen, Carlsbad, CA, USA; cat. 13778-150)
- ETS proto-oncogene 1 (ETS1) siRNAs used for the transfection experiments are displayed in Table 2.3

2.1.13 Cytokines, growth factors and lipopolysaccharide

All cytokines and growth factor were recombinant human proteins. Interferon = IFN; interleukin = IL; lipopolysaccharide = LPS; tumour necrosis factor = TNF; vascular endothelial growth factor = VEGF.

- CCL2 (Peprotech, Rocky Hill, NJ, USA; cat. 300-04-20)
- IFN-γ (R&D Systems, Minneapolis, MN, USA; cat. 285-IF-100)
- IL-17 (Peprotech, Rocky Hill, NJ, USA; cat. 200-07-25)
- IL-1β (R&D Systems, Minneapolis, MN, USA; cat. 201-LB-005)
- IL-22 (R&D Systems, Minneapolis, MN, USA; cat. 782-IL-010)
- IL-6 (Peprotech, Rocky Hill, NJ, USA; cat. 200-06-20)
- IL-8 (Peprotech, Rocky Hill, NJ, USA; cat. 200-08M-25)
- LPS from *Escherichia coli* 055:B5 (Merck-Sigma Aldrich, St. Louis, MO, USA; cat. L2880)
- TNF-α (R&D Systems, Minneapolis, MN, USA; cat. 210-TA-020)

Table 2.3. ETS proto-oncogene 1 (ETS1) small interfering RNAs (siRNAs), manufacturer information and targeted ETS1 isoforms

Small interfering RNAs (siRNAs)	Manufacturer and catalogue number	Targeted isoforms and GenBank ID
ETS1 siRNA s4847 (ETS1 siRNA 1)	Thermo Fisher Scientific-Gibco, Grand Island, NY, USA; cat. 4392420	Transcript variant 1 (NM_001143820.1) Transcript variant 2 (NM_005238.3) Transcript variant 3 (NM_001162422.1)
ETS1 siRNA s4848 (ETS1 siRNA 2)	Thermo Fisher Scientific-Gibco, Grand Island, NY, USA; cat. 4392420	Transcript variant 1 (NM_001143820.1) Transcript variant 2 (NM_005238.3) Transcript variant 4 (NM_001330451.3)

- VEGF-A (VEGF-A isoform 165) (Peprotech, Rocky Hill, NJ, USA; cat. 100-20-100)

2.1.14 Primers

Lyophilized primers were dissolved in nuclease-free H_2O to achieve a stock solution of 100 µM. Then, further dilution to 10 mM was done with nuclease-free H_2O for primer aliquots that were stored at -20 °C. Primers were either sourced from GeneWorks, Adelaide, SA, Australia or Sigma Aldrich-Sigma, St. Louis, MO, USA. Table 2.4 shows the primers used in standard and real-time PCR reactions.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Cell isolation

2.2.1.1.1 Human retinal endothelial cells

The human retinal endothelial cell line was produced by our laboratory, through an immortalization process using LXSN16E6E7 (kindly provided by Dr Denise A. Galloway, Fred Hutchinson Cancer Institute Seattle, WA, USA), an amphotropic retrovirus that encodes the papilloma virus E6 and E7.[608] Characterization was based on microscopic evaluation of the expected cobblestone pattern, CD31 and von Willebrand factor expression, and development of a capillary-like net on a Matrigel (BD Biosciences Discovery Labware, Franklin Lakes, NJ, USA). Further description of the method is detailed by Bharadwaj et al.[38] The human retinal endothelial cell line was cultured in MCDB-131 medium, supplemented with 10% FBS and endothelial growth factors at 37 °C and 5% CO_2 in air.

For primary cell acquisition, cadaveric human eyes were obtained from the Eye Bank of South Australia (Southern Adelaide Clinical Human Research Ethics Committee – protocol number: 175.13; date of latest approval: March 1, 2021). Retinal endothelial cells were obtained through dissection of the retinae from the posterior cups of the eye and digestion with collagenase type II 0.25 to 1 mg/mL. During the first seven days, cells were cultured in MDCB-131 medium, with 2% FBS and growth factors (EGM-2 SingleQuots supplement, hydrocortisone and gentamicin; Clonetics-Lonza). Cells were purified over Dynal magnetic beads (Thermo Fisher Scientific-Invitrogen, Oslo, Norway) coated with anti-CD31 mouse monoclonal antibody (BD Biosciences

Table 2.4. List of primer pairs used for standard PCR and real-time quantitative PCR

Gene transcript	Manufacturer	GenBank ID	Primer set	Sequence	Product size (bp)
ALAS1[609]	Sigma-Aldrich	NM_000688.6	Forward	5'-GGCAGCACAGATGAATCAGA-3'	150
			Reverse	5'-CCTCCATCGGTTTTCACACT-3'	
CTNBB1[610]	GeneWorks	NM_001098209.2	Forward	5'-AAAGCGGCTGTTAGTCACTGG-3'	132
			Reverse	5'-GACTTGGGAGGTATCCACATCC-3'	
B2M[611]	GeneWorks	NM_004048.4	Forward	5'-ACTGAATTCACCCCCACTGA-3'	114
			Reverse	5'-CCTCCATGATGCTGCTTACA-3'	
Claudin-5[610]	GeneWorks	NM_001130861.1	Forward	5'-GCAGCCCCTGTGAAGATTGA-3'	106
			Reverse	5'-GTCTCTGGCAAAAAGCGGTG-3'	
ETS1[612]	Sigma-Aldrich	NM_001143820.2	Forward	5'-GTGCTGACCTCAATAAGGA-3'	134
			Reverse	5'-GCTGATAAAAGACTGACAGGAT-3'	
GAS5[613]	Sigma-Aldrich	NR_002578.3	Forward	5'-TCTTGCCTCACCCAAGCTAGAG-3'	127
			Reverse	5'-TTGTGCCATGAGACTCCATCAG-3'	
ICAM-1[614]	Sigma-Aldrich	NM_000201.3	Forward	5'-TAAGCCAAGAGGAAGGAGCA-3'	282
			Reverse	5'-CATATCATCAAGGGTTGGGG-3'	

Gene transcript	Manufacturer	GenBank ID	Primer set	Sequence	Product size (bp)
IL-6R[615]	GeneWorks	NM_000565.4 NM_181359.3 NM_001206866.2 NM_001382769.1 NM_001382770.1 NM_001382771.1 NM_001382772.1 NM_001382773.1 NM_001382774.1	Forward	5'-GAGGGAGACAGCTCTTTCTAC-3'	240
			Reverse	5'-CCGTTCAGCCCGATATCTGAG–3'	
JAM3[610]	GeneWorks	NM_032801.5	Forward	5'-CCCTGTCTGTAGAGTGCCGAAG-3'	258
			Reverse	5'-GAGCCTGCGTCATTGGAAGC-3'	
KCNQ1OT1[613]*	Sigma-Aldrich	NR_002728.3	Forward	5'-AATATGGATTCCTAACTGAGCCC-3'	567
			Reverse	5'-TGCCTTCTGCCAACACTTGGC-3'	
KCNQ1OT1[616]	Sigma-Aldrich	NR_002728.3	Forward	5'-CCTCCCTCACTGAGCTTTGG-3'	352
			Reverse	5'-GTGCGGACCCTATACGGAAG-3'	
LINC00294[617]	Sigma-Aldrich	NR_015451	Forward	5'-TGTGTTGTCCTCCAGAATCG-3'	225
			Reverse	5'-CCAACCAAGAGCCAACAAAG-3'	

Gene transcript	Manufacturer	GenBank ID	Primer set	Sequence	Product size (bp)
MALAT1[618]*	Sigma-Aldrich	NR_002819.4	Forward	5'-ATTCCGGTGATGCGAGTTGT-3'	396
			Reverse	5'-ATTCGGGGCTCTGTAGTCCT-3'	
MALAT1[491]	Sigma-Aldrich	NR_002819.4	Forward	5'-AAGCAAGGTCTCCCCACAAG-3'	170
			Reverse	5'-AACCCACCAAAGACCTCGAC-3'	
MEG3[613]	Sigma-Aldrich	NR_002766.2	Forward	5'-GAGTGTTTCCCTCCCCAAGG-3'	187
			Reverse	5'-GCGTGCCTTTGGTGATTCAG-3'	
mIL-6R[289]	GeneWorks	NM_000565.4 NM_001382769.1 NM_001382770.1 NM_001382771.1 NM_001382772.1 NM_001382774.1	Forward	5'-CTCCTCTGCATTGCCATTGT-3'	202
			Reverse	5'-TGTGGCTCGAGGTATTGTCA-3'	
MIR155HG[619]	Sigma-Aldrich	NR_001458.3	Forward	5'-GTCCCAAATCTAGGTTCAAGTTCA-3'	149
			Reverse	5'-TCTCTATCTCATCTAAGCCTCACAA-3'	
Mycoplasma[620]	Sigma-Aldrich	EU596508.1	Forward	5'-GGGAGCAAACAGGATTAGATACCCT-3'	269
			Reverse	5'-TGCACCATCTGTCACTCTGTTAACCTG-3'	

Gene transcript	Manufacturer	GenBank ID	Primer set	Sequence	Product size (bp)
NEAT1[618]	Sigma-Aldrich	NR_131012.1	Forward	5'-CTTGGCACTGGTACTGGGAG-3'	137
			Reverse	5'-ACCCACGCACTAAATTCCCC-3'	
NORAD[621]	Sigma-Aldrich	NR_027451	Forward	5'-CCTGGAAGGTGAGCGAAGT-3'	157
			Reverse	5'-AGAGGGTGGTGGGCATTT-3'	
OIP5-AS1[622]	Sigma-Aldrich	NR_026757.2	Forward	5'-TACTCAGATGGACCAGGAT-3'	198
			Reverse	5'-TTACGGGACATAACAAGG-3'	
PPIA[618]	Sigma-Aldrich	NM_021130.5	Forward	5'-GAGCACTGGAGAGAAAGGATTT-3'	355
			Reverse	5'-GGTGATCTTCTTGCTGGTCTT-3'	
RPLP0[618]	Sigma-Aldrich	NM_001002.4	Forward	5'-GCAGCATCTACAACCCTGAA-3'	235
			Reverse	5'-GCAGATGGATCAGCCAAGAA-3'	
SENCR[623]	Sigma-Aldrich	NR_038908	Forward	5-GCGCATTGTTAGGAGAAGGG-3'	240
			Reverse	5-CCTGCTGACTGTCCTAGAGG-3'	
sIL-6R[289]	GeneWorks	NM_181359.3 NM_001382773.1	Forward	5'-CGACAAGCCTCCCAGGTTCA-3'	195
			Reverse	5'-CGGTTGTGGCTCGAGGTATT-3'	

Gene transcript	Manufacturer	GenBank ID	Primer set	Sequence	Product size (bp)
TUG1[624]	Sigma-Aldrich	NR_110492.1	Forward	5'-CTGAAGAAAGGCAACATC-3'	140
			Reverse	5'-GTAGGCTACTACAGGATTTG-3'	
VE-cadherin	GeneWorks	NM_001795.5	Forward	5'-CTTCACCCAGAGCAAGTACACA-3'	281
			Reverse	5'-GGCTCATGTATCGGAGGTCG-3'	
YWHAZ[625]	GeneWorks	NM_001135699.2	Forward	5'-ACTTTTGGTACATTGTGGCTTCAA-3'	94
			Reverse	5'-CCGCCAGGACAAACCAGTAT-3'	
ZO-1[610]	GeneWorks	NM_001301025.3	Forward	5'-GAACGAGGCATCATCCCTAA-3'	218
			Reverse	5'-CCAGCTTCTCGAAGAACCAC-3'	

Abbreviations: ALAS1 = 5'-aminolevulinate synthase 1; bp = base pairs; B2M = beta-2-microglobulin; CTNBB1 = β-catenin; ETS1 = ETS proto-oncogene 1; GAS5 = growth arrest specific 5; ICAM-1 = intercellular adhesion molecule-1; IL-6R = transmembrane and soluble interleukin-6 receptors; JAM3 = junctional adhesion molecule 3; KCNQ1OT1 = potassium voltage-gated channel subfamily Q member opposite strand/antisense transcript 1; LINC00294 = long intergenic non-protein coding RNA 00294; MALAT1 = metastasis associated lung adenocarcinoma transcript 1; MEG3 = maternally expressed 3; MIR155HG = microRNA 155 host gene; mIL-6R = transmembrane interleukin-6 receptor or interleukin-6 receptor α; NEAT1 = nuclear paraspeckle assembly transcript 1; NORAD = non-coding RNA activated by DNA damage; OIP5-AS1 = opa interacting protein 5 antisense RNA 1; PPIA = peptidylprolyl isomerase A; RPLP0 = ribosomal protein lateral stalk subunit P0; SENCR = smooth muscle and endothelial cell enriched migration/differentiation-associated lncRNA; sIL-6R = soluble interleukin-6 receptor; TUG1 = taurine up-regulated 1; VE-cadherin = vascular endothelial cadherin; YWHAZ = tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; ZO-1 = zona occludens-1. *primer pair only used for standard PCR. Where not indicated, primer pair was used for standard and real-time quantitative PCR or real-time quantitative PCR only.

Pharmingen) and were cultured at 37 °C in 5% CO_2 in air in MCDB-131 medium with 10% FBS.[38]

2.2.1.1.2 THP-1 cell line

A monocyte cell line derived from the peripheral blood of a patient with acute monocytic leukaemia,[626] THP-1, was cultured with RPMI 1640, 10% FBS, and 2-ME 0.05 mM in 75 cm^2 tissue culture flasks. Cultures were maintained by addition of fresh medium. Sub-cultures were made when cell concentration reached around 800,000 cells/mL.

2.2.1.2 Cell culture maintenance and sub-culture

Cells were checked daily under the microscope. Every 48-72 hours, cells were washed once with PBS and the medium was replenished. When fully confluent, the cell monolayer was dissociated by incubation with trypsin 0.05% for 5 minutes at 37 °C and 5% CO_2. Then, cells were checked under the microscope to confirm intercellular dissociation, pre-warmed fresh medium at 37 ºC was added to quench trypsinization, and cells were aspirated to a 15 mL tube and centrifuged at 280 x *g* for 5 minutes. Supernatants were discarded and cells were resuspended in warm fresh medium and passaged to a new sterile cell culture dish or flask. For THP-1 cells, medium with floating THP-1 cells was aspirated into a new 75 cm^2 tissue culture flasks and cells were incubated at 37 °C in 5% CO_2 in air.

2.2.1.3 Cell counting

A volume of 10 µL of resuspended cells was added to a 0.2 mL microcentrifuge tube containing 10 µL of 0.4% trypan blue and mixed by pipetting. Then, 10 µL of the mixture was added to a counting chamber, counted by the TC20™ Automated Cell Counter twice, and the counts averaged.

2.2.1.4 Freezing cells

The cell monolayer was dissociated by trypsinization for 5 minutes at 37 °C in 5% CO_2 in air. Cells were checked under the microscope to confirm dissociation and pre-warmed medium (37 ºC) was added to quench trypsinization and cells were aspirated to a 15 mL tube and centrifuged at 280 x *g* for 5 minutes. Supernatant was discarded and cells were resuspended in cold medium (4 ºC) containing DMSO 10% vol/vol, added to cryogenic storage vials and placed into a freezing container

containing isopropanol, previously cooled to 4 ºC. The container was left overnight in a -80 ºC freezer, after which the cryogenic storage vials were transferred to the liquid nitrogen tank.

2.2.1.5 Raising cells

Cryogenic storage vials were withdrawn from the nitrogen tank, transported on ice, and placed for 1 minute in a warm bead bath, doused with 70% ethanol, dried, and cells were transferred to a 15 mL tube containing 9 mL of pre-warmed medium at 37 ºC. Cells were centrifuged at 280 x *g* for 5 minutes, the supernatant was discarded, cells were resuspended in fresh medium and transferred to a sterile cell culture dish. Cells were then checked under the microscope to assess even distribution in the dish and placed inside the incubator at 37 ºC in 5% CO_2 in air.

2.2.1.6 Mycoplasma testing

Mycoplasma is a frequent contaminant found in cell culture.[627] Testing for *Mycoplasma* cell culture contamination by qPCR was performed when freezing cells, after primary cells isolation and thereafter on a weekly or biweekly basis.

Genomic DNA isolation was performed using PureLink Genomic DNA Mini Kit, according to manufacturer's guidelines. Two hundred µL of each sample was loaded into a 1.7 mL tube, with further addition of 20 µL of Proteinase K and 20 µL of RNAse A, rocked and incubated at room temperature for 2 minutes. An aliquot of 200 µL of Genomic Lysis Buffer was added, rocked, and incubated for 10 minutes at 55 ºC on the Corning LSE digital dry bath. A volume of 200 µL of 100% ethanol was added to each sample, rocked, transferred to a spin column which was placed into a new tube, and centrifuged for 1 minute at 10,000 x *g*. The flow-through was discarded and the spin column was transferred to a new collection tube. Five hundred µL of the Wash Buffer was loaded into the spin column, centrifuged for 1 minute at 10,000 x *g*, the flow-through was discarded and the column was transferred to a new collection tube. An additional 500 µL of the Wash Buffer was loaded into the spin column and centrifuged for 3 minutes at 10,000 x *g*. The column was transferred to a new 1.7 mL tube, 20 µL of Elution Solution was added to the spin column, incubated for 1 minute at room temperature, and centrifuged for 1 minute at 10,000 x *g*, resulting in the genomic DNA.

Preparation of PCR reactions was performed in a UV-decontaminated BioAir Aura PCR cabinet, with each PCR reaction containing:

- 2 µL of genomic DNA (diluted 1:10 in nuclease-free H_2O) or nuclease-free H_2O only in the case of the no-template control
- 4 µL of Sso Advanced universal SYBR Green Supermix
- 375 µM of forward and reverse *Mycoplasma* primers at 10 µM
- 11 µL of nuclease-free H_2O

Triplicates of each sample were loaded into the wells of the PCR plate, as well as one positive and one negative control sample. Plates were sealed, placed in the Allegra X-12R Centrifuge and centrifuged for 1 minute at 280 x *g*. The PCR plate was inserted into the Bio-Rad CFX Connect Real-Time PCR Detection System Thermocycler and qPCR was run by CFX Manager version 3.1.

The qPCR protocol consisted of:

1. Amplification: pre-cycling for 5 minutes at 95 °C, denaturation in 40 cycles at 94 °C for 30 seconds.
2. Annealing: 60 seconds at 60 °C
3. Extension: 30 seconds at 72 °C
4. Post-extension: 1 second at 72 °C
5. Melt-curve: melting curve was generated by a 1-second hold at 0.5 ºC increments from 70 ºC and 95 ºC.

If cells were contaminated by *Mycoplasma*, they were discarded.

2.2.2 Stimulation of cells

Human retinal endothelial cells were stimulated with specific molecules at different time points.

Retinal endothelial cells were seeded at 200,000 cells/well in 12-well plates and grown to confluence. Vascular endothelial growth factor (VEGF-A isoform 165), cytokines (CCL2, IL-1β, IL-6, IL-8, IL-17, IL-22, TNF-α and IFN-γ), and LPS were diluted in medium and used to stimulate the cells, while medium was used as the negative control. Working concentrations of cytokines and growth factor are seen in Table 2.5. After stimulation, cells were lyzed with RNA lysis buffer (GenElute Mammalian Total RNA Extraction Miniprep Kit) and frozen at -80 °C.

2.2.3 RNA extraction

GenElute Mammalian Total RNA Extraction Miniprep Kit was used for RNA extraction from the lyzed cells, following the manufacturer's guidelines. Lyzed cells were scraped and aspirated from the plates and pipetted into individual filtration columns for each well and centrifuged at 14,000 x *g* for 2 minutes. The filtration column was then discarded, with the filtrate remaining in the collecting tubes. An equal volume of 70% ethanol solution was added and mixed with the filtered lysate. The lysate/ethanol mixture was transferred to an individual binding column and centrifuged for 15 seconds at 14,000 x *g*. The flow-through was discarded and the lysate/ethanol mixture was loaded into a new binding column, where 250 µL of Washing Solution I from the kit was added and centrifuged for 15 seconds at 14,000 x *g*. An aliquot of 80 µL of the DNase/Digest Buffer mixture was pipetted into the binding column and incubated for 15 minutes at room temperature. A volume of 250 µL of the Washing Solution I was added and centrifuged for 15 seconds at 14,000 x *g*. Each binding column was transferred to a new 2 mL collection tube, the flow-through was discarded, and 500 µL of Washing Solution II containing ethanol was added into the binding column and centrifuged for 2 minutes at 14,000 x *g*. Subsequently, the binding column was transferred to a new 2 mL collection tube, and 50 µL of Elution Solution was loaded into the binding column and centrifuged for 1 minute at 14,000 x *g*. The flow-through after centrifugation was the purified RNA that was stored at -80 °C.

2.2.4 Complementary DNA synthesis

Prior to complementary DNA (cDNA) synthesis, the RNA concentration of each sample was verified by spectrophotometry with Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Fisher

Table 2.5. Stimulatory molecules, doses, manufacturers, and references to active concentrations in the literature

Molecule (concentration)	Manufacturer	Reference
IL-1β (10 ng/mL)	R&D Systems	Yun et al.[138], Kowluru et al.[254], Martinez-Fabregas et al.[628], and Ramírez-Pérez et al.[629]
IL-6 (20 ng/mL)	Peprotech	Li et al.[205], Chruewkamlow et al.[630] and Liu et al.[631]
IL-8 (50 ng/mL)	Peprotech	Dwyer et al.[355], Cockx et al.[632] and Kremer et al.[633]
IL-17 (100 ng/mL)	Peprotech	Maertzdorf et al.[634] and von Palffy et al.[635]
IL-22 (100 ng/mL)	R&D Systems	Shang et al.[636]
IFN-γ (10 ng/mL)	R&D Systems	Lopez-Ramirez et al.[637]
CCL2 (100 ng/mL)	Peprotech	Yao et al.[638] and Hu et al.[639]
TNF-α (10 ng/mL)	R&D Systems	Li et al.[207] and Bharadwaj et al.[640]
LPS (10 μg/mL)	Merck-Sigma Aldrich	You et al.[641]
VEGF-A 165 (100 ng/mL)	Peprotech	Petreaca et al.[358], Huang et al.[642], and Park et al.[643]

Abbreviations: IL = interleukin; IFN-γ = interferon-γ; CCL2 = C-C motif chemokine ligand 2; TNF-α = tumour necrosis factor-α; LPS = lipopolysaccharide; VEGF-A = vascular endothelial growth factor-A.

Scientific Inc., Wilmington, DE, USA). This process used 2 µL drops of RNA. For the cDNA synthesis, the input of RNA template for retinal endothelial cells was 100 or 200 ng/µL. Variable volumes of nuclease-free H_2O and RNA template were loaded into 0.2 mL tubes with 4 µL of iScript Reverse Transcription Supermix and 16 µL of nuclease-free H_2O for RT-PCR, achieving a total volume of 20 µL for each tube (2 tubes for each sample). The tubes were centrifuged and loaded into the CFX Connect Real-Time PCR Detection System Thermocycler, using a reaction protocol as follows: priming – 5 minutes at 25 °C; reverse transcription – 20 minutes at 46 °C; reverse transcription inactivation – 1 min at 95 °C. Later, the pair of tubes from each sample was combined into one and the cDNA was diluted in 1:10 with nuclease-free H_2O in a separate 0.65 mL tube. Neat cDNA was used for a set of RT-qPCR reactions, where appropriately indicated.

2.2.5 Standard polymerase chain reaction

For IL-6R, mIL-6R and sIL-6R primer sets, singlet PCR reactions for each sample comprised of nuclease-free H_2O and 1X PCR buffer, plus 0.4 mM dNTP mix, 2 mM $MgCl_2$, 0.8 µM forward and reverse primers, 0.625 unit of Taq DNA polymerase and 1.5 µL of cDNA template, in a total volume of 25 µL. For IL-6R and mIL-6R primer sets, the standard PCR protocol consisted of two phases: amplification: pre-cycling for 5 min at 95 °C for 5 minutes, denaturation for 10 cycles of 30 seconds at 95 °C, annealing: 30 seconds at 66 °C (decreasing by 1 °C per cycle); extension: 1.5 minute at 72 °C; followed by 30 cycles of denaturation for 30 seconds at 95 °C, annealing: 30 seconds at 56 °C; extension: 1.5 minute at 72 °C; post-extension: 5 minutes at 72 °C for IL-6R and mIL-6R primers. For sIL-6R primer set, cycling conditions were as the following: 5-minute pre-amplification hold at 95 °C, followed by 40 cycles of denaturation for 30 seconds at 95 °C; annealing for 30 seconds at 62 °C; extension for 60 seconds at 72 °C; and a post-amplification hold of 72 °C for 5 minutes. The product sizes of the PCR reactions were confirmed by 2% agarose gel electrophoresis. Peptidylprolyl isomerase A (PPIA) was used as the reference gene.

For lncRNA primer sets, each PCR reaction contained 0.2 mM dNTP mix, 2.0 mM $MgCl_2$, 0.6 mM each of forward and reverse primer, 1 unit of HotStarTaq Plus DNA polymerase and 1.5 µL of cDNA template, in a total volume of 25 µL. Cycling conditions were as follows: 5-minute pre-amplification

hold at 95 °C, followed by 40 cycles of denaturation for 30 seconds at 95 °C; annealing for 30 seconds at up to 63.3 °C; extension for 60 seconds at 72 °C; and a post-amplification hold of 72 °C for 5 minutes. The product sizes from the PCR reactions were confirmed by 2% agarose gel electrophoresis. PPIA or tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) were used as reference genes.

2.2.6 Agarose gel electrophoresis

The separation of DNA occurred by electrophoresis in a 2% agarose gel. To make the gel, 2 g of agarose was added to 100 mL of TAE buffer and dissolved by heating, followed by addition and mixing of 10 µL of SYBR Safe DNA gel stain. The mixture was poured into an acrylic tray (Bio-Rad) containing a 15 or 20-well comb and left to cool until room temperature before being inserted inside the Bio-Rad Wide Mini-Sub Cell GT Cell containing TAE buffer covering the surface of the gel. Gel loading dye was mixed with neat DNA in a 1:6 proportion and loaded into the wells of the gel, while 6 µL of the DNA molecular weight 100 bp ladder was added to adjacent wells. The gel was run for 25-35 minutes at 100 V, to allow migration of the dye to the bottom of the gel. Gel photographs were taken with a Canon DS126181 digital camera inside a black box on the Safe Imager 2.0 Blue-Light Transilluminator with a 470 nm filter. Photographs were processed by Canon EOS Utility version 2.14 and Canon Digital Photo Professional version 3.14 software.

2.2.7 DNA extraction from agarose gel

DNA bands from the agarose gel were visualized under the Safe Imager 2.0 Blue-Light Transilluminator with a 470 nm filter, excised using individualized razor blades for each product, loaded into 2 mL tubes and weighed. DNA extraction was performed using the GenElute Gel Extraction Kit according to the manufacturer's instructions. For gel solubilization, three gel volumes of the Gel Solubilization Solution were added to each tube and incubated at 55 °C for 10 minutes with alternating vortexing. While incubating the gels, the binding columns were prepared by addition of 500 µL of Column Preparation Solution to each column, centrifugation at 16,000 x *g* for 1 minute and discarding of the flow-through. In the gel-containing tubes, one gel volume of 100% isopropanol was added and mixed thoroughly. The solubilized solution was then loaded into the binding column

in 700 µL aliquots, centrifuged at 16,000 x *g* at room temperature for 1 minute, and the flow-through was discarded. Two washes by addition of 700 µL of Wash Solution to each tube and centrifugation at 16,000 x *g* for 1 minute at room temperature were done to confirm complete removal of ethanol. Then, the binding column was relocated to a new collection tube, and 30 µL of Elution Solution was loaded into the centre of the binding column membrane and incubated for 1 minute. Finally, the column was centrifuged for 1 minute at 16,000 x *g* and the eluted material was stored at -20 °C or sent immediately for genetic sequencing.

When DNA extraction was obtained for sequencing purposes, before the addition of the Wash Solution, a second addition of three gel volumes of the Gel Solubilization Solution was loaded into each tube and centrifuged for 1 minute at 16,000 x *g*, at room temperature, before proceeding to the washing column step.

2.2.8 Real-time quantitative polymerase chain reaction

In addition to nuclease-free H_2O, each qPCR reaction contained 4 µL of SsoAdvanced Universal SYBR Green Supermix, 375 µM of each of forward and reverse primer, and 2 µL of cDNA template. The qPCR protocol was the following: amplification – pre-cycling for 5 minutes at 95 °C, denaturation in 40 cycles at 95 °C for 30 seconds; annealing – 30 seconds at 60 °C (or 61.4 °C for LINC00294, TUG1, NORAD, OIP5-AS1, SENCR; and 20 seconds at 62 °C for sIL-6R); extension – 30 seconds at 72 °C; post-extension – 1 second at 75 °C. Each primer set generated an individual melt curve fluorescent peak by a 1-second hold at each 0.5 °C between 70 °C and 95 °C. The quantification cycle (Cq) was obtained using regression mode. The relative expression was assessed and normalized against stable reference genes as defined by Pfaffl et al.[644] A combination of two reference genes was used for normalization, depending on the experiment, including: ribosomal protein lateral stalk subunit P0 (RPLP0), PPIA, beta-2-microglobulin (B2M), 5'-aminolevulinate synthase 1 (ALAS1) and YWHAZ. Coefficient of variance (CV) of qPCR ranged from 0.11 to 0.246, whereas expression stability measurement (M) values varied from 0.34 to 0.578. Primer set efficiency was determined by standard curves, produced with serial dilution of the product, yielding an efficiency of 84.4% or greater; with the exception of the ZO-1 primer set, which reached an

efficiency of 73.5%. Product size was confirmed by 2% agarose gel electrophoresis.

2.2.9 Genetic Sequencing

Genetic sequencing from purified IL-6R, mIL-6R and sIL-6R PCR products was performed in the Flinders Sequencing Facility in SA Pathology, Adelaide, Australia. The basic local alignment search tool (BLAST) from the National Center for Biotechnology Information (NCBI)[645] was used to confirm alignment with consensus sequences.

2.2.10 Immunocytochemistry

The presence of ZO-1 in human retinal endothelial cells was investigated via immunolabelling at different time points. First, human retinal endothelial cells were seeded at a density of 12,000 cells/well in 96-well plates and left in the incubator for 24 hours (n = 2 per condition). Subsequently, cells were treated with fresh medium only or cytokine – IL-1β (10 ng/mL), IL-6 (20 ng/mL), TNF-α (10 ng/mL) – diluted in fresh medium for 24 and 48 hours. Then, cells were fixed with 4% PFA in PBS for 10 minutes, followed by removal and washing with PBS twice. A blocking solution containing 2% normal goat serum, 0.05% Triton X-100 and PBS was made and a volume of 100 µL was added to each well for 30 minutes. Rabbit anti-human ZO-1 diluted to 2.5 µg/mL in the blocking solution was added to half of the wells and incubated for 2 hours. At the same time, the other half of the wells was treated with rabbit IgG diluted to 2.5 µg/mL. After 2 hours, all wells were washed twice with PBS, and goat anti-rabbit Alexa Fluor 488 diluted to 5 µg/mL in the blocking solution was added to the wells, followed by incubation in the dark for 1 hour. Subsequently, wells were washed twice with PBS and cells were treated with DAPI 300 nM and incubated in the dark for 5 minutes. Then, Fluoromount mounting media was added and cells were photographed with Olympus IX53 Inverted Microscope using appropriate fluorescence modes with the same magnification (400x) and exposure as controls. Images were saved in tagged file image format (TIFF) and were merged using Adobe Photoshop Creative Cloud version 20.0.6 using matched adjustments. Colour saturation of the photographs differed between 24- and 48-hour experiments because experiments were independently conducted, resulting in less saturated DAPI labelling and more saturated ZO-1 labelling in the 48-hour treatment experiment.

2.2.11 Electrical impedance assay

Real-time changes in transcellular electrical resistance were evaluated using the iCELLigence system. The iCELLigence is a biosensor that measures real-time transcellular electrical impedance *in vitro* via gold electrode arrays of individual electronic plates (E-plates), providing non-invasive and label-free impedance monitoring. Cellular adhesion depends on several factors, such as cell type and viability, migration, spreading, growth and proliferation. The impedance values measured by the iCELLigence instrument are transformed by the iCELLigence software into the cell index, a unit-less value, calculated as (Rn-Rb)/15, where Rn is the impedance measurement of the well when cells are present and Rb is the background measurement calculated for medium only (without cells) at any specific time point before cell addition.[646] The higher the number of cells that adhere to the bottom of the well, the higher the impedance and cell index. The degree of cell-cell and cell-plate adhesion also affects the impedance values; therefore, the cell index reflects cell number, capacity for adhesion, morphology and viability.

First, a resistor plate verification was conducted to verify the connection status of the iCELLigence instrument with the E-plates. Then, 150 µL of medium was added to each well of the E-plate and left for 30 minutes at 37 °C in 5% CO_2 in air before E-plate insertion into the iCELLigence instrument for background measurement. Following the background measurement, retinal endothelial cells were seeded into the plate (well area of 64 mm^2) at a concentration 50,000 cells per well in 500 µL of medium. Cells were allowed to settle for 30 minutes before the plates were inserted into the cradle of the instrument, which was kept inside the incubator, and monitored hourly for 24 hours. Then, 250 or 500 µL of medium was carefully aspirated and 250 or 500 µL of fresh medium containing cytokine or VEGF-A (to obtain a final concentration of TNF-α 10 ng/mL, IL-1β 10 ng/mL, IL-6 20 ng/mL, IL-8 50 ng/mL, IL-17 100 ng/mL, CCL2 100 ng/mL, VEGF-A 100 ng/mL), or fresh medium for controls, was added to the wells. After treatment or medium addition, the cell index was assessed hourly for further 72 hours, with no additional manipulation.

The iCELLigence E-plates were reused, following a protocol reported by a publication demonstrating reproducible results after E-plate reuse.[647] At the conclusion of the experiment, the content of each

well was aspirated and the E-plate was washed twice with PBS. Then, 100 µL of 0.05% trypsin was added to each well and the E-plate was left inside the incubator for 5 minutes. Trypsin solution was aspirated, wells were washed twice with PBS and each well was examined at the microscope to confirm the removal of cells. Wells were then washed twice with sterile nuclease-free H_2O, the absence of cells was confirmed under the microscope and the E-plates were left to dry inside the tissue culture hood. At the beginning of a new experiment with a reused E-plate, the cell index measured during the background check was expected to be zero, confirming the removal of cells.

2.2.12 Cell survival assay

Cell survival was assessed using CyQuant NF Cell Proliferation Assay Kit via a cell-permeant DNA-binding dye associated with a plasma membrane permeabilization reagent. Human retinal endothelial cells were plated at different densities (3,000 and 5,000 cells/well) in 100 µL/well of medium in 96-well plates and left inside the incubator for 24 hours (6 wells/density/condition). Then, cells were treated either with TNF-α (10 ng/mL), IL-1β (10 ng/mL), IL-6 (20 ng/mL), IL-17 (100 ng/mL) or fresh medium for 48 hours. Subsequently, culture medium was aspirated and 50 µL of dye-binding solution was added to each well. After 60 minutes of incubation, fluorescence was measured in relative fluorescence units (RFU) in the microplate reader VICTOR X3, at excitation and detection wavelengths of 485 nm and 535 nm, respectively. Fluorescence of empty wells was also obtained and averaged, with subsequent subtraction of the mean value of empty wells from each well containing the samples.

Additionally, 5,000 cells/well were plated in 96-well plates (6 wells/condition) and titrated doses of TNF-α (0.1 ng/mL; 0.5 ng/mL; 1 ng/mL; 5 ng/mL; 10 ng/mL) and IL-1β (0.1 ng/mL; 0.5 ng/mL; 1 ng/mL; 5 ng/mL; 10 ng/mL) or fresh medium were used to treat human retinal endothelial cells at 24 hours post-plating. After 48 hours, the impact of different dosing in cell proliferation was assessed via fluorescence measurement.

2.2.13 Flow cytometry

2.2.13.1 Necrosis and apoptosis assays

Fluorescein isothiocyanate-conjugated annexin V and propidium iodide were used to evaluate

apoptosis and necrosis of human retinal endothelial cells treated with TNF-α (10 ng/mL), IL-1β (10 ng/mL), IL-6 (20 ng/mL) or IL-17 (100 ng/mL). One well for each condition was seeded with 200,000 cells in a 12-well plate and placed in the incubator for 24 hours. Then, wells were replenished with TNF-α, IL-1β, IL-6 or IL-17 for treatment samples and fresh medium for controls. Each experiment was replicated independently at least three times. After 24 and 48 hours of treatment, cells were incubated with trypsin 0.05% for 5 minutes at 37 °C in 5% CO_2 in air. Cells were then harvested and centrifuged with fresh medium at 280 x *g* for 5 minutes. Supernatant was discarded and 1X Annexin V Buffer (previously diluted in deionized H_2O from the 10X Annexin Binding Buffer) was added and centrifuged again at 280 x *g* for 5 minutes. The supernatant was discarded and 3 µL of FITC-Annexin V was added to the cells and incubated for 15 minutes in the dark. Subsequently, another wash with 1X Annexin V Buffer was performed by a new centrifugation at 280 x *g* for 5 minutes. Supernatant was then discarded, 200 µL of 1X Annexin V Buffer and 5 µL of propidium iodide was added. Flow cytometry was performed on the CytoFLEX S and analyzed with CytExpert software. The gating strategy analyzed single cells, which were sorted based on their side scatter area (SSC-A), forward scatter area (FSC-A) and forward scatter height (FSC-H) characteristics. Cells were classified as viable (propidium iodide and FITC-Annexin V negative), early apoptotic (propidium iodide negative, FITC-Annexin V positive), necrotic (propidium iodide positive and FITC-Annexin V negative) or late apoptotic (propidium iodide and FITC-Annexin V positive).

2.2.13.2 Interleukin-6 receptor immunolabeling assay

Human retinal endothelial cells were seeded in 12-well plates at a density of 200,000 cells/well in medium and incubated at 37 °C in 5% CO_2 in air. Medium was replenished after 24 hours. Forty-eight hours after seeding, cells were dissociated with trypsin 0.05% for 5 minutes at 37 °C in 5% CO_2 in air, transferred to a flow cytometry tube containing PBS with 1% FBS and centrifuged for 5 minutes at 280 x *g*. Then, cells were washed and centrifuged twice with PBS containing 1% FBS and 0.05% Triton X-100 (in the case of permeabilization) or omission of Triton X-100 in non-permeabilization conditions. After discarding the supernatant and resuspending the cells, APC-anti-CD126 antibody and FITC-anti-CD31 antibody were added to each tube, except for controls, and

incubated on ice in the dark for 30 minutes. Cells were fixed with PFA 4% for 5 minutes and subsequently washed and centrifuged for 5 minutes at 280 x *g* with PBS twice, before being resuspended in PBS and read with CytoFLEX S Flow cytometer and analyzed with FlowJo Software version 10.7.1.

In preliminary experiments, a monocyte cell line derived from the peripheral blood of a patient with acute monocytic leukaemia (THP-1 cells) was used for immunolabeling and flow cytometry as described above in human retinal endothelial cells, and also with different immunolabeling protocols consisting of changing permeabilization reagent (Triton X-100 or methanol), paraformaldehyde concentration (4% or 0.05% w/v), procedural step order (staining-fixation or fixation-staining) and skipping the fixation step, to optimize the procedure. After harvesting, cells were transferred to a flow cytometry tube with 3 mL of PBS and centrifuged for 5 minutes at 280 x *g* for 5 minutes. Then, cells were fixed with paraformaldehyde 4% for 5 minutes, washed and centrifuged twice with 3 mL of PBS containing 1% FBS with or without 0.05% Triton X-100. Following discarding the supernatant and resuspending the cells, 3 µL of APC-anti-CD126 antibody and 3 µL of FITC-anti-CD31 antibody were loaded into each tube (except for controls) and incubated on ice in the dark for 30 minutes. Then, cells were washed in 3 mL of PBS and centrifuged for 5 minutes at 280 x *g* for 5 minutes, and the supernatant was discarded and resuspended in 500 µL of PBS for flow cytometric analysis.

Alternatively, the same procedure was done using PFA 0.05% instead of 4% for 5 minutes in one set of experiments and also skipping the fixation step in another set of experiments. Methanol was included for permeabilization in one set of experiments, as follows: cells were harvested and washed with 3 mL of PBS by centrifuging at 280 x *g* for 5 minutes; cells were fixed with PFA 4% for 5 minutes and centrifuged twice with PBS containing 1% FBS with or without 0.05% Triton X-100; 1 mL of cold 100% methanol was added to the tubes which contained 0.05% Triton X-100, and incubated overnight at 4 °C. The next day, cells were washed and centrifuged twice with PBS containing 1% FBS at 280 x *g* for 5 minutes, the supernatant was discarded, and cells were resuspended; 3 µL of APC-anti-CD126 antibody and 3 µL of FITC-anti-CD31 antibody were added to each tube (except for controls) and incubated on ice in the dark for 30 minutes; subsequently, cells were washed in 3

mL of PBS, centrifuged at 280 x *g* for 5 minutes, the supernatant was discarded, the cells were resuspended in 500 µL of PBS and analyzed by flow cytometry.

2.2.14 Bioinformatics analyses

To identify potential transcription factors implicated in human IL-6R gene regulation in human retinal endothelial cells, the IL-6R transcript sequence was obtained from the NCBI "GenBank" database (Reference Sequence: NG_012087.1 on chromosome 1) in March 2021, and the regulatory sequence was established as the first exon plus 1000 bp of sequence immediately upstream the first exon. The sequence was inputted into the JASPAR database[648] – an open-access database of matrix-based nucleotide transcription factor binding profiles – to search for putative binding sites of human transcription factors, using a profile score threshold of 90%. The results were compared against a human retinal endothelial cell transcriptome published by Smith et al.[483] to search for transcription factors expressed by these cells. Transcription factors were ranked based on the number of putative binding sites provided by the JASPAR analysis and by the normalized average RNA counts per million reads.

2.2.15 Transfection of human retinal endothelial cells using small interfering RNA

Human retinal endothelial cells were seeded into a 12-well plate at a density of 125,000 cells/well in medium and incubated at 37 °C in 5% CO_2 in air to be transfected the following day after achieving 80% confluency. Cells from each well were transfected with 12 pmol of each siRNA (ETS1 siRNA 1, ETS1 siRNA 2 and non-targeted negative siRNA; n = 4 wells/condition) with 2 µL of lipofectamine RNAiMAX and Opti-MEM™ in a total volume of 200 µL/well on top of 1 mL of medium already present in each well. Before transfection, equal volumes of suspensions containing siRNA in Opti-MEM™ and lipofectamine in Opti-MEM™ were mixed and incubated for 5 minutes. Each condition consisted of 4 wells. After transfection, cells were incubated at 37 °C in 5% CO_2 in air for 24 hours, the medium was exchanged, and cells were incubated for an additional 24 hours. Upon the completion of a total transfection time of 48 hours, medium was aspirated, cells were washed with PBS, and lyzed with lysis solution (GenElute Mammalian Total RNA Extraction Miniprep Kit) containing 1% of 2-ME and stored at -80 °C for subsequent RNA extraction. Other conditions

consisted of addition of Opti-MEM™ and lipofectamine only, which were included as additional controls.

2.2.16 Statistical analyses

GraphPad Prism v6.04 (GraphPad Software, La Jolla, CA) was used for statistical analyses, using unpaired Student's t-tests when comparing two independent variables, or analysis of variance (ANOVA) when comparing three or more independent values. Correlations were measured using Pearson correlation coefficient (r). The significance value was set at 0.05.

CHAPTER 3: EFFECT OF INFLAMMATORY CYTOKINES ON ELECTRICAL IMPEDANCE OF HUMAN RETINAL ENDOTHELIAL CELL MONOLAYERS

3.1 Introduction

The posterior segment of the eye is protected from the free influx of cells, proteins and fluid by the inner and outer blood-retinal barriers, which are based around tightly interconnected retinal endothelial cells and retinal pigment epithelial cells, respectively.[36, 649] During non-infectious uveitis, breakdown of the inner blood-retinal barrier is associated with the release of numerous inflammatory mediators. These molecules include inflammatory cytokines, which have the potential to disrupt intercellular junctional complexes and to be cytotoxic.[25, 99, 109-126, 133] There is *in vivo* and *in vitro* evidence suggesting involvement of TNF-α,[115-122, 124, 126, 133, 205-210, 212-215] IL-1β,[99, 116, 117, 124, 126, 133, 205, 206, 211, 250-252, 254-258] IL-6,[125, 192, 193, 205, 209, 210, 284-287] IL-8,[194, 196, 197, 355-358] IL-17,[117, 199, 306, 308, 311-324, 336-340] and CCL2[194, 371, 385, 387-389] in the impairment of the inner blood-retinal barrier during inflammation.

The main methods for assessing the barrier function of cell monolayers include macromolecule permeability assays, which demonstrate solute flux across the monolayer, and transcellular electrical resistance assays, which measure the resistance to ionic flow by direct current or single frequency alternate current systems.[598, 599, 650-652] Some of the disadvantages of macromolecular permeability assays are their end-point nature, vulnerability to changes in cell-substratum contact and the possibility of cell toxicity related to the tracers.[598, 599] Macromolecular permeability assays and classical transcellular electrical resistance assays use transwells, which consist of culture inserts in which cells sit on a semi-permeable membrane separating two compartments.[599, 652] A more modern approach to transcellular electrical resistance analysis is the monitoring of electrical impedance, measured by alternating electrical currents throughout an array of frequencies, permitting label-free, real-time monitoring.[598, 653]

The iCELLigence instrument is one of the several biosensors that measure electrical impedance in real-time across cell monolayers seeded in multi-well plates using basal microelectrode arrays.[653-655] The iCELLigence assesses the well impedance at three different frequencies (10, 25 and 50 kHz), and combines these measurements to generate the cell index, a unit-less value adjusted against wells that do not contain cells. The cell index is associated with cell number, surface area, morphology and attachment.[598, 654] Cell adhesion depends on characteristics that include cell

type and viability, spreading and migration, as well as proliferation and stability of intercellular junctions.[656] Changes in the cell index are observed even after cells reach confluency, which indicate fluctuations in cell adhesion.[654] Outputs from the iCELLigence display good correspondence with results from standard transcellular electrical resistance assays.[601]

The aim of the research presented in this chapter was to assess the effect of the inflammatory cytokines, TNF-α, IL-1β, IL-6, IL-8, IL-17 and CCL2, on the electrical impedance of the human retinal endothelial cell monolayers by real-time electrical impedance monitoring in an iCELLigence. The work undertaken also addressed technical considerations related to the use of the system.

3.2 Results

3.2.1 Technical considerations for the measurement of electrical impedance of the human retinal endothelial cell monolayers

All experiments presented in this chapter made use of a human retinal endothelial cell line that was produced in the home laboratory and has been extensively characterized in previous publications.[38, 657, 658] In considering measurement of the electrical impedance of human retinal endothelial cell monolayers, concerns that first needed to be addressed were how the medium was exchanged in the E-plates, how to accomodate the edge effect, and how many times the E-plates could be used. These topics present methodological challenges when using electrical impedance biosensors, such as the iCELLigence. In evaluating the electrical impedance of treated cell monolayers and controls, only experiments with comparable cell indices between treated and control groups ($p \geq 0.05$) at the time point immediately prior to addition of a test compound or fresh medium, as applicable, were considered valid. This rule was established to prevent biased results due to significant differences between cell indices of different groups at baseline.

3.2.1.1 Manipulation of the medium in the biosensor plates

Initial experiments showed a plunge in the cell index of human retinal endothelial cell monolayers following aspiration and replenishment of the medium, likely attributed to the dislodgement of the cells within the wells. Figure 3.1A depicts an experiment with unstimulated human retinal endothelial cells, performed by exchanging the total volume of medium in the wells, showing the plunge in the

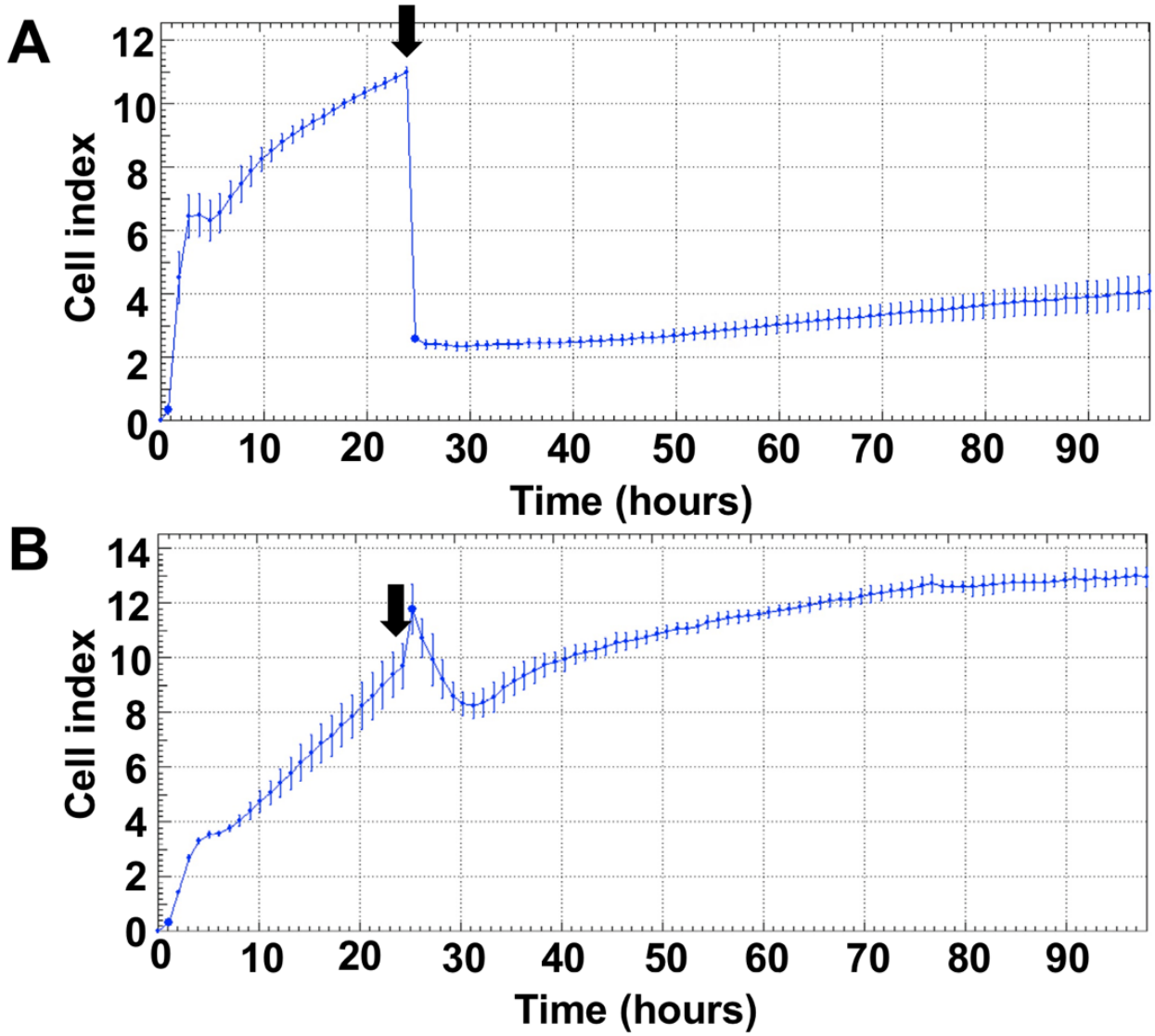


Figure 3.1. Effect of different manipulations of culture medium on the electrical impedance of human retinal endothelial cells

Plots of cell index of human retinal endothelial cell monolayers measured by iCELLigence wells in representative experiments involving (A) aspiration and replenishment of entire volume of medium in each well (B) aspiration and replenishment of one-half of the volume of medium in each well. Plots show measurements made hourly. Arrows indicate time of medium exchange. Error bars represent standard deviation for groups of at least 3 wells.

cell index, followed by partial recovery. The method was adjusted such that only half of the volume of each well was aspirated and replenished at one time, to limit disturbance of the cells. The outcome of this modified technique is observed in Figure 3.1B, where the sudden fall in cell index is no longer seen. The marked differences in the cell index traces between the two techniques could produce differences in experimental outcomes (data not shown). Therefore, standard practice for this work became replacement of half of the volume of medium in a well at any manipulation.

3.2.1.2 The edge effect

The edge effect is a term to characterize the altered behaviour of cells cultured in edge wells in relation to inner wells of a plate and is one of the challenges when working with cell culture. The edge effect results from increased evaporation from the edge wells of the plate in comparison with the inner wells, commonly reported in multi-well microplates and also recognized in real-time cell analysing techniques.[659, 660] The recommendation of the iCELLigence manufacturer is to incubate the E-plate at room temperature for 30 minutes after cell seeding before inserting the plate inside the incubator and starting the experiment. However, since human retinal endothelial cells are temperature-sensitive, leaving them at room temperature for 30 minutes could be deleterious. Therefore, it was decided to place the E-plate inside the incubator for 30 minutes immediately after seeding, ahead of placing it into the iCELLigence instrument and initializing the experiment. However, promptly inserting the cells in the incubator after seeding increased the chance of microcirculation inside the wells, with the possibility of uneven allocation of the cells across the wells, possibly resulting in a variation of the cell index in the edge wells when compared to the inner wells.[661]

The edge effect in these assays is demonstrated in Figure 3.2. Human retinal endothelial cells were seeded in one E-plate and after 24 hours the medium was refreshed. Individually, E-plates have 8 wells, separated as two groups of 4 wells each. The graphs in Figure 3.2 A and D show the cell index for each of two groups of four wells in the same E-plate. It was noted that the cell index traces of the two edge wells deviated from the rest of the group. When analysing the well group (Figure 3.2 B and E), the standard deviation for cell index was obviously smaller when the edge well was excluded

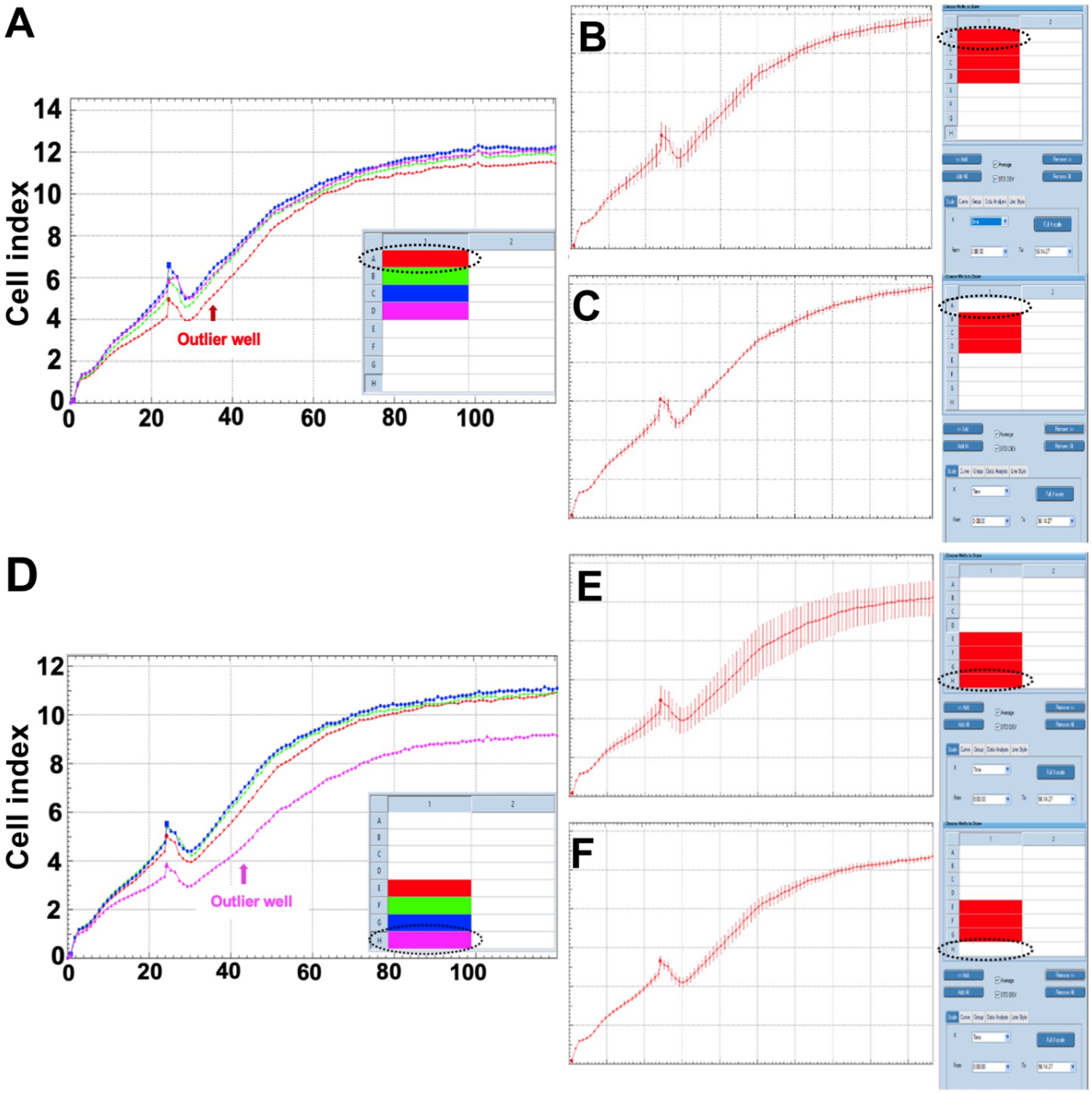


Figure 3.2. The edge effect in E-plates with human retinal endothelial cell monolayers

Plots of cell index of human retinal endothelial cell monolayers measured by iCELLigence wells in two 4-well groups (A-C and D-F) in the same E-plate. A and D show cell index for individual wells (red, green, blue and pink). B and E show mean cell index for four wells (including edge well) with standard deviation bars. C and F show mean cell index for three wells (excluding edge well) with standard deviation bars. Dashed circle indicates the edge wells.

(Figure 3.2 C and F).

In some experiments, an extreme deviation was observed in one well from one or both groups, mostly in edge wells. Hence, when an outlier value was present, the following tenet was applied to assess if the well would be excluded from the analysis: if one well within the group displayed a cell index value at the final time point that fell outside four standard deviation values of the group mean calculated without the outlier well, that well was discarded from the analysis, respecting a minimum of three wells per group per experiment. With this rule, 99.9% of the values within the Gaussian distribution were considered, allowing variation within the group, without including wells with extreme variation in the cell index, which could lead to erroneous results from an assay.

3.2.1.3 Reuse of the biosensor single-use plates

The iCELLigence manufacturer recommends that E-plates should be used once and discarded. However, a study by Stefanowicz-Hajduk et al.[647] demonstrated that iCELLigence E-plates could be used up to three times, achieving comparable results when analysing vinblastine sulphate treatment of human cervical adenocarcinoma cells and human breast adenocarcinoma cells. The reuse of E-plates seeded with human retinal endothelial cells was evaluated, adopting the protocol described by the investigators.[647] The protocol for preparing the E-plates for reuse included cell dissociation by trypsinization and copious washing, with removal of the cells confirmed by light microscopy at the end of this process, plus a null cell index at the beginning of each new experiment. Three E-plates were evaluated in five individual experiments conducted with human retinal endothelial cells, comparing the cell index of each experiment at approximately 24 hours after seeding, prior to treatment or medium exchange (Figure 3.3).

The correlation between the cell indices at 24 hours after seeding and the number of uses of the E-plate was non-significant for each E-plate (E-plate 1 – r = -0.75, confidence interval, CI, -0.9825 to 0.3870, p = 0.1; E-plate 2 – r = 0.4, CI -0.7584 to 0.9446, p = 0.5; E-plate 3 – r = -0.008, CI -0.8840 to 0.8805, p = 0.1). These experiments proved that that the E-plates could be used up to five times. Of note, some E-plates were used successfully up to seven times (data not shown).

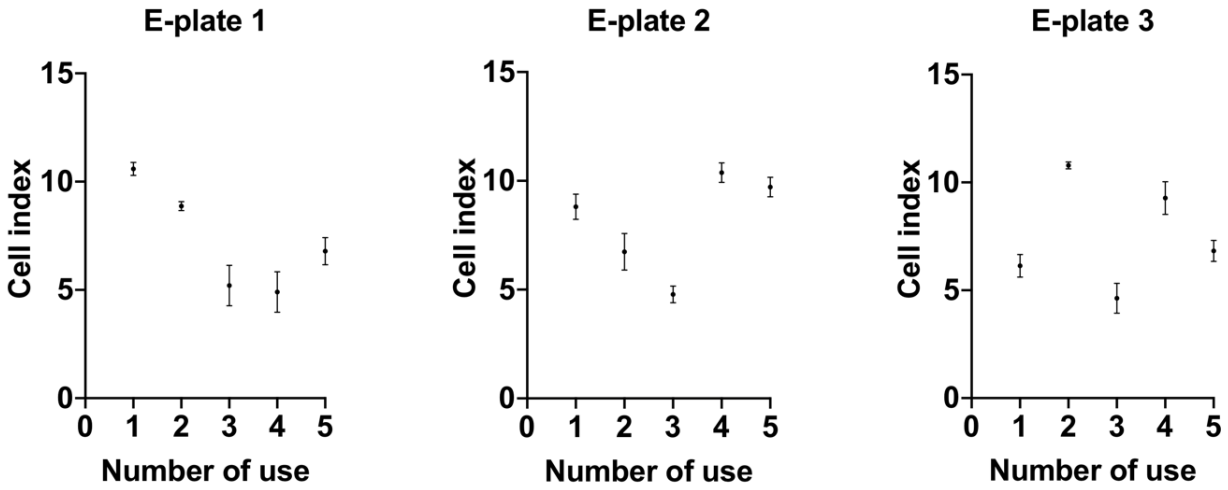


Figure 3.3. Cell index measured after reuse of single-use E-plates

Each iCELLigence E-plate was used in up to five experiments with human retinal endothelial cells. Mean cell index for each E-plate was recorded 24 hours after cell seeding. Dots indicate mean cell index and error bars represent standard deviation for groups of 8 wells.

3.2.2 Electrical impedance of human retinal endothelial cell monolayers and reduction in the presence of vascular endothelial growth factor-A

First, the iCELLigence biosensor was used to evaluate the electrical impedance of human retinal endothelial cells under non-stimulated conditions. Figure 3.4 shows tracings for the cell index over time in six independent experiments. In all experiments, a gradual increase in the cell index was observed until the medium was exchanged. A slight escalation followed by a short decline in the cell index was observed at this time, before the cell index began to rise steadily again, reaching a peak and plateau phase approximately 56 hours later. Averaged over all experiments, the mean and standard error of the mean for the cell indices were 8.8 ± 0.7 at 24 hours after seeding, 11.9 ± 0.1 at 56 hours post-medium exchange (plateau) and 11.9 ± 0.2 at 72 hours. Overall, the experiments achieved good reproducibility, and the overall pattern of the human retinal endothelial cell impedance tracing was established.

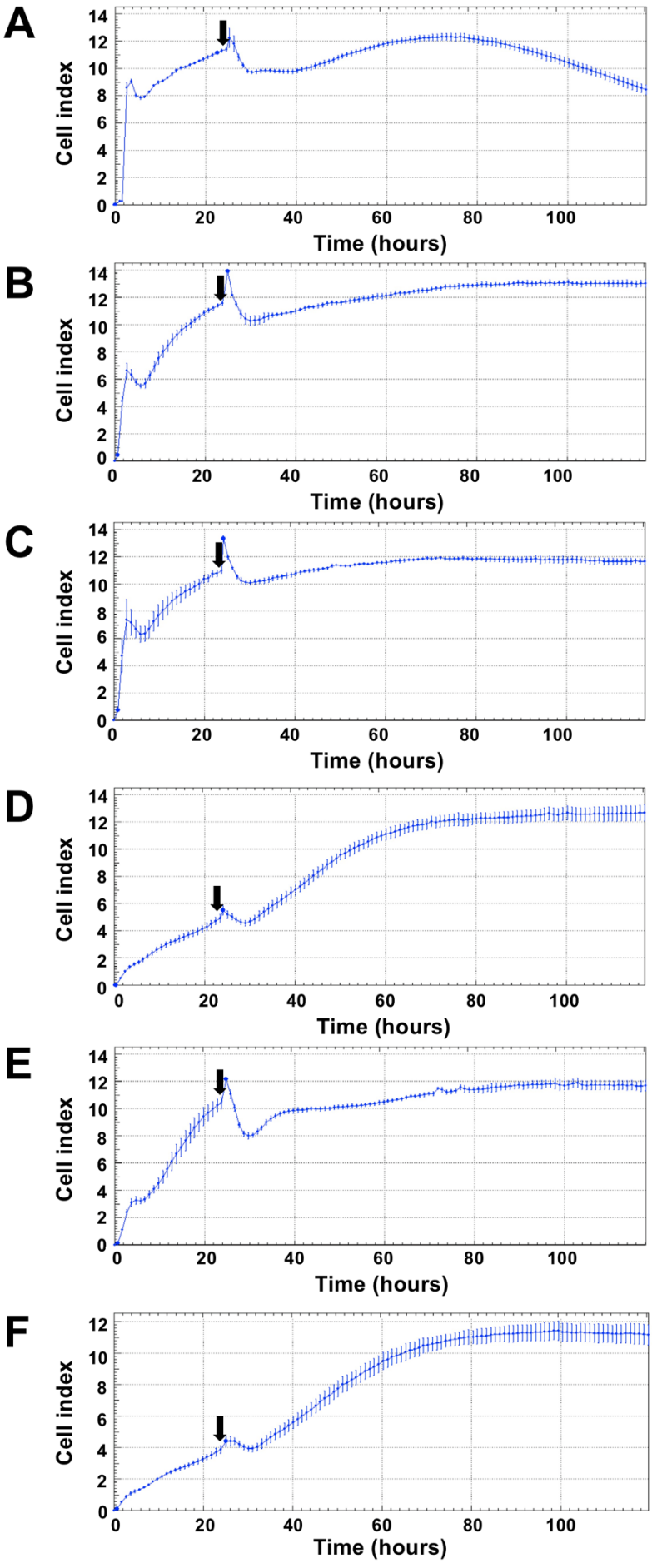
Vascular endothelial growth factor-A is known for its angiogenic properties and for inducing endothelial permeability, including in retinal endothelial cell monolayers.[437, 438, 662-664] After assessing the baseline cell index traces of human retinal endothelial cells and adjusting technical issues, cells were stimulated with VEGF-A to demonstrate that the experimental system was useful to show the changes in the electrical impedance of the cell monolayer. Human retinal endothelial cells were plated into E-plates, and treated with VEGF-A at 24 hours thereafter. The electrical impedance of the cell monolayer was monitored for a total of 96 hours. A significant reduction in the cell index ($p < 0.001$) was observed at 24-, 48-, and 72 hours in the VEGF-A-treated group in two independent experiments (Figure 3.5), with the highest reduction measuring 62% (mean cell index difference 7.9, Figure 3.5A and B). In summary, the administration of VEGF-A induced a fall in the cell index, showing the usefulness of this method for evaluating the effect of inflammatory cytokines on the barrier function of human retinal endothelial cell monolayers.

3.2.3 Effect of inflammatory cytokines on the electrical impedance of human retinal endothelial cell monolayers

The iCELLigence was used to identify inflammatory cytokines that could reduce the electrical impedance of human retinal endothelial cell monolayers. Tumour necrosis factor-α, IL-1β, IL-6,

Figure 3.4. Electrical impedance of human retinal endothelial cell monolayers

Plots of mean cell index of human retinal endothelial cell monolayers recorded hourly for 120 hours by an iCELLigence in 5 independent experiments (A through E). Error bars represent standard deviation for groups of 4 wells. Arrows indicate the time of medium exchange.


A
B
C
D
E
F
Cell index
Time (hours)
0
2
4
6
8
10
12
14
20
40
60
80
100

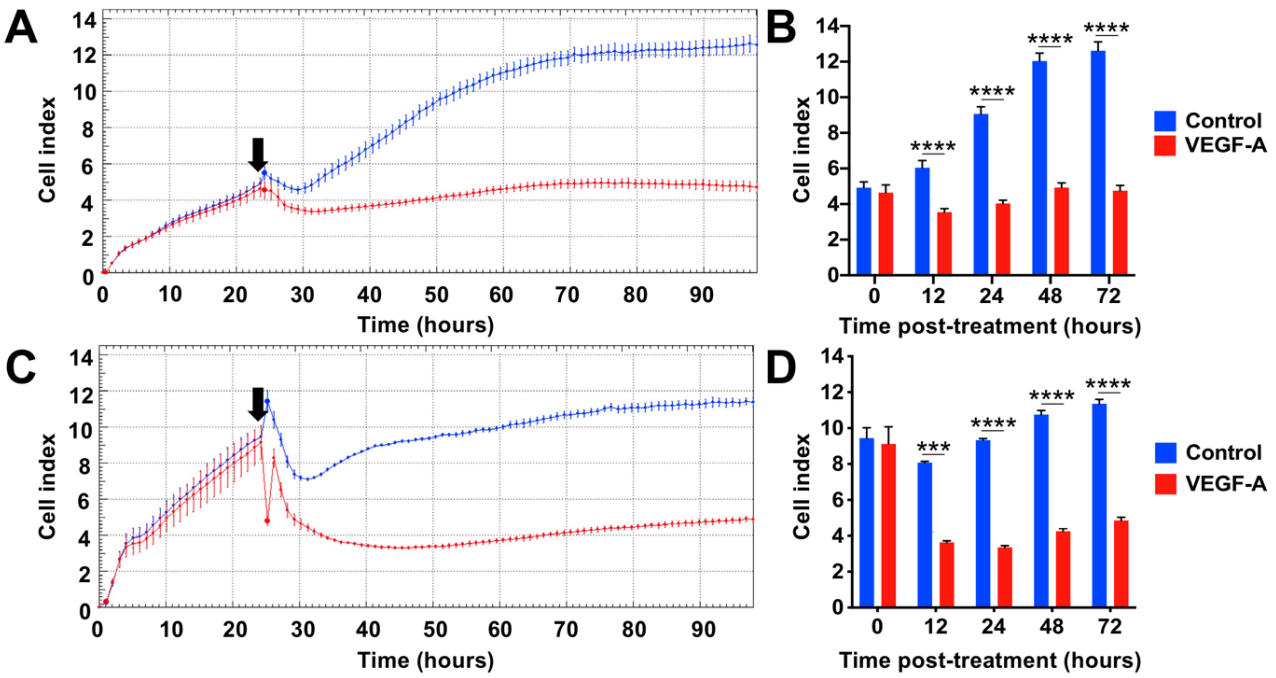


Figure 3.5. Effect of vascular endothelial growth factor-A on the electrical impedance of the human retinal endothelial cell monolayers

Plots (A and C) and graphs (B and D) of cell index of human retinal endothelial cell monolayers measured by iCELLigence in two independent experiments (A and B, C and D). VEGF-A (100 ng/mL) diluted in fresh medium or fresh medium alone was added to wells 24 hours after cell seeding. Plots show measurements made hourly, and graphs present results for pre-specified time intervals post-treatment. Blue indicates the medium alone control wells and red indicates wells treated with VEGF-A. Time of treatment is indicated by arrow on plot. Error bars represent standard deviation for groups of 4 wells. Statistical analysis was performed using an unpaired Student's t-test. ***$p < 0.001$; ****$p < 0.0001$.

IL-8, IL-17, and CCL2 were investigated in these studies. Concentrations of these cytokines were within the recommended concentrations provided by the manufacturers, unless higher concentrations had been suggested by prior published work.[205, 207, 254, 355, 628-635, 638-640] Treatment and control wells exhibited comparable cell indices immediately before the addition of cytokine in all instances ($p \geq 0.05$).

3.2.3.1 Tumour necrosis factor-α

Tumour necrosis factor-α was added to human retinal endothelial cells 24 hours following cell seeding on E-plates, and the cell indices were monitored for 72 hours after treatment. A statistically significant reduction in the cell index of the TNF-α-treated group was observed almost throughout all time points in two independent experiments (Figure 3.6), except for the 72-hour time point in the experiment showed in Figure 3.6 C and D ($p = 0.06$). Tumour necrosis factor-α led to a maximal reduction in the cell index of 42% in comparison with controls (maximal mean cell index difference of 5.1; $p < 0.0001$). Additionally, TNF-α decreased the cell index of the human retinal endothelial cell monolayer across all time points in two additional experiments performed by plating control and treatment groups into separate E-plates (data not shown). Based on these results, TNF-α was shown to impair the electrical impedance of human retinal endothelial cell monolayers.

3.2.3.2 Interleukin-1β

Interleukin-1β was applied to human retinal endothelial cells 24 hours subsequent to the plating. The cell indices of the treated and control groups significantly differed 12 hours after treatment ($p < 0.001$) in two independent experiments (Figure 3.7), and throughout the remainder of the experiment, the treated groups persisted with a lower cell index than the controls. A maximal drop of 44% in the cell index (maximal mean cell index difference of 5.1; $p < 0.0001$) was recorded 72 hours after treatment with IL-1β when compared with controls. This outcome demonstrated that IL-1β effectively diminished the human retinal endothelial cell monolayer electrical impedance, presenting similar results as treatment with TNF-α.

3.2.3.3 Interleukin-6

The effect of IL-6 on the electrical impedance of human retinal endothelial cell monolayers was

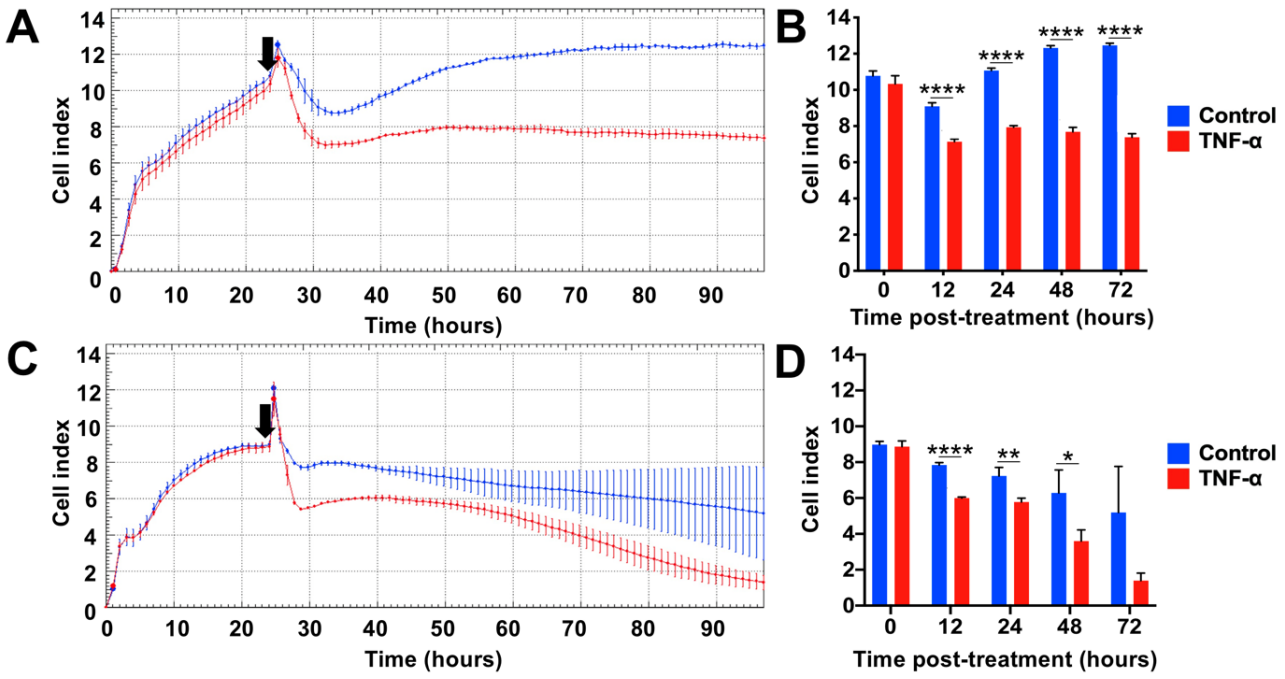


Figure 3.6. Effect of tumour necrosis factor-α on the electrical impedance of human retinal endothelial cell monolayers

Plots (A and C) and graphs (B and D) of cell index of human retinal endothelial cell monolayers measured by iCELLigence in two independent experiments (A and B, C and D). TNF-α (10 ng/mL) diluted in fresh medium or fresh medium alone was added to wells 24 hours after cell seeding. Plots show measurements made hourly, and graphs present results for pre-specified time intervals post-treatment. Blue indicates the medium alone control wells and red indicates wells treated with TNF-α. Time of treatment is indicated by arrow on plot. Error bars represent standard deviation for groups of at least 3 wells. Statistical analysis was performed using an unpaired Student's t-test. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

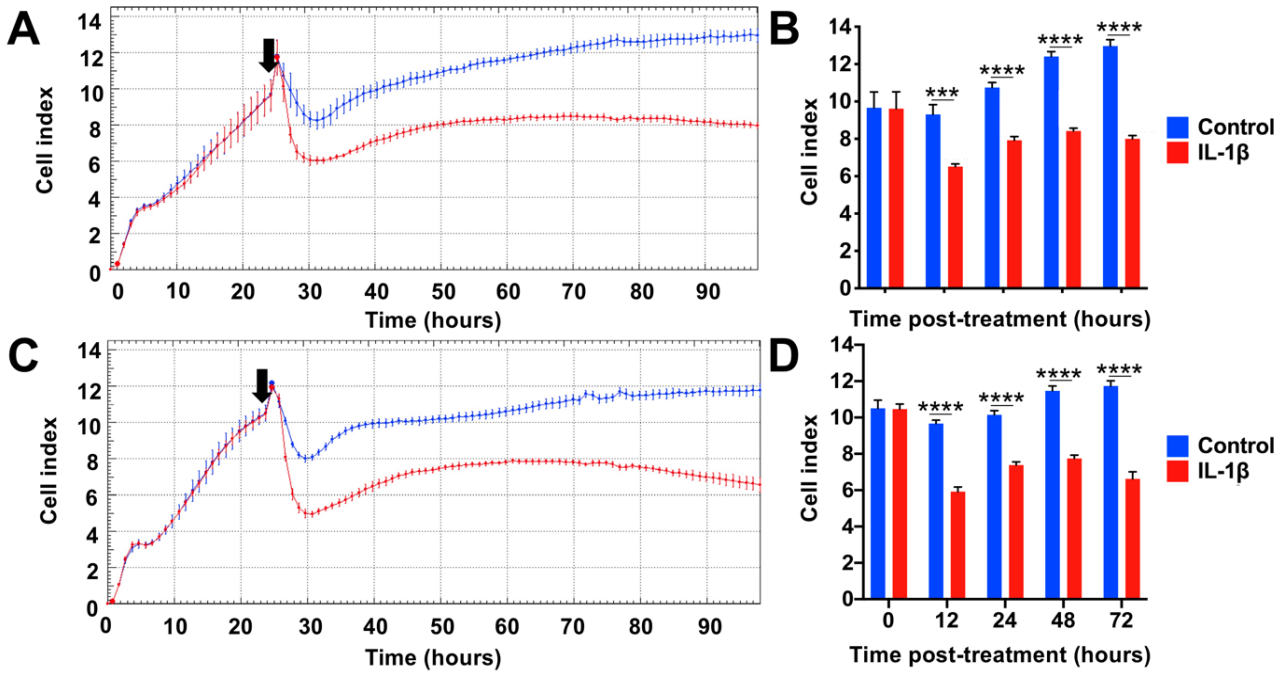


Figure 3.7. Effect of interleukin-1β on the electrical impedance of human retinal endothelial cell monolayers

Plots (A and C) and graphs (B and D) of cell index of human retinal endothelial cell monolayers measured by iCELLigence in two independent experiments (A and B, C and D). IL-1β (10 ng/mL) diluted in fresh medium or fresh medium alone was added to wells 24 hours after cell seeding. Plots show measurements made hourly, and graphs present results for pre-specified time intervals post-treatment. Blue indicates the medium alone control wells and red indicates wells treated with IL-1β. Time of treatment is indicated by arrow on plot. Error bars represent standard deviation for groups of at least 3 wells. Statistical analysis was performed using an unpaired Student's t-test. ***$p < 0.001$; ****$p < 0.0001$.

evaluated 24 hours after plating. Since it was not clear in the literature if human retinal endothelial cells expressed the IL-6R, and due to previous contrasting findings on the impact of IL-6 on the endothelial barrier integrity,[117, 284, 285, 288, 289] the experiment was independently performed five times to substantiate the results. All five replications of the experiment showed a reduction in the cell index of the IL-6-treated group across at least two pre-defined time points (mean cell index reduction of 7.3%). In two experiments (Figure 3.8 A to D), a decrease in cell index was observed only at 48 and 72 hours in IL-6-treated cells, while in another experiment (Figure 3.8 E and F), a persistent decrease in cell index was observed throughout all time points following IL-6 exposure. In two final experiments (Figure 3.8 G to J), retinal endothelial cells treated with IL-6 had a drop in cell index starting at 12 hours post-treatment, but the difference became non-significant towards the end of the experiment. The maximum difference in cell index was observed at 48 hours post-treatment in the experiment showed in Figure 3.8 C and D, representing a 13% reduction in the cell index of treated cells (mean cell index difference 1.42; p = 0.03). In summary, IL-6 reduced the human retinal endothelial electrical impedance, which persisted for approximately 24 hours or more, but was of smaller magnitude than that induced by TNF-α or IL-1β.

3.2.3.4 Interleukin-8

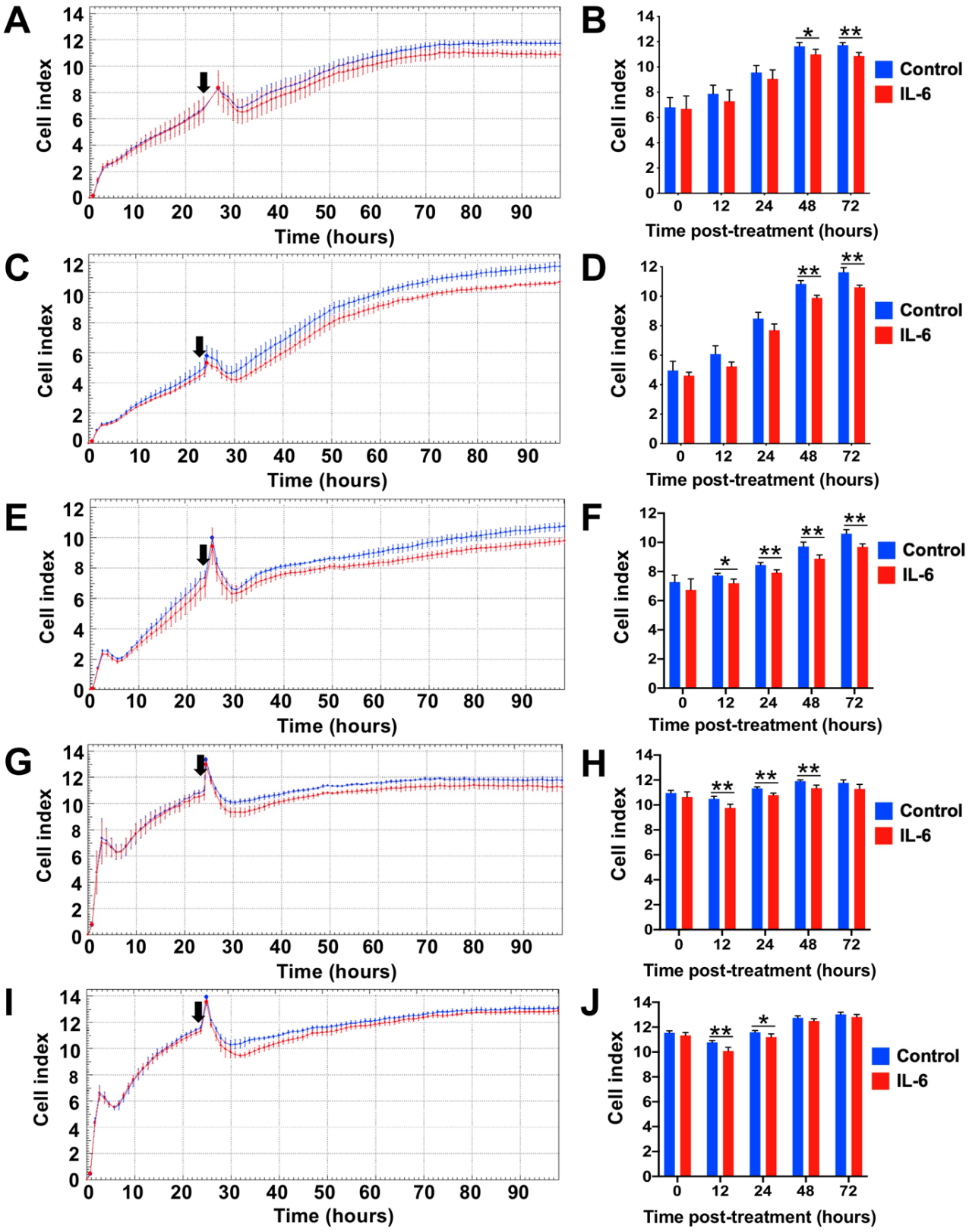
Human retinal endothelial cells were seeded into E-plates and half of the wells received only fresh medium after 24 hours, while the other half was treated with IL-8. The cell index was monitored over a total period of 96 hours. Interleukin-8 did not induce a statistically significant reduction of the cell index of the retinal endothelial cell monolayer in either of two independent experiments, although a wide standard deviation was observed in the experiment showed in Figure 3.9 C and D. These findings suggest that IL-8 does not provoke the disruption of the barrier function of the human retinal endothelial cell monolayer.

3.2.3.5 Interleukin-17

Interleukin-17 was applied to human retinal endothelial cells 24 hours following cell seeding into the E-plates. The cell index was similar between controls and IL-17-treated cells for all time points in two of three experiments (Figure 3.10 A to D), while only the experiment represented in Figure 3.10 E and F showed a significant 12% reduction in the cell index of the treated group at 72 hours (mean

Figure 3.8. Effect of interleukin-6 on the electrical impedance of the human retinal endothelial cell monolayers

Plots (A, C, E, G and I) and graphs (B, D, F, H and J) of cell index of human retinal endothelial cell monolayers measured by iCELLigence in five independent experiments (A and B, C and D, E and F, G and H, J and I). IL-6 (20 ng/mL) diluted in fresh medium or fresh medium alone was added to wells 24 hours after cell seeding. Plots show measurements made hourly, and graphs present results for pre-specified time intervals post-treatment. Blue indicates the medium alone control wells and red indicates wells treated with IL-6. Time of treatment is indicated by arrow on plot. Error bars represent standard deviation for groups of at least 3 wells. Statistical analysis was performed using an unpaired Student's t-test. *$p < 0.05$; **$p < 0.01$.


A
Cell index
Time (hours)
B
Cell index
Time post-treatment (hours)
Control
IL-6
C
D
E
F
G
H
I
J

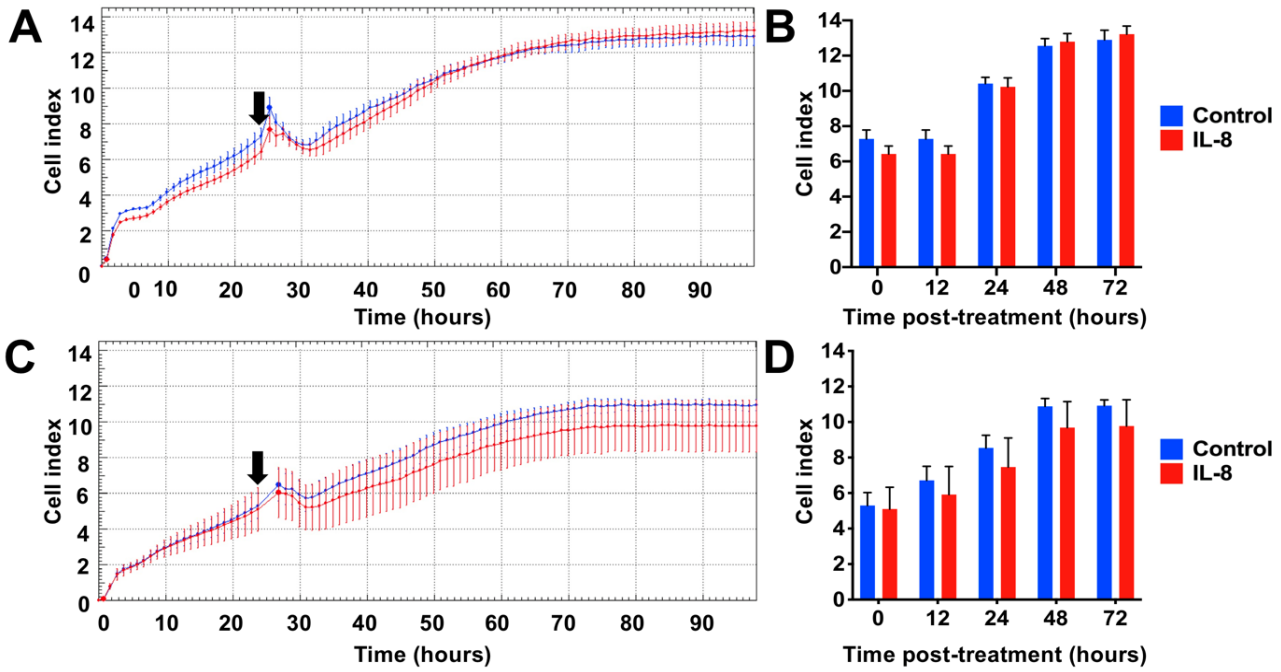


Figure 3.9. Effect of interleukin-8 on the electrical impedance of the human retinal endothelial cell monolayers

Plots (A and C) and graphs (B and D) of cell index of human retinal endothelial cell monolayers measured by iCELLigence in two independent experiments (A and B, C and D). IL-8 (50 ng/mL) diluted in fresh medium or fresh medium alone was added to wells 24 hours after cell seeding. Plots show measurements made hourly, and graphs present results for pre-specified time intervals post-treatment. Blue indicates the medium alone control wells and red indicates wells treated with IL-8. Time of treatment is indicated by arrow on plot. Error bars represent standard deviation for groups of at least 3 wells. Statistical analysis was performed using an unpaired Student's t-test.

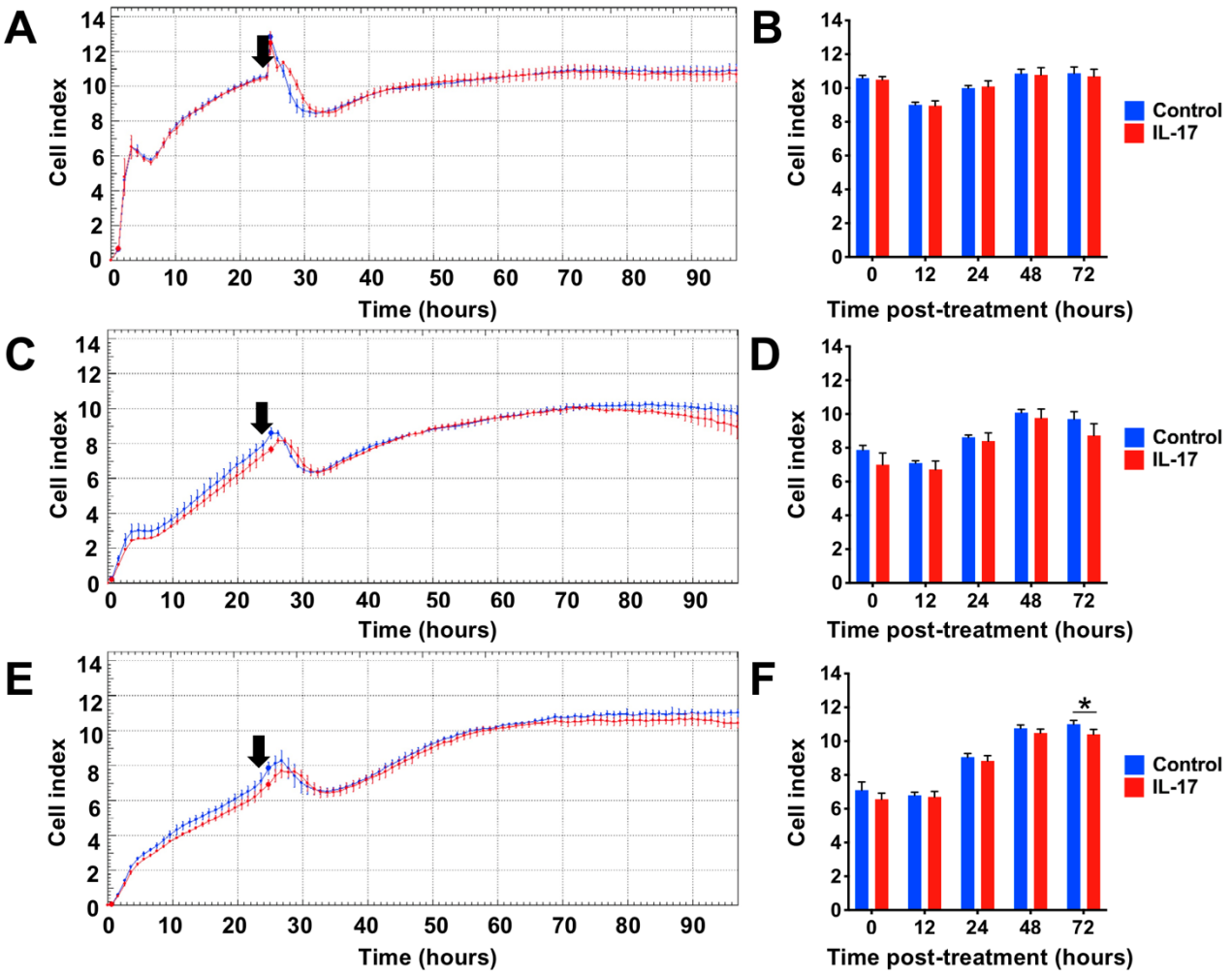


Figure 3.10. Effect of interleukin-17 on the electrical impedance of the human retinal endothelial cell monolayers

Plots (A, C and E) and graphs (B, D and F) of cell index of human retinal endothelial cell monolayers measured by iCELLigence in three independent experiments (A and B, C and D, E and F). IL-17 (100 ng/mL) diluted in fresh medium or fresh medium alone was added to wells 24 hours after cell seeding. Plots show measurements made hourly, and graphs present results for pre-specified time intervals post-treatment. Blue indicates the medium alone control wells and red indicates wells treated with IL-17. Time of treatment is indicated by arrow on plot. Error bars represent standard deviation for groups of at least 3 wells. Statistical analysis was performed using an unpaired Student's t-test. *$p < 0.05$.

cell index difference 0.9; $p = 0.008$). Although not depicted in the graphs, in the experiment presented as Figure 3.10 A and B, a transient increase in the cell index of endothelial cells treated with IL-17 was observed from 4 to 6 hours post-exposure (12% increase at 5 hours post-treatment; mean cell index difference 1.2; $p = 0.009$), while a 14% decrease in cell index in IL-17-treated cells was verified at 1 hour after treatment in the experiment showed in Figure 3.10 C and D (mean cell index difference 1.2; $p = 0.01$). Overall, IL-17 did not consistently alter the electrical impedance of the human retinal endothelial cell monolayer.

3.2.3.6 C-C motif chemokine ligand 2

Human retinal endothelial cells were plated into E-plates and subsequently treated with CCL2 after 24 hours. The similarity between the cell index of treatment and control groups was confirmed by both experiments shown in Figure 3.11, with that showed in A and B presenting quite a wide standard deviation bar on the tracing of the treatment group. Based on these observations, CCL2 did not affect the electrical impedance of the human retinal endothelial cell monolayers.

3.2.3.7 Combination of selected inflammatory cytokines

Among all the assessed cytokines, TNF-α, IL-1β and IL-6 reduced the retinal endothelial electrical impedance, with TNF-α and IL-1β displaying a greater magnitude of effect on the cell index than IL-6. Hence, combinations of TNF-α and IL-6, and IL-1β and IL-6 were added to human retinal endothelial cells and the cell indices were compared against TNF-α and IL-1β, respectively, to assess for a possible IL-6 additive effect on the TNF-α- or IL-1β-induced electrical impedance reduction.

Figure 3.12 depicts three independent experiments of electrical impedance monitoring of human retinal endothelial cells treated either with TNF-α plus IL-6 or TNF-α alone at 24 hours after cell seeding. In the experiments showed in Figure 3.12 A to D, at 72 hours, the TNF-α plus IL-6 treatment group presented a maximal increase in cell index of 4.6% when compared to TNF-α-only group (mean cell index difference 0.4; $p = 0.02$), while no differences were observed between the groups in a third experiment shown in Figure 3.12 E and F. Overall, IL-6 did not have an additive effect on the TNF-α-induced reduction of the electrical impedance of the human retinal endothelial

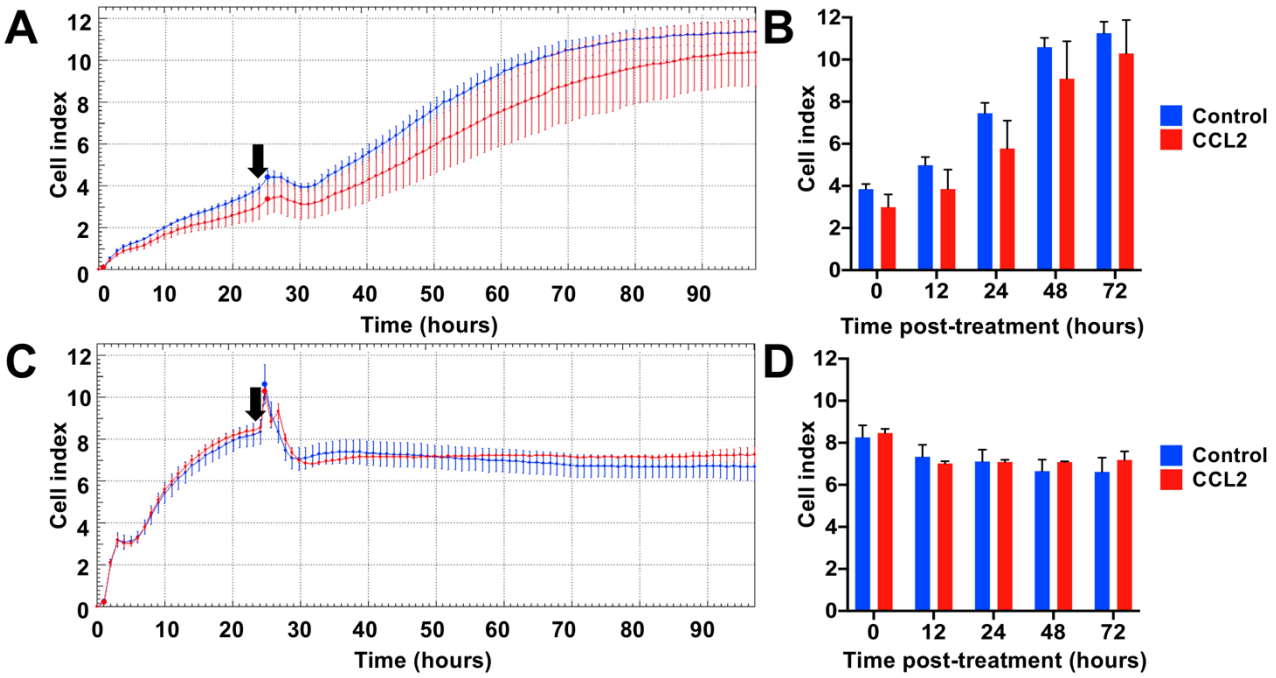


Figure 3.11. Effect of C-C motif chemokine ligand 2 on the electrical impedance of the human retinal endothelial cell monolayers

Plots (A and C) and graphs (B and D) of cell index of human retinal endothelial cell monolayers measured by iCELLigence in two independent experiments (A and B, C and D). CCL2 (100 ng/mL) diluted in fresh medium or fresh medium alone was added to wells 24 hours after cell seeding. Plots show measurements made hourly, and graphs present results for pre-specified time intervals post-treatment. Blue indicates the medium alone control wells and red indicates wells treated with CCL2. Time of treatment is indicated by arrow on plot. Error bars represent standard deviation for groups of at least 3 wells. Statistical analysis was performed using an unpaired Student's t-test.

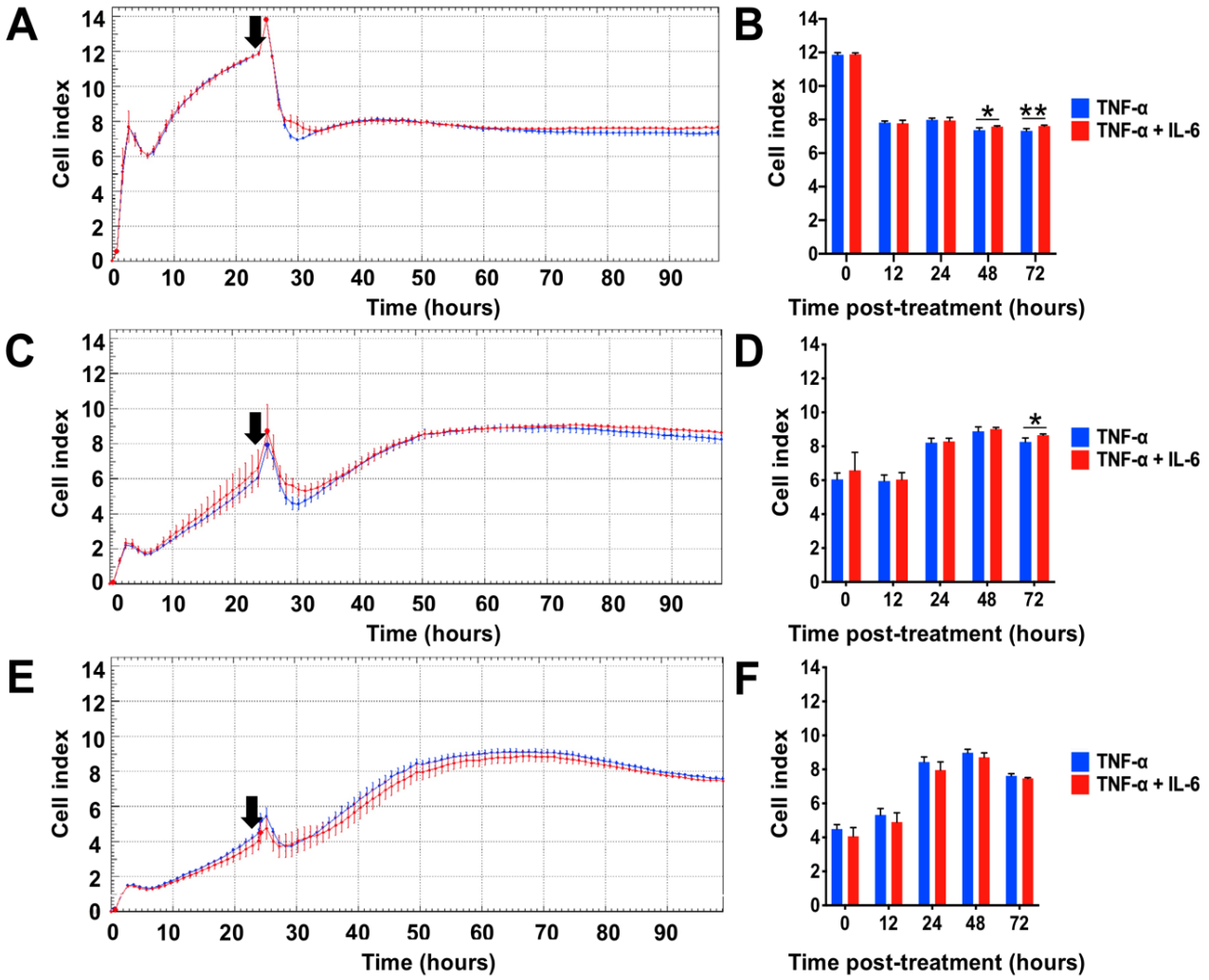


Figure 3.12. Combined effect of tumour necrosis factor-α and interleukin-6 on the electrical impedance of the human retinal endothelial cell monolayers

Plots (A, C and E) and graphs (B, D and F) of cell index of human retinal endothelial cell monolayers measured by iCELLigence in three independent experiments (A and B, C and D, E and F). TNF-α (10 ng/mL) plus IL-6 (20 ng/mL) or IL-6 alone, both diluted in fresh medium, was added to wells 24 hours after cell seeding. Plots show measurements made hourly, and graphs present results for pre-specified time intervals post-treatment. Blue indicates wells treated with IL-6 and red indicates wells treated with TNF-α plus IL-6. Time of treatment is indicated by arrow on plot. Error bars represent standard deviation for groups of 4 wells. Statistical analysis was performed using an unpaired Student's t-test. *$p < 0.05$; **$p < 0.01$.

monolayer. On the contrary, IL-6 slightly reduced the impairment of the electrical impedance of the retinal endothelial cell monolayer produced by TNF-α in two of three experiments.

To analyze if IL-6 would have an additive effect in the reduction of the endothelial impedance caused by IL-1β, a combination of IL-1β and IL-6 was applied to human retinal endothelial cells 24 hours after cell plating and compared against IL-1β alone. Among the three experiments, a subtle but significant reduction in cell index was seen at 48 and 72 hours for the IL-1β plus IL-6 treatment group solely in the experiment depicted in Figure 3.13 A and B (p = 0.02 and p = 0.04, respectively), reaching a 2.6% difference (mean cell index difference 0.1) at 72 hours, whereas the remaining experiments did not demonstrate any significant differences in cell index between the IL-1β and IL-1β plus IL-6 conditions (Figure 3.13 C to F). Overall, IL-6 did not have an additive effect on the IL-1β-driven impairment of the human retinal endothelial cell monolayer electrical impedance, as shown in two of three experiments.

3.2.4 Summary of results

The analyses of the electrical impedance of the human retinal endothelial monolayer showed that TNF-α, IL-1β and IL-6 consistently decreased the electrical impedance, whereas IL-8, IL-17 and CCL2 generally did not affect the impedance of the monolayer. The difference between the cell index for treated and control groups increased over time for TNF-α or IL-1β stimulation, and achieved a peak at 72 hours post-treatment. Human retinal endothelial cells treated with TNF-α and IL-1β had a similar maximal percentage reduction in the cell index, at approximately 40%. A smaller reduction in the cell index was observed in retinal endothelial cells treated with IL-6, at roughly 10%. The addition of IL-6 to TNF-α or IL-1β did not enhance the decrease in electrical impedance of the cell monolayer, and possibly slightly increased it in the case of TNF-α plus IL-6.

3.3 Discussion

The work described in this chapter demonstrated that TNF-α, IL-1β and IL-6 impaired the electrical impedance of human retinal endothelial cell monolayers, while IL-8, IL-17 and CCL2 did not affect it. These experiments represent the most comprehensive evaluation of the effects of cytokines on

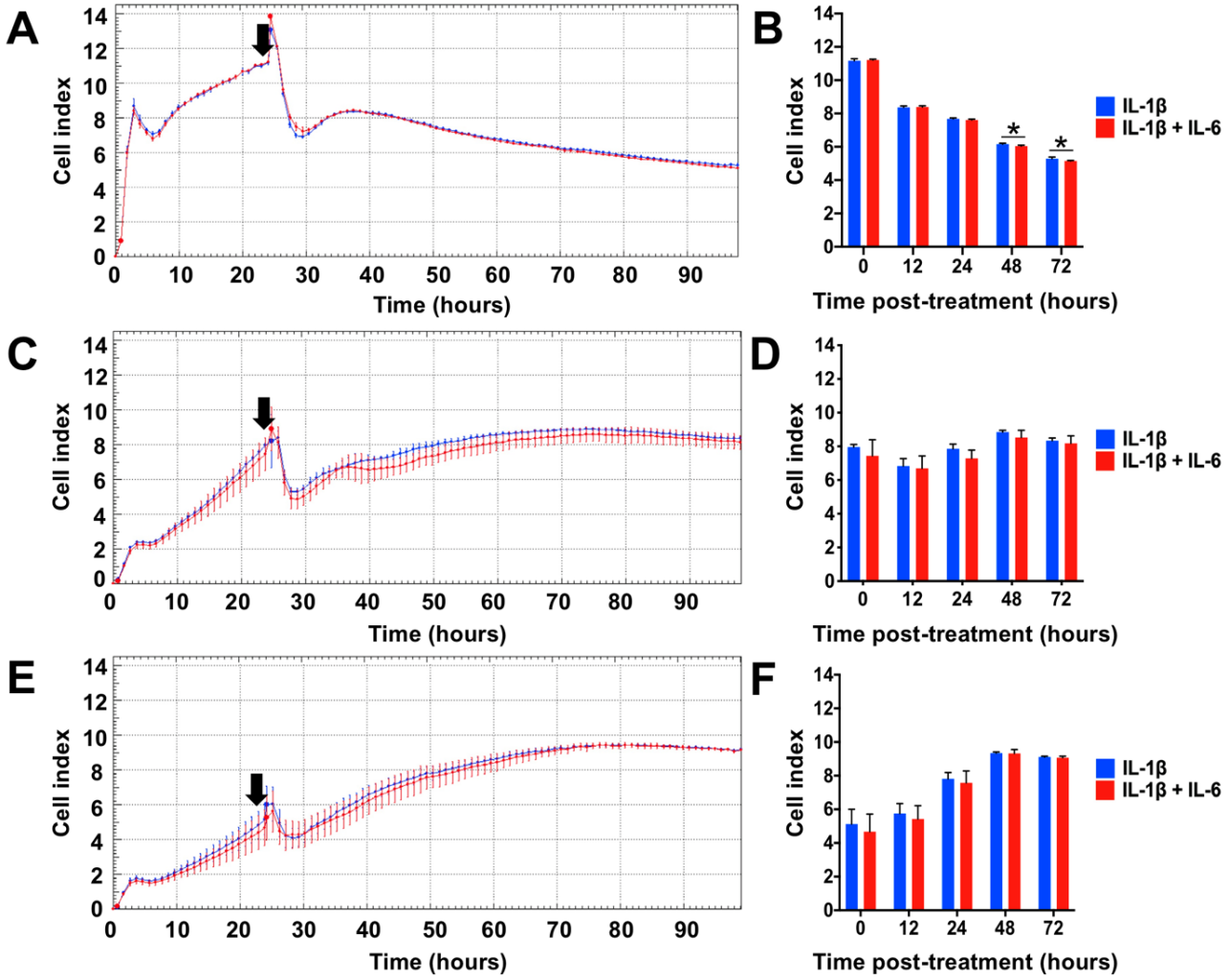


Figure 3.13. Combined effect of interleukin-1β and interleukin-6 on the electrical impedance of the human retinal endothelial cell monolayers

Plots (A, C and E) and graphs (B, D and F) of cell index of human retinal endothelial cell monolayers measured by iCELLigence in three independent experiments (A and B, C and D, E and F). IL-1β (10 ng/mL) plus IL-6 (20 ng/mL) or IL-6 alone, both diluted in fresh medium, was added to wells 24 hours after cell seeding. Plots show measurements made hourly, and graphs present results for pre-specified time intervals post-treatment. Blue indicates wells treated with IL-6 and red indicates wells treated with IL-1β plus IL-6. Time of treatment is indicated by arrow on plot. Error bars represent standard deviation for groups of 4 wells. Statistical analysis was performed using an unpaired Student's t-test. *$p < 0.05$.

the electrical impedance of the human retinal endothelial cell monolayer using a real-time biosensor ever undertaken. Another unique aspect of all experiments was the use of a human retinal endothelial cell line made and characterized in-house, maintaining endothelial phenotypic hallmarks, whereas commercial cell lines were used by others who have evaluated cytokine effects on retinal endothelial barrier function. The possibility of reusing the biosensor plate up to 5 times had a substantial impact on costs of these experiments.

Real-time monitoring over a total of 96 hours demonstrated that TNF-α achieved roughly a 40% reduction in the electrical impedance of human retinal endothelial cell monolayers. Several *in vivo* and *in vitro* studies have corroborated the findings of retinal endothelial cell monolayer dysfunction caused by TNF-α.[99, 115, 117, 126, 139, 209, 211, 213, 214] Other authors have reported up to 50% reduction in retinal endothelial impedance in short duration assays that used commercial cell lines and lower concentrations of TNF-α.[117, 122, 124] Reduced transcellular electrical resistance and increased permeability has also demonstrated with higher TNF-α concentrations.[205, 665] Only one group of investigators, conducting short duration studies, reported that TNF-α had no effect on retinal endothelial impedance.[119] The impairment of the barrier function by TNF-α was reported also in human brain endothelial cells,[209, 210] astrocytes,[139] and bovine retinal endothelial cells.[115, 120] Several *in vivo* and *in vitro* studies corroborate the findings of retinal endothelial cell monolayer dysfunction caused by TNF-α.[99, 115, 117, 126, 139, 209, 211, 213, 214] Interestingly, one study showed an acute reduction in barrier impedance in brain endothelial cells followed by a progressive impedance strengthening, suggesting that endothelial cells from the brain react differently to those from the retina when challenged with inflammatory cytokines.[211]

Interleukin-1β induced a similar substantial reduction in the electrical impedance of the human retinal endothelial cell monolayer as TNF-α. Effects of the two cytokines were not directly compared in the experiments presented here, but Aveleira et al.[126] noted a more potent effect of IL-1β than TNF-α in enhancing the permeability of bovine retinal endothelial cells. The systemic use of an IL-1β blocking drug reportedly reduces macular oedema in uveitis,[265] which is conceivable based on the findings reported here. Other groups have reported diminished barrier resistance of human retinal endothelial

cells in varying magnitudes, assessing the barrier function for shorter periods and using commercial cell lines and diverse concentrations of IL-1β,[117, 124, 205, 253] Conversely, in brain endothelial cells, IL-1β induced a transient reduction with a subsequent increase in barrier impedance, suggesting a tissue-specificity response to inflammatory stimuli.[211]

A reduction in the electrical impedance of the human retinal endothelial cell monolayer was induced by IL-6, and this observation was consistently replicated. The science is controversial regarding this finding. Two research groups[117, 284] did not find any impact of IL-6 on a human retinal endothelial barrier when applying IL-6 to a commercial cell line, even when using high concentrations, although assessing the barrier for just 2 days post-IL-6 exposure,[284] and with one of the studies not showing the data.[117] However, decreased transcellular electrical resistance and increased macromolecular permeability were observed up to 18 hours[205] and 48 hours[125] after IL-6 treatment of human retinal endothelial commercial cell lines, using a concentration of IL-6 similar to which was used in the experiments here reported. Valle et al.[285] showed an impairment of the electrical impedance of human retinal endothelial cell monolayers after concomitant exposure to IL-6 and sIL-6R, but did not test IL-6 alone. Some reports indicate that IL-6 induced macromolecular permeability[209, 210] and decreased transcellular electrical resistance[287] of human non-ocular endothelium. Alsaffar et al.[286] reported that the umbilical vein endothelial electrical impedance was impaired by the simultaneous addition of IL-6 and sIL-6R, but not by IL-6 alone; however, the monitoring time was approximately 24 hours, and thus possible late changes would not have been detected.

The lack of effect of IL-6 reported by some authors on the barrier function of human retinal endothelial cells could be explained by the fact that transmembrane IL-6R protein has not been demonstrated in human retinal endothelial cells,[284, 285, 288] although transcript expression has been reported,[289] and the assumption that IL-6 actions would be exerted by trans-signalling via sIL-6R,[285, 286] found in increased concentrations in the eye during uveitis[290] and diabetic retinopathy.[666] Evidence of clinical improvement of macular oedema secondary to uveitis by anti-IL-6 drugs,[293-296] allied to the observation that IL-6 decreases the retinal endothelial electrical impedance presented here, suggest that IL-6R *is* expressed by the human retinal endothelium, a controversy that should

be explored further.

Interleukin-17, IL-8 and CCL2 did not consistently affect the electrical impedance of the human retinal endothelial cell monolayer. A lack of effect of IL-17 on the barrier function of the human retinal endothelial cells was reported by Da Cunha et al.[117] who used a commercial cell line with a short period of monitoring. However, an increase in macromolecular permeability was reported using IL-17 in an analogous dose.[308] Apart from different cell sources, this discrepancy might be attributed to different growth substrates altering cell adhesion to the well base, which can influence cell differentiation, polarization and nutrition.[599] The receptors of IL-17 include the IL17-RA and IL17-RC subunits.[308] The expression of IL17-RA has been documented in the retina of diabetic mice, while the expression of IL17-RC has been described in human retinal endothelial cells, and therefore it is likely that IL-17 would act on the human retinal endothelial cell monolayer.[308, 667]

This is the first report of the IL-8 effect on the electrical impedance of the human retinal endothelial cell monolayer, with previous work evaluating the influence of IL-8 on astrocyte impedance and showing a lack effect,[139] and on non-ocular endothelial cells, where IL-8 induced hyperpermeability.[358] The absence of impact of CCL2 on the electrical impedance of retinal endothelial cells is in agreement with a study using a commercial cell line and monitoring the impedance for a short period.[107] These data suggest that IL-17, IL-8 and CCL2 do not have a direct effect on the breakdown of the retinal endothelial barrier, but might affect the barrier when other inflammatory mediators are present,[117, 339] or by the promotion of chemotaxis and leukocyte infiltration.[194, 197] C-X-C receptor 1 and CXCR2 bind to IL-8, while CXCR2 binds also to CCL2. The expression of CXCR1 and CXCR2 has been documented in brain endothelial cells, while the latter has also been reported in the retina of diabetic mice.[355, 668] Hence, it is possible that the lack of effect of IL-8 and CCL2 upon the human retinal endothelial monolayer is owed to the possible absence of their receptors, as their expression has not been documented in the human retinal endothelium.

Interleukin-6 did not enhance the retinal endothelial barrier impairment when applied in combination with IL-1β or TNF-α. Although minimal transient changes in electrical impedance were observed in one of the experiments combining IL-1β and IL-6, the changes were small and not consistently

present in all experimental replications, pointing out the difficulties that can arise when interpreting the data of a real-time impedance monitoring system. The impact of combined cytokines on the electrical impedance was reported in one study using a human retinal endothelial commercial cell line, demonstrating an absence of additive effect of IL-6 to TNF-α and IL-1β with a monitoring duration of only 12 hours after cytokine stimulation and without showing the data.[117] Tumour necrosis factor-α and IL-1β individually induced potent reductions in the electrical impedance of human retinal endothelial cell monolayers. These dramatic effects may have precluded any measurable additive impact of IL-6, which produced a limited decrease in the impedance when applied alone. Moreover, it could be hypothesized that TNF-α and IL-1β impact IL-6 signalling on retinal endothelial cells, for example by altering IL-6R expression on the cell monolayer, although the expression of IL-6R by human retinal endothelial cells is a controversial topic in the literature.[284, 285, 288, 289]

The evaluation of cytokine concentrations in intraocular fluids provides insights into ocular tissue biological processes; however, it does not necessarily represent what is occurring at the retinal cellular level. Intravitreal levels of TNF-α range from 15 pg/mL[171] to over 4,000 pg/mL[166] in patients with non-infectious uveitis, while intraocular concentrations of IL-1β vary from circa 2 pg/mL[169, 178] to 80 pg/mL.[149] Curnow et al.[160] showed IL-6 levels as high as 800 ng/mL in the aqueous humour of patients with idiopathic uveitis, whereas IL-8 vitreous levels reached around 1,800 pg/mL.[162] Intraocular levels of IL-17 ranged from 158 to 462 pg/mL in patients with birdshot chorioretinopathy,[169] while CCL2 was found in concentrations above 1,000 pg/mL in idiopathic non-infectious uveitis.[160] Although higher than the intraocular concentrations during uveitis, the selection of the cytokine concentrations for cell stimulation in the work described here was based on previous publications[205, 207, 254, 355, 628-635, 638-640] and/or were within the range of the suggested concentrations provided by the manufacturers.

The possibility of reusing of iCELLigence E-plates up to three times was reported in a study with human cervical carcinoma and breast adenocarcinoma cell lines, using the half-maximum inhibitory concentration as the measure of comparison among E-plates.[647] In the work presented here, the cell index was measured 24 hours after seeding human retinal endothelial cells in order to investigate

the feasibility of reusing the E-plates beyond this number. Variation in the cell index was observed across different experiments for each E-plates, and there was no association between the cell index and the number of uses, implying that the E-plates could be reused up to five times at least. This opportunity for considerable reuse of E-plates translates to conservation of an expensive resource, bringing environmental and economic responsibility into research.

The findings presented in this chapter focus on a single element of the inner blood-retinal barrier: the retinal endothelial cell. The research does not take account of the endothelial basement membrane, or various neighbouring cells, including pericytes, glial cells and neurons, which may also contribute to the barrier *in vivo*. However, it was intentional to isolate the human retinal endothelial cell for this work, as it is the principal cellular component of the inner blood-retinal barrier. These cells were generated from human eyes by the home laboratory, and when used in an extended assessment of monolayer impedance following multiple cytokine treatments, they represent a robust proxy to study the inner blood-retinal barrier under conditions of inflammation.

These experiments, involving a range of inflammatory cytokines with multiple replications, constitute a thorough analysis of the effect of cytokines on the electrical impedance of human retinal endothelial cell monolayers, pointing to the main cytokines responsible for the retinal endothelial barrier breakdown during inflammation: TNF-α, IL-1β and IL-6. Questions raised by this series of experiments include what mechanisms are involved in the endothelial barrier impairment by these cytokines and whether the IL-6R is expressed by human retinal endothelial cells.

Some of the work of this chapter was included in a manuscript submitted to Eye and Vision: Ferreira LB, Ashander LM, Appukuttan B, Ma Y, Williams KA, Best G, Smith JR. Human retinal endothelial cells express functional interleukin-6 receptor. 2023 (under review).

CHAPTER 4: EXPRESSION AND REGULATION OF INTERLEUKIN-6 RECEPTOR IN HUMAN RETINAL ENDOTHELIAL CELLS

4.1 Introduction

Interleukin-6 is a major inflammatory cytokine secreted by T-cells and monocytes amongst other cells.[144, 192, 669] It has a broad range of activities, such as promoting cell proliferation, differentiation and inducing vascular permeability, also being an important cytokine in the defence against infections. Increased IL-6 activity is linked to several inflammatory and autoimmune conditions, as well as cancer, and the blockade of IL-6 signalling has been used clinically for the treatment of autoimmune disorders, including non-infectious posterior uveitis.[670]

Interleukin-6 signal transduction occurs mainly via classical signalling, mediated by the binding of IL-6 to mIL-6R, which is expressed by limited cell types, such as hepatocytes and leukocytes, and trans-signalling, achieved by ligation of IL-6 with the sIL-6R.[291, 671, 672] Interleukin-6 is thought to have an anti-inflammatory effect when binding to mIL-6R, but an inflammatory action through binding to sIL-6R.[277-279] An additional pathway of IL-6 signal transduction occurs via trans-presentation, in which IL-6 activates mIL-6R from dendritic cells and is trans-presented to T-cells.[673] All pathways require the association of the IL-6/IL-6R complex with the signal-transducing glycoprotein, gp130, which is widely expressed across different cell populations, including human retinal endothelial cells.[285, 288] A soluble form of gp130 (sgp130) also exists and blocks IL-6-sIL-6R signalling.[144] The downstream result of IL-6 signal transduction involves the activation of JAK/STAT, MAPK and PI3K pathways.[674] As the expression of the transmembrane receptor is restricted, the sIL-6R generated by shedding of mIL-6R or alternative RNA splicing enhances the reach of IL-6 actions.[291]

Interleukin-6 has been implicated in the development of uveitis *in vivo,*[192, 193] and has been found in increased concentrations in the intraocular fluids of patients with uveitis.[152, 154, 157, 159, 160, 166, 167, 169-174, 178, 180] The impairment of the electrical resistance of human retinal endothelial cell monolayers observed in Chapter 3 suggests that IL-6R is expressed by human retinal endothelial cells, although this remains a controversial topic in the literature.[284, 285, 288, 289] Protein expression of IL-6R has not been demonstrated in human retinal endothelial cells,[284, 285, 288] but the transcript has been detected.[289]

To understand the effect of IL-6 on human retinal endothelial cells, IL-6R expression was assessed in primary cells from multiple donors using standard RT-PCR and real-time RT-qPCR, as well as flow cytometry. Having showed the presence of IL-6R on these cells, the impact of inflammatory cytokines on the differential expression of IL-6R was analyzed, and IL-6R gene regulation was investigated.

4.2 Results

4.2.1 Expression of interleukin-6 receptor in human retinal endothelial cells

The presence of IL-6R transcript was verified in primary human retinal endothelial cell isolates prepared from seven cadaveric donors (one male and six females, aged 35 to 64 years with a median of 55 years at death) and a human retinal endothelial cell line. Three different primer sets were used to perform standard RT-PCR: (1) IL-6R, which targeted all 9 IL-6R transcript variants; (2) mIL-6R, targeting all transcript variants possessing the receptor transmembrane domain (part of the 9th exon), that is, all variants except 2, 3 and 8; and (3) sIL-6R, targeting transcript variants 2 and 8. The IL-6R amplicon was detected in all primary cell isolates and the cell line (Figure 4.1A). An extra product of around 100 bp was amplified in isolate 1 and may correspond to an off-target product, sortin nexin 32 (SNX32 – 94 bp). Amplification of this extra product was not isolate-specific, being detected for other cell isolates when the experiment was replicated (data not shown). The mIL-6R product was present in all primary cell isolates and the cell line (Figure 4.1A), while the sIL-6R product was expressed in the cell line and six of the seven primary cell isolates. All PCR products were confirmed by sequencing.

After confirming the presence of the transcripts, RT-qPCR was employed to measure expression levels of IL-6R, mIL-6R and sIL-6R in the same samples assessed in standard RT-PCR. Interleukin-6R, mIL-6R and sIL-6R were present at variable levels in all primary human retinal endothelial cell isolates and the cell line (Figure 4.1B). Isolates 1 and 3 showed the highest expression levels of IL-6R and mIL-6R, whereas sIL-6R expression was higher in isolate 1 and the cell line. Differences in transcript levels were observed across the three molecules, thus being presented in separate graphs. These findings confirm that mIL-6 and sIL-6R transcripts are present in human retinal

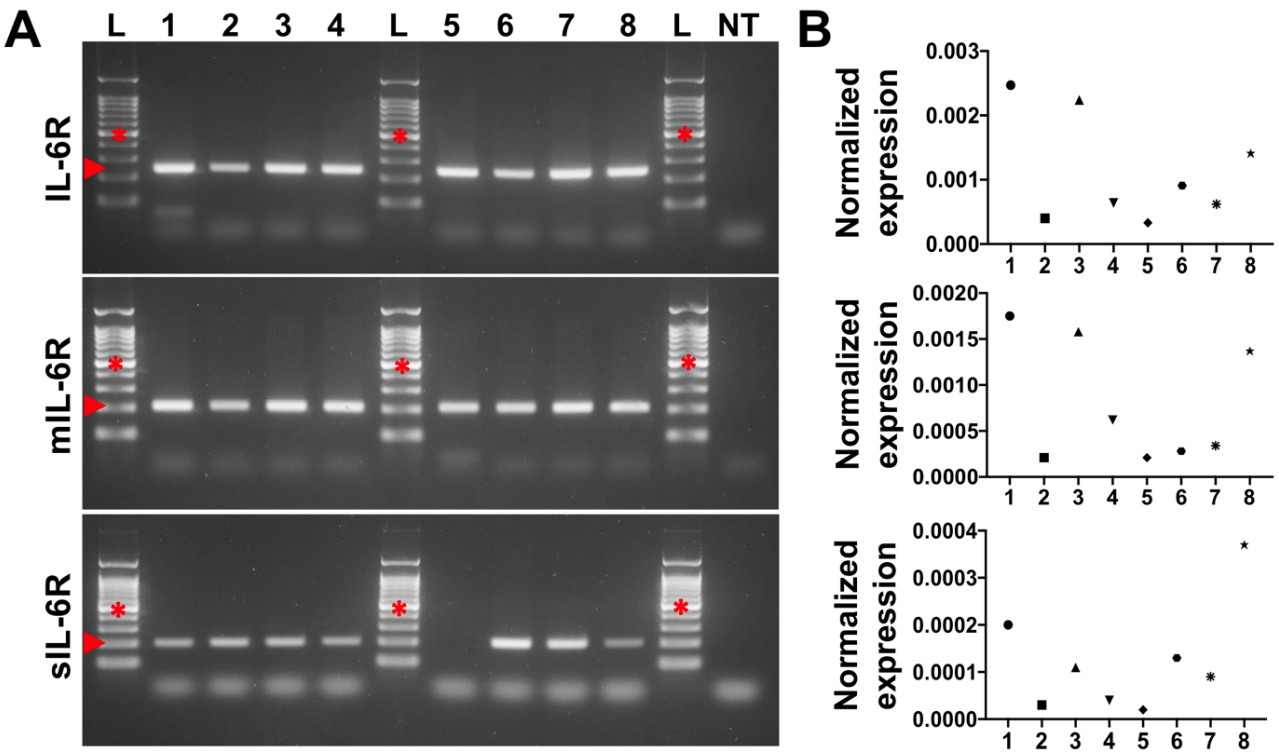


Figure 4.1. Interleukin-6 receptor transcript expression in human retinal endothelial cells

(A) Images showing IL-6R amplicons run on 2% agarose gel. L = DNA ladder (500 base pairs indicated by red cross); 1-7 = primary retinal endothelial cell isolates from individual donors; 8 = expanded retinal endothelial cell isolate; NT = no cDNA template control. Expected product sizes (indicated by red arrows): IL-6R = 240 bp; mIL-6R = 202 bp; sIL-6R = 195 bp. (B) Graphs showing relative normalized expression of corresponding IL-6R transcripts in the same cell isolates showed in (A). Reference genes were RPLP0 and PPIA.

endothelial cells, with relatively low levels of sIL-6R expression.

A cell line derived from monocytes known to express the IL-6R,[675, 676] THP-1 cells, was selected for optimizing an immunolabeling protocol to detect the protein expression of IL-6R by flow cytometry. Different protocols consisted of altering the permeabilization reagent (Triton X-100 or methanol), the PFA concentration (4% or 0.05%), the sequence of steps in the procedure (labelling followed by fixation or fixation followed by labelling), and use of fixation. The expression of IL-6R in THP-1 cells using different immunolabeling protocols is displayed in Figure 4.2, in which controls consisted of unstained cells. Fixation was conducted before immunolabeling in the experiments from Figure 4.2 A through D, while Figure 4.2E corresponds to experiments in which cells were first immunolabeled and then fixed, and Figure 4.2F demonstrates experiments in which cells were only stained, but not fixed. All experiments used the permeabilizing agent Triton X-100, except the experiments showed in Figure 4.2A, in which cells were not permeabilized, and in Figure 4.2D, in which cells were permeabilized with methanol.

For the majority of the tested protocols, the expression of IL-6R was low, with a maximum of approximately 5% of THP-1 cells labelled. Among these protocols, the highest IL-6R expression was detected when the sequence consisted of immunolabeling prior to fixation, achieving an IL-6R positivity of 21.8%. This observation suggested that the fixation stage could affect the IL-6R-anti-IL-6R antibody interaction or partially degrade the IL-6R protein. Most of the experiments in which the cells were permeabilized showed a higher rate of IL-6R positivity.

Following optimization of the immunolabelling protocol in THP-1 cells, flow cytometry was used to assess the protein expression of IL-6R in primary human retinal endothelial cells isolates from five donors (three males and two females, aged 40 to 77 years, with a median of 55 years at death) and a human retinal endothelial cell line. Non-permeabilized and permeabilized conditions were employed to detect surface and intracellular IL-6R expression, respectively. The percentage of IL-6R was calculated on cells that were positive for CD31, an endothelial cell marker. For the cell line, mean IL-6R expression from four independent experiments was 11.1% (range of 0.9% to 26.6%) in non-permeabilized cells, and 41.3% (range of 4.5% to 89.6%) in permeabilized cells (Figure 4.3).

Figure 4.2. Interleukin-6 receptor protein expression in THP-1 monocytes

Flow cytometry plots showing THP-1 monocytes immunolabelled with protocol modifications: (A) non-permeabilized cells fixed with 4% PFA; (B) Triton X-100-permeabilized cells fixed with 4% PFA; (C) Triton X-100-permeabilized cells fixed with 0.5% PFA; (D) methanol-permeabilized cells fixed with 4% PFA; (E) Triton X-100-permeabilized cells post-fixed with 4% PFA; and (F) permeabilized cells that were not fixed. **Abbreviation**: APC-A = allophycocyanin area.

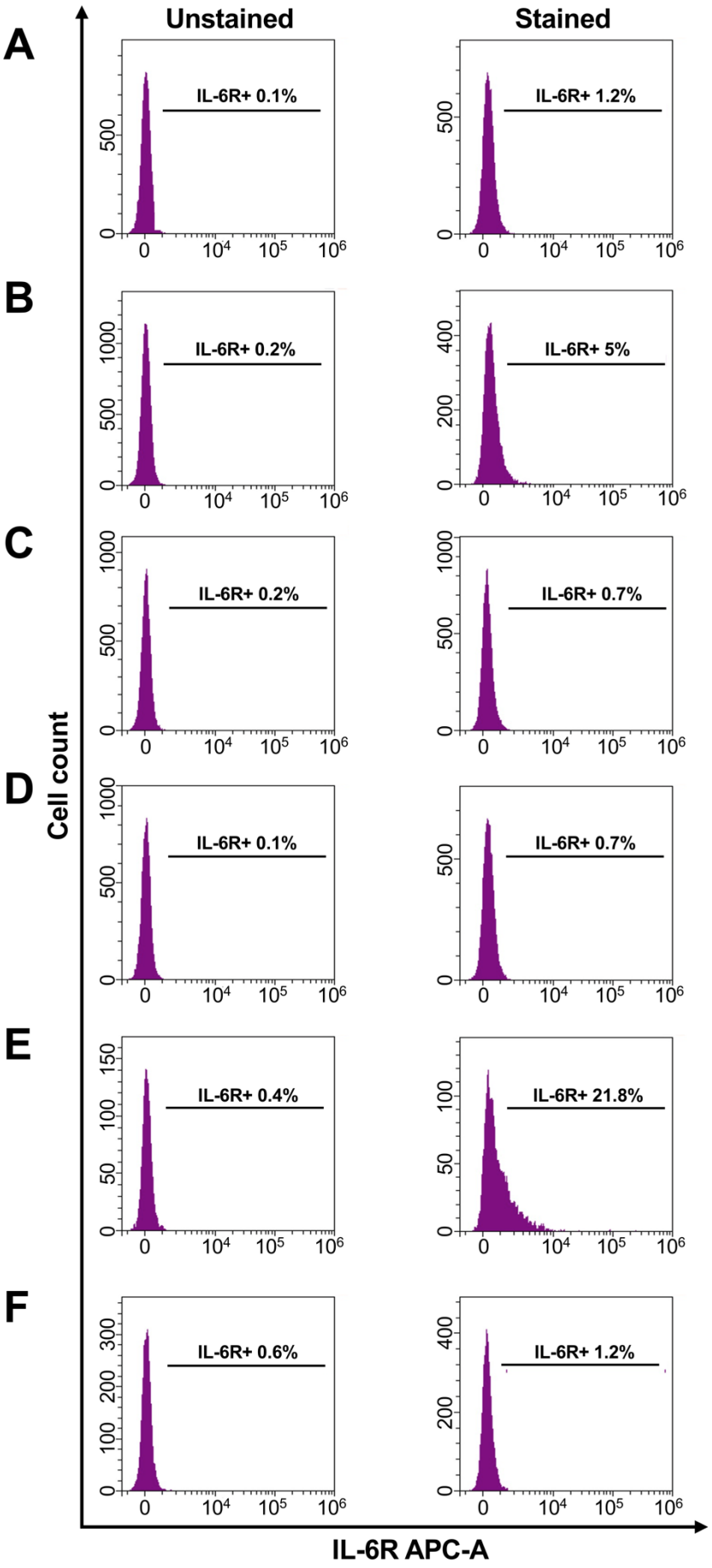
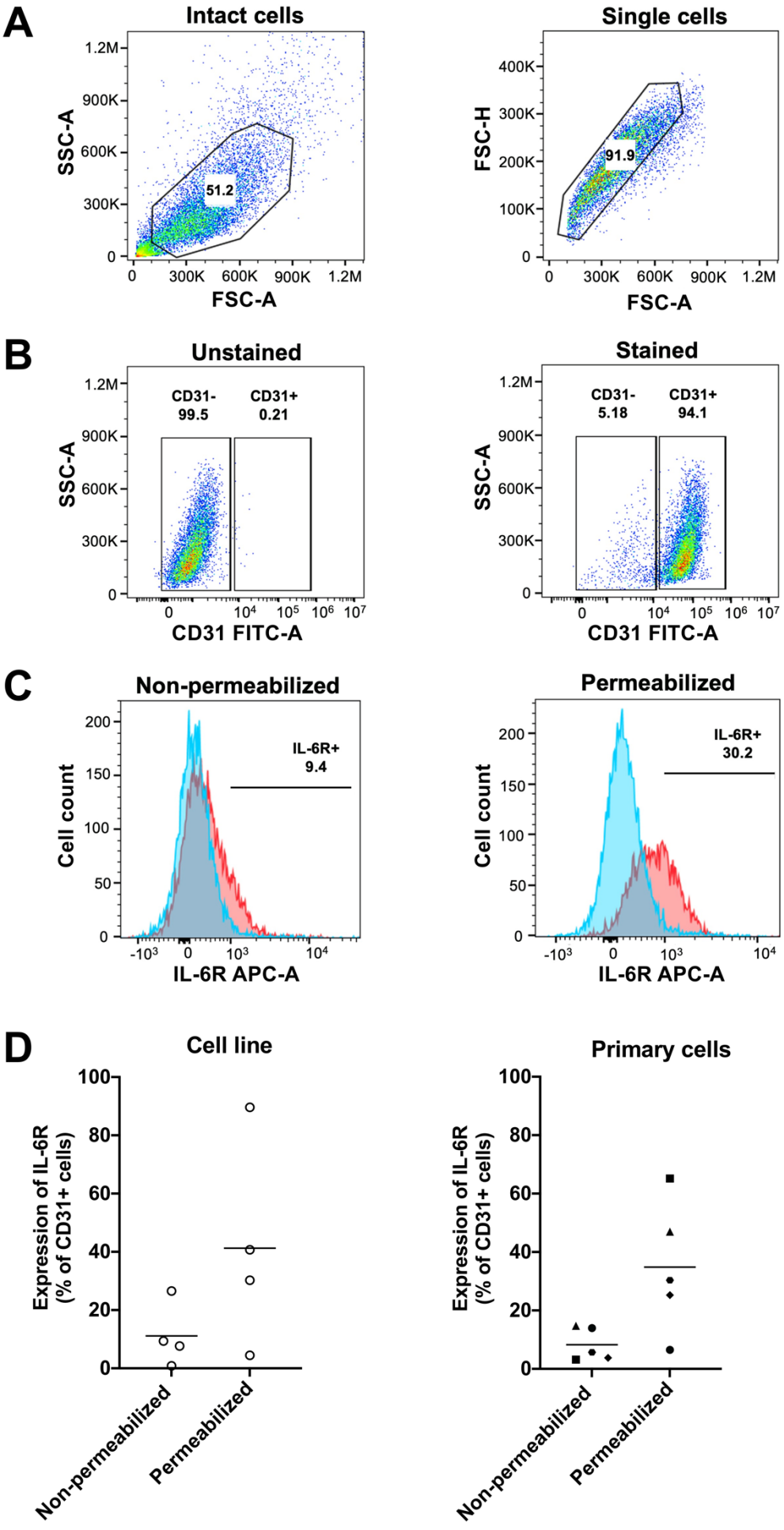

A
Unstained
Stained
IL-6R+ 0.1%
IL-6R+ 1.2%
B
IL-6R+ 0.2%
IL-6R+ 5%
C
IL-6R+ 0.2%
IL-6R+ 0.7%
D
IL-6R+ 0.1%
IL-6R+ 0.7%
E
IL-6R+ 0.4%
IL-6R+ 21.8%
F
IL-6R+ 0.6%
IL-6R+ 1.2%
Cell count
IL-6R APC-A

Figure 4.3. Interleukin-6 receptor protein expression in human retinal endothelial cells

IL-6R protein expression in human retinal endothelial cells. (A-C) Representative flow cytometry plots: (A) Debris and doublets were excluded based on forward scatter (FSC) and side scatter (SSC) properties. (B) Expression of IL-6R was assessed for the CD31-positive cell population relative to unstained controls. (C) Non-permeabilized and permeabilized cells CD31-positive cells were studied: red histograms represent IL-6Rα-positive CD31-positive cells and blue histograms represent unstained controls. (D) Percentage of CD31-positive cells expressing IL-6R for the expanded retinal endothelial cell isolate and primary retinal endothelial cell isolates from 5 individual donors. Crossbars indicate mean. **Abbreviations**: APC-A = allophycocyanin area; FITC-A = fluorescein isothiocyanate area; FSC-A = forward scatter area; FSC-H = forward scatter height; SSC-A = side scatter area.


A
Intact cells
SSC-A
FSC-A
51.2
Single cells
FSC-H
FSC-A
91.9
B
Unstained
CD31-
99.5
CD31+
0.21
SSC-A
CD31 FITC-A
Stained
CD31-
5.18
CD31+
94.1
SSC-A
CD31 FITC-A
C
Non-permeabilized
IL-6R+
9.4
Cell count
IL-6R APC-A
Permeabilized
IL-6R+
30.2
Cell count
IL-6R APC-A
D
Cell line
Expression of IL-6R
(% of CD31+ cells)
Non-permeabilized
Permeabilized
Primary cells
Expression of IL-6R
(% of CD31+ cells)
Non-permeabilized
Permeabilized

In non-permeabilized primary cells, mean IL-6R expression was 8.3%, ranging from 3.15% to 14.8%, while mean expression in permeabilized primary cells was 34.8%, varying from 6.5% to 65.2%. Overall, these results indicate that IL-6R protein is produced by human retinal endothelial cells, with generally lower expression on the cell surface and a higher level intracellularly.

4.2.2 Differential expression of the interleukin-6 receptor in activated human retinal endothelial cells

To study the effect of inflammatory mediators on the expression of IL-6R in human retinal endothelial cells, TNF-α, IL-1β, IL-6, IL-17, IL-22, IFN-γ, VEGF-A and LPS were applied to the human retinal endothelial cell line for 4 and 24 hours. The expression of ICAM-1 transcript was assessed as a marker of endothelial activation following each treatment, as ICAM-1 has previously been shown to be upregulated on human retinal endothelial cells by TNF-α and IL-1β.[251, 490, 677]

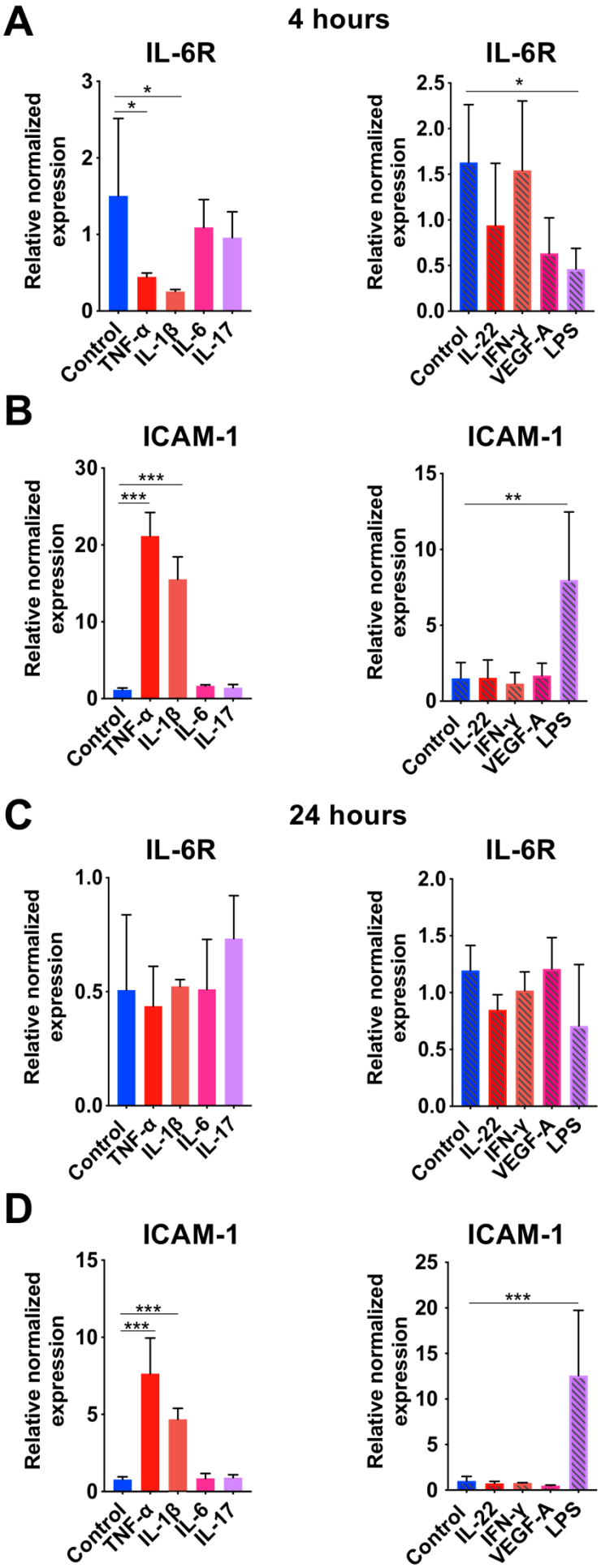
After 4 hours, IL-6R expression decreased with TNF-α (p = 0.03) and IL-1β (p = 0.01) exposure, as well as with LPS (p = 0.04), while ICAM-1 upregulation occurred with the same treatments (Figure 4.4 A and B). At 24 hours, no differences in IL-6R expression were observed between treatment and controls, whereas the upregulation of ICAM-1 induced by TNF-α, IL-1β and LPS persisted (Figure 4.4 C and D). Interleukin-6, IL-17, IL-22, IFN-γ and VEGF-A did not alter IL-6R expression at either time point, nor did they lead to an increase in ICAM-1 expression. These data demonstrate an early downregulation of IL-6R by TNF-α, IL-1β and LPS in activated human retinal endothelial cells.

4.2.3 *In silico* prediction of transcription factors that regulate interleukin-6 receptor gene expression

The methodological approach for identifying transcription factors involved in IL-6R gene regulation in human retinal endothelial cells is presented in Figure 4.5. The IL-6R promoter region was defined as the first exon plus 1000 bp upstream of this exon. Using JASPAR 2020, a database of curated transcription factor binding profiles,[648] 152 binding sites for different transcription factors were localized to this regulatory region (Appendix, Table). A list of transcription factors corresponding to these binding sites was cross-referenced with the transcriptome of the human retinal endothelial cell (RNA-seq with read depth of approximately 60 million)[483] to search for transcription factors that could be involved in the regulation of IL-6R in human retinal endothelial cells. This identified 66

Figure 4.4. Expression of interleukin-6 receptor and intercellular adhesion molecule-1 transcript in activated human retinal endothelial cells

Graphs showing normalized expression of IL-6R and ICAM-1, calculated relative to stable reference genes, in human retinal endothelial cells treated with TNF-α (red; 10 ng/mL), IL-1β (light red; 10 ng/mL), IL-6 (pink; 20 ng/mL), IL-17 (violet; 100 ng/mL), IL-22 (red with stripes; 100 ng/mL), IFN-γ (light red with stripes; 10 ng/mL), VEGF-A (pink with stripes; 100 ng/mL), LPS (violet with stripes; 10 μg/mL), or fresh medium control (blue) for 4 hours (A and B) or 24 hours (C and D). Bars indicate mean, and error bars indicate standard deviation (n = 3-4 endothelial cell monolayers per condition). Reference genes were RPLP0 and YHWAZ for TNF-α, IL-1β, IL-6, IL-17 treatments, and B2M and ALAS1 for IL-22, IFN-γ, VEGF-A and LPS treatments. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.


A
4 hours
IL-6R
Relative normalized expression
Control
TNF-α
IL-1β
IL-6
IL-17
IL-22
IFN-γ
VEGF-A
LPS
B
ICAM-1
C
24 hours
D

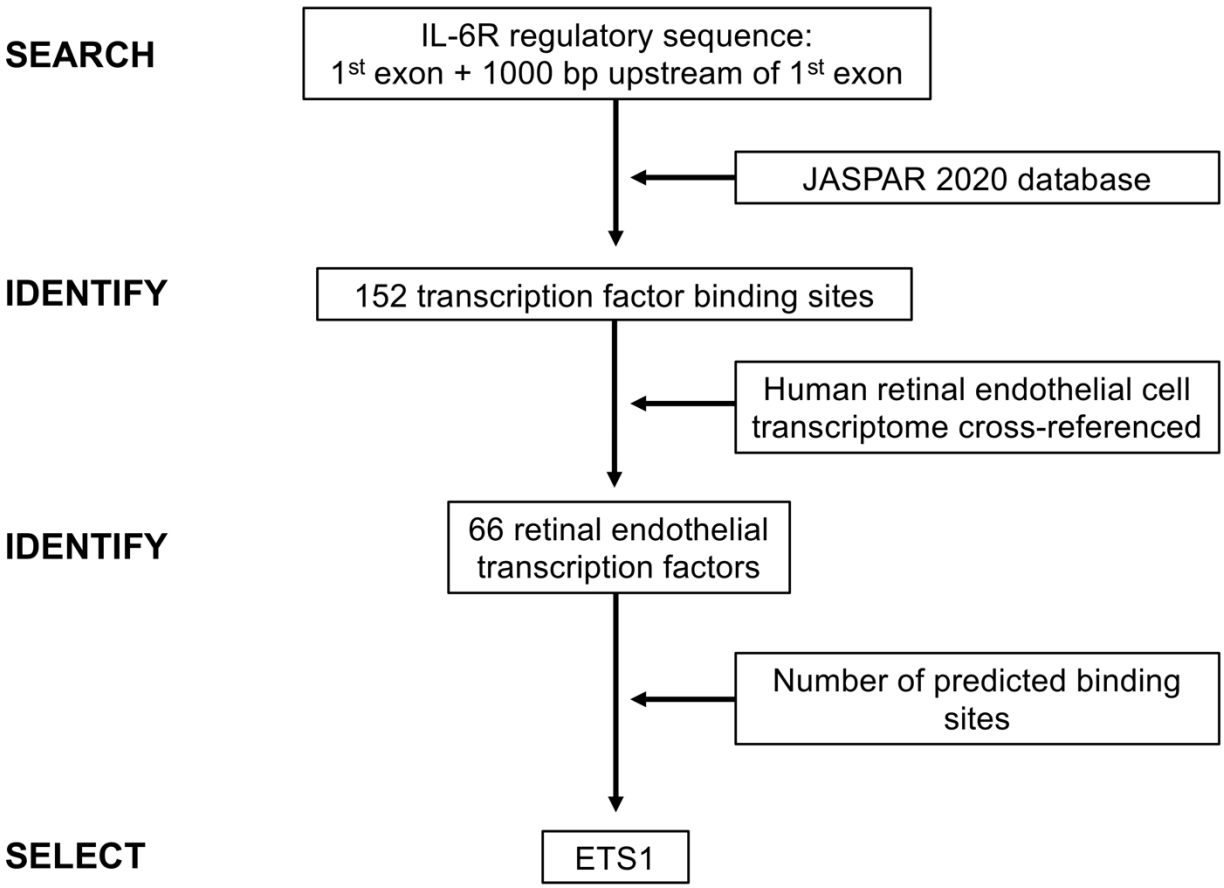


Figure 4.5. Identification of ETS1 as a transcription factor potentially involved in interleukin-6 receptor gene regulation in human retinal endothelial cells

The regulatory sequence of IL-6R was inputted into JASPAR 2020 and results were cross-referenced with a human retinal endothelial cell transcriptome generated by RNA sequencing. Transcription factors were ranked based on number of binding sites predicted by JASPAR 2020 and read count in the transcriptome, leading to the selection of ETS1 for *in vitro* evaluation.

transcription factors (Table 4.1).

A ranking of the transcription factors was performed, based on the number of putative binding sites obtained from JASPAR 2020. If two or more transcription factors had the same number of predicted binding sites, they were sorted by the read counts from the human retinal endothelial transcriptome. The top-ranked transcription factor was ETS1, with 10 transcription binding sites predicted by JASPAR 2020, and an average read count of 186 from the RNA-seq transcriptome. On this basis, as well as the reported association with inflammation,[678-683] ETS1 was selected for an *in vitro* study of IL-6R gene regulation. Table 4.2 shows the predicted ETS1 binding sites in the IL-6R gene promoter.

4.2.4 Effect of ETS1 RNA interference on interleukin-6 receptor gene expression in human retinal endothelial cells

Knockdown of ETS1 in the human retinal endothelial cell line was performed using siRNA to assess if the *in silico* projection of IL-6R regulation by ETS1 would correspond to the situation *in vitro*. Two siRNAs targeting ETS1 (ETS1 siRNA 1, targeting transcript variants 1, 2 and 3; and ETS1 siRNA 2, targeting transcript variants 1, 2 and 4) and a negative control non-targeted siRNA were transfected into human retinal endothelial cells. Additional controls included the Opti-MEM™ medium alone and the transfection reagent, lipofectamine, but the primary comparison of the ETS1-targeted siRNA was against the non-targeted siRNA. Following the transfection, expression of ETS1 and IL-6R was assessed via RT-qPCR. In three independent experiments, human retinal endothelial cells transfected with ETS1 siRNA 1 and 2 had decreased IL-6R expression in comparison with the negative control siRNA, achieving statistical significance in two experiments (Figure 4.6).

In the experiments depicted in Figure 4.6 A and B, there was a 2.3-fold reduction in the expression of IL-6R in cells transfected with ETS1 siRNA 1 and a 2.1-fold reduction in cells transfected with ETS1 siRNA 2, in comparison with cells transfected with the negative control siRNA. A non-significant decrease in IL-6R expression in both ETS1 siRNA groups was observed in the experiment presented in Figure 4.6C. Differential gene expression was presented as fold change relative to negative control siRNA. This was done to account for variations in expression levels across experiments,

Table 4.1. List of transcription factors predicted to regulate the interleukin-6 receptor promoter in human retinal endothelial cells

Transcription factors are ranked firstly on the basis of binding site count predicted by JASPAR 2020, and secondly by the read counts from the RNA-seq transcriptome.

Abbreviation	Transcription factor	Number of binding sites	Read count
ETS1	ETS proto-oncogene 1, transcription factor	10	185.8
GATA2	GATA binding protein 2	7	32.7
NFIX	Nuclear factor I X	7	7.9
THAP1	THAP domain containing 1	6	11.2
SP1	Sp1 transcription factor	5	88.5
SOX18	SRY-box 18	5	19
ZEB1	Zinc finger E-box binding homeobox 1	4	167.6
SP3	Sp3 transcription factor	4	81.7
TCF3	Transcription factor 3	4	72.3
NFIC	Nuclear factor I C	4	23.4
KLF2	Krüppel-like factor 2	4	7.2
CEBPB	CCAAT enhancer binding protein beta	4	5.7
JUNB	JunB proto-oncogene, AP-1 transcription factor subunit	3	33.2
FOSL1	FOS like 1, AP-1 transcription factor subunit	3	31
MEIS1	Meis homeobox 1	3	7.3
ETV4	ETS variant 4	3	6
TCF4	Transcription factor 4	2	531.9
KLF6	Krüppel-like factor 6	2	221.3
MAZ	MYC associated zinc finger protein	2	81
YY1	YY1 transcription factor	2	79.2
TFE3	Transcription factor binding to IGHM enhancer 3	2	47.2

Abbreviation	Transcription factor	Number of binding sites	Read count
NFKB2	Nuclear factor kappa B subunit 2	2	43.2
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region	2	42.3
JUND	JunD proto-oncogene, AP-1 transcription factor subunit	2	29.7
ZBTB14	Zinc finger and BTB domain containing 14	2	15.6
CEBPD	CCAAT enhancer binding protein delta	2	11.9
SNAI1	Snail family transcriptional repressor 1	2	11.7
MZF1	Myeloid zinc finger 1	2	11.2
ZNF354C	Zinc finger protein 354C	2	10.9
SNAI2	Snail family transcriptional repressor 2	2	9.4
EGR1	Early growth response 1	2	2.7
FLI1	Fli-1 proto-oncogene, ETS transcription factor	1	185.2
ELK4	ETS transcription factor ELK4	1	177
FOSL2	FOS like 2, AP-1 transcription factor subunit	1	175.5
ETS2	ETS proto-oncogene 2, transcription factor	1	91.2
KLF3	Krüppel-like factor 3	1	83.9
ZNF148	Zinc finger protein 148	1	82
E2F4	E2F transcription factor 4	1	61.7
KLF10	Krüppel-like factor 10	1	53.8
SREBF2	Sterol regulatory element binding transcription factor 2	1	41.5
CEBPG	CCAAT enhancer binding protein gamma	1	40.6
NFATC2	Nuclear factor of activated T-cells 2 interacting protein	1	38.2
USF1	Upstream transcription factor 1	1	36
RFX7	Regulatory factor X7	1	31.5

Abbreviation	Transcription factor	Number of binding sites	Read count
FOXO3	Forkhead box O3	1	27.4
ZNF263	Zinc finger protein 263	1	27.2
SREBF1	Sterol regulatory element binding transcription factor 1	1	26.4
NR2C1	Nuclear receptor subfamily 2 group C member 1	1	25.8
TFEC	Transcription factor EC	1	25.6
IRF7	Interferon regulatory factor 7	1	22.5
ZNF75D	Zinc finger protein 75D	1	20.4
ELK1	ETS transcription factor ELK1	1	15.9
FOXC1	Forkhead box C1	1	15.1
KLF11	Krüppel-like factor 11	1	14.3
KLF16	Krüppel-like factor 16	1	12.2
MSX1	Msh homeobox 1	1	9.3
NRF1	Nuclear respiratory factor 1	1	8.6
FOXL1	Forkhead box L1	1	8.5
E2F6	E2F transcription factor 6	1	8.2
HOXB3	Homeobox B3	1	6.7
ZBTB26	Zinc finger and BTB domain containing 26	1	6.3
E2F1	E2F transcription factor 1	1	5.8
EBF1	EBF transcription factor 1	1	4.9
HOXD8	Homeobox D8	1	4.5
HOXB6	Homeobox B6	1	2.9
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	1	1.1

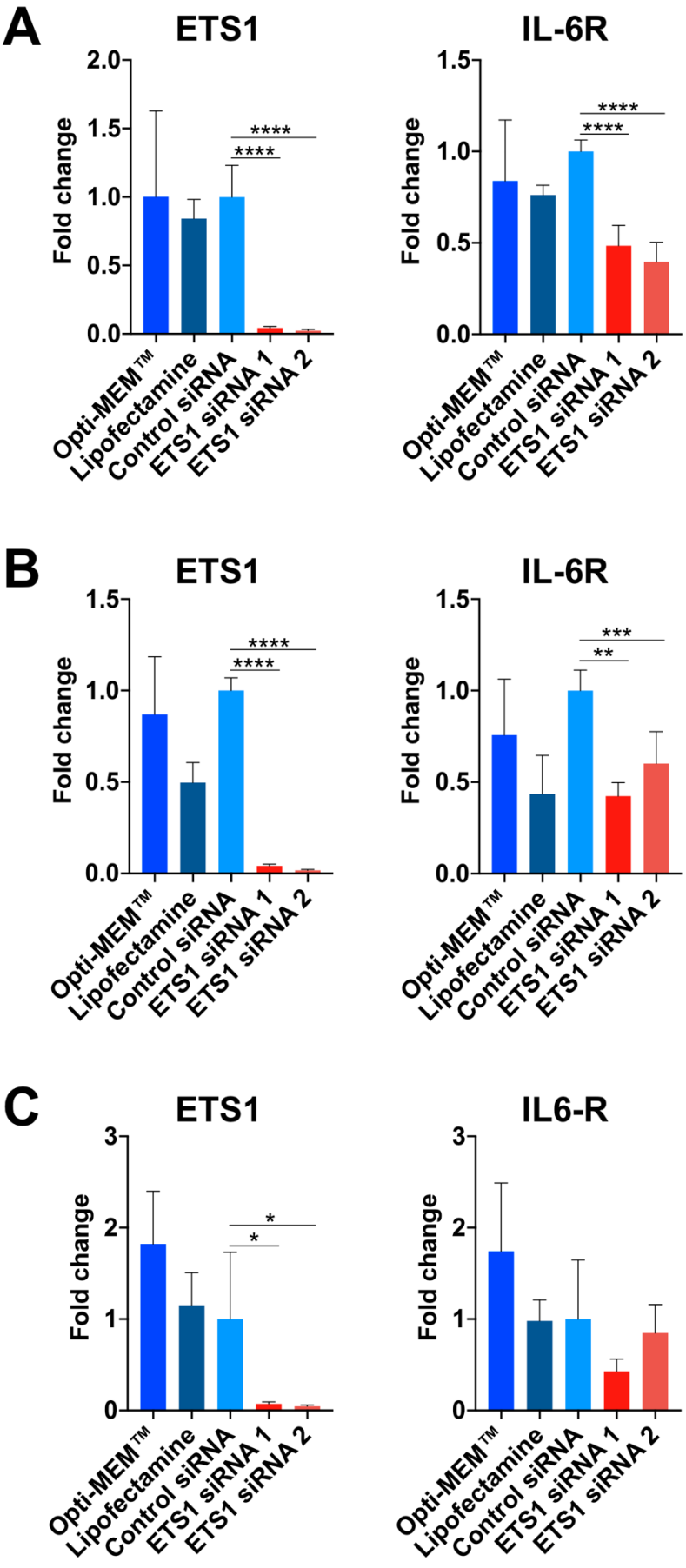
Table 4.2. List of predicted ETS proto-oncogene 1 (ETS1) binding motifs in the interleukin-6 receptor promoter region

The IL-6R promoter region was defined as the first exon plus 1000 bp upstream of this exon, and interrogated with JASPAR 2020.

Predicted motifs of ETS1	Start	End	Strand
CTTCCG	7	12	+
CTTCCT	280	285	-
CTTCCT	342	347	-
TTTCCT	457	462	+
CTTCCT	731	736	-
CATCCG	386	391	+
CATCCG	602	607	-
GTTCCT	148	153	-
GTTCCT	444	449	+
CTTCCC	504	509	-

Figure 4.6. Expression of ETS1 and interleukin-6 receptor expression in human retinal endothelial cells following knockdown of ETS1

Graphs showing fold change for ETS1 and IL-6R transcripts, calculated relative to B2M and PPIA, in human retinal endothelial cells transfected with siRNA targeting ETS1 (red and light red) or control non-targeted siRNA (light blue) in three independent experiments (A, B and C). Expression of transcripts in cells treated with Opti-MEM™ medium only (blue) or lipofectamine (dark blue) without siRNA is also shown. Bars indicate mean, and error bars indicate standard deviation (n = 3-4 endothelial cell monolayers per condition). Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test. *$p < 0.5$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.


A
ETS1
IL-6R
Fold change
2.0
1.5
1.0
0.5
0.0
****
****
Opti-MEM™
Lipofectamine
Control siRNA
ETS1 siRNA 1
ETS1 siRNA 2
B
ETS1
IL-6R
***
**
C
ETS1
IL6-R
3
2
1
0
*
*

which may have been due to slight differences in RNA input across the experiments. The efficiency of the ETS1 knockdown ranged from 93% to 96% for ETS1 siRNA 1, and from 95% to 98% for ETS1 siRNA 2.

In showing that the expression of IL-6R was downregulated by the silencing of ETS1 *in vitro*, these results indicate that ETS1 is involved in IL-6R gene regulation, confirming the *in silico* prediction.

4.3 Discussion

In the work described in this chapter, expression of IL-6R transcript and protein was confirmed in primary human retinal endothelial cell isolates from multiple donors, as well as a retinal endothelial cell line. These results, taken together with the findings reported in Chapter 3, where IL-6 was shown to induce a decrease in monolayer impedance, confirm the presence of functional IL-6R in human retinal endothelial cells. The differential expression of IL-6R in human retinal endothelial cells upon stimulation with inflammatory molecules was demonstrated, and *in silico* prediction of ETS1 as a regulator of IL-6R gene expression in human retinal endothelial cells was verified *in vitro*.

Interleukin-6 receptor is essential to trigger the IL-6 signalling cascade, being expressed on the cell surface, or existing in a soluble form that can activate cells lacking transmembrane receptor.[291] There are 9 transcript variants of the IL-6R gene; the transmembrane domain is encoded by exon 9,[672, 684] which is part of the transcript variants 1, 4, 5, 6, 7 and 9, whereas transcript variants 2, 3 and 8 lack the transmembrane domain. The sIL-6R is generated mostly by limited proteolysis of the transmembrane receptor, but also via alternative RNA splicing.[268-276] The main proteases involved in the generation of sIL-6R via proteolytic cleavage are ADAM17 and ADAM10.[271, 272] It has been proposed that IL-6 impacts the retinal endothelial barrier via trans-signalling[285] during inflammation, when there is an increase in the intraocular levels of sIL-6R associated with infiltrating leukocytes, such as reported in uveitis[290] and diabetic retinopathy.[666]

Some recent studies have demonstrated low levels of IL-6R expression in extraocular human vascular endothelial cells,[685, 686] which were thought not to express it.[291, 687, 688] Also, Ye et al.[289] reported the transcript expression of the mIL-6R in human retinal endothelial cells, suggesting that

sIL-6R was generated from alternative splicing from mIL-6R during hyperglycaemia. The fact that all primary cell isolates studied here expressed mIL-6R and sIL-6R by real-time RT-qPCR, but sIL-6R was not amplified for one isolate in standard RT-PCR, may be attributed to the higher sensitivity of qPCR. In agreement with these findings, Mesquida et al.[284] detected low levels of sIL-6R protein in human retinal endothelial cell-conditioned medium, which were increased under certain growth conditions, but insufficient to induce IL-6 trans-signalling. Here, it was demonstrated that retinal endothelial cells isolated from multiple human donors, as well as a human retinal endothelial cell line, expressed IL-6R, mIL-6R and sIL-6R transcripts at variable levels.

Other authors[284, 285, 288] had attempted to find IL-6R protein in human retinal endothelial cells, but until this work, the finding had not been made. The relatively low mean percentage of IL-6R across the different human retinal endothelial cell isolates is consistent with observations made by Montgomery et al.,[686] who reported relatively low levels of IL-6R expression in human umbilical vein and dermal microvascular endothelial cells. Higher levels of protein expression in permeabilized cells suggests the presence of an intracellular pool, likely in the cytoplasm or endoplasmic reticulum. The downregulation of IL-6R by LPS, added to the low basal expression levels of IL-6R, as well as differences in cell culture techniques and in immunolabeling protocols, might explain why other authors could not demonstrate the IL-6R protein in the human retinal endothelium.

To obtain optimal results when performing flow cytometric analysis, the protocols for fixation, permeabilization and immunolabeling need to be individually adjusted for each cell type, including the best fixation and detergent agents.[689, 690] Time of incubation, the concentration of the reagents and the order of steps all may affect the experimental outcome. Therefore, different protocols were tested to obtain an enhanced identification of the IL-6R protein, and the optimal option involved immunolabelling the cells prior to fixation. Denaturation of antigens may complicate the use of cross-linking fixatives such as paraformaldehyde,[690] and might explain the low detection of IL-6R when fixation preceded immunolabeling. Given that IL-6R levels were found to be highest intracellularly, an optimized identification is achieved by immunolabeling prior to fixation.[691]

The assessment of the differential IL-6R expression in human retinal endothelial cells following treatment with inflammatory stimuli showed an early downregulation by TNF-α, IL-1β and LPS. These results agree with Zegeye et al.,[685] who observed that human umbilical vein endothelial cells treated with TNF-α or LPS downregulated IL-6R. The effect of IL-1β on IL-6R expression has not been evaluated on endothelial cells, and contrasting results have been reported in other cell types, with IL-1β downregulating IL-6R in human peripheral monocytes, and upregulating IL-6R expression in hepatocytes.[692] The decrease of IL-6R expression by TNF-α, IL-1β and LPS may represent a protective mechanism against enhancement during inflammation, since TNF-α and LPS were shown to promote IL-6 secretion in endothelial cells,[693] while IL-1β was shown to stimulate IL-6 secretion in other cell types.[694, 695] Hence, the activation of human retinal endothelial cells by inflammatory stimuli may preclude the detection of the IL-6R expression.

Treatment of retinal endothelial cells with LPS downregulated IL-6R in the short-term, which agrees with previous work that showed decreased expression of IL-6R in human umbilical vein endothelial cells and human peripheral monocytes exposed to LPS.[685, 692] In contrast, LPS upregulated IL-6R in the spleen, liver, kidney and skeletal muscle[696, 697] in animal models, and in a monocytic cell line.[698] Lipopolysaccharide derives from the outer cell membrane of the gram-negative bacteria, and is a common contaminant of cell culture which may impact experimental results.[699] The early downregulation of IL-6R by LPS implies that optimal experimental conditions are required to ensure the detection of IL-6R protein.

The expression of IL-6R was not altered by IL-6 in human retinal endothelial cells; however, IL-6 knockdown upregulated IL-6R and gp130[685] in umbilical vein endothelial cells, and IL-6 exposure downregulated IL-6R in some cell types,[692, 700-702] while increasing its expression in hepatocytes.[692] The impact of IFN-γ on IL-6R expression has been assessed in leukocytes, where decreased IFN-γ diminished IL-6R on a multiple myeloma cell line,[702] and upregulated IL-6R on a monocytic cell line.[698, 703] The effect of IL-17 and VEGF-A on IL-6R differential expression has not been previously established, but the blockage of IL-6R has been associated with a decreased Th17 response,[704-706] reduced IL-17 production[707, 708] and VEGF-A suppression.[289, 709, 710] Classical signalling of IL-6 is

associated with Th22 differentiation,[704] but whether IL-22 alters IL-6R expression has not been previously investigated.

A long list of transcription factors that potentially regulate IL-6R expression was determined *in silico*, and overlapping with a published transcriptome identified over 60 transcription factors potentially involved in IL-6R regulation in human retinal endothelial cells. Based on the number of *in silico* predicted motifs and human retinal transcriptome counts, ETS1 was selected for knockdown in human retinal endothelial cells, with subsequent assessment of IL-6R expression. The two ETS1 siRNAs that were used decreased the expression of IL-6R, both targeting transcript variants 1 and 2 of the 4 existing ETS1 isoforms. The *in vitro* confirmation of the *in silico* prediction indicates that IL-6R is regulated by ETS1, and produces additional evidence that IL-6R is expressed and functional in human retinal endothelial cells.

The archetype of the ETS family of transcription factors is ETS1, which is highly expressed in lymphoid cells, and involved in promotion of Th1 responses and suppression of Th17 differentiation.[711-714] Dysregulation of ETS1 is linked to the development of cancer and autoimmune diseases, such as rheumatoid arthritis, systemic erythematous lupus, and multiple sclerosis.[712, 715, 716] The regulation of numerous cytokines, including IL-2,[713, 717] IL-4,[718] IL-5,[719] IL-8,[720] IL-10,[718] IL-13,[718] IL-17,[718] IL-22,[718] TNF-α,[721] IFN-γ,[721] and TGF-β,[722, 723] has been linked with ETS1, as well as the regulation of signalling pathways following cytokine-receptor ligation.[724-727] ETS1 was shown to promote IL-7Rα[728] and IL-12Rβ2[729] – a sub-component of the IL-12R – expression, regulating the proliferation of and differentiation of T-cells. ETS1 upregulated the chemokine receptor CCR7, involved in tumour progression and metastasis.[730] Interestingly, ETS1 has been implicated in the maintenance of non-ocular endothelial cell barriers upon TNF-α stimulation.[683] Contrasting with the downregulation of IL-6R observed in retinal endothelial cells following ETS1 knockdown, mature Th cells lacking ETS1 maintained IL-6R expression, while gp130 was upregulated in atopic dermatitis.[718] Thus, modulation of ETS1 may be a future therapeutic approach for autoimmune diseases.

New research to build upon the results presented in this chapter include protein studies of IL-6R following ETS1 knockdown experiments, and functional assays to evaluate if IL-6 classical signalling or trans-signalling pathways would be altered following ETS1 silencing in human retinal endothelial cells. For confirmation of ETS1 binding at the IL-6R promoter site, chromatin immunoprecipitation with subsequent sequencing (ChIP-seq) analysis might be used. *In vitro* options to explore the role of ETS1 in IL-6R gene transcription could include protein-binding microarrays, systematic evolution of ligands by exponential enrichment (SELEX), and mechanically-induced trapping of molecular interactions (MITOMI).[731, 732]

In conclusion, the work presented in this chapter confirms that IL-6R is variably expressed by human retinal endothelial cells, and downregulated by some inflammatory mediators. Furthermore, ETS1 is involved in the regulation of IL-6R gene expression in human retinal endothelial cells. It is therefore likely that IL-6R is functional in the retinal endothelium, and that classical IL-6 signaling contributes to disruption of the retinal endothelial barrier during intraocular inflammation. These *in vitro* observations provide a pathophysiological basis for the clinical improvements of refractory uveitic macular oedema and retinal vasculitis that are reported in patients treated with tocilizumab, a monoclonal antibody against IL-6R.[230, 293-296] Future work should explore the downstream results of classical and trans-signalling pathways in human retinal endothelial cells, as well as IL-6R regulation by ETS1 under inflammatory conditions, to evaluate whether targeting ETS1 would be an effective therapy for macular oedema and vascular leakage in uveitis.

Some of the work of this chapter was included in a manuscript submitted to Eye and Vision: Ferreira LB, Ashander LM, Appukuttan B, Ma Y, Williams KA, Best G, Smith JR. Human retinal endothelial cells express functional interleukin-6 receptor. 2023 (under review).

CHAPTER 5: IMPACT OF INFLAMMATORY CYTOKINES ON JUNCTIONAL MOLECULE EXPRESSION AND VIABILITY OF HUMAN RETINAL ENDOTHELIAL CELLS

5.1. Introduction

Inflammatory cytokines involved in the promotion of uveitis, such as TNF-α, IL-1β and IL-6, have been demonstrated to foster the dysfunction of retinal endothelial cells, including affecting their barrier function.[117, 124, 125, 205, 206, 208, 250, 251, 253, 285] In Chapter 3, TNF-α, IL-1β and IL-6 were shown to impair the electrical impedance of human retinal endothelial cell monolayers. Retinal endothelial cells were previously shown to express TNF-α and IL-1β receptors, while the expression of the IL-6R by these cells was demonstrated in Chapter 4.[245, 246, 733] Questions remain on what are the mechanisms through which TNF-α, IL-1β and IL-6 disrupt the barrier function of the human retinal endothelial cell monolayers.

Among the potential molecular mechanisms behind impairment of the retinal endothelial barrier, a major one to be considered is the impact of the inflammatory cytokines on the intercellular junctions, possibly altering the expression, phosphorylation or localization of the junctional components.[25, 36] Cellular mechanisms responsible for the breakdown of the retinal endothelial barrier by the cytokines include effects on cell viability, potentially eliciting cellular death processes, such as apoptosis and necrosis.[25, 109] Changes in junctional complexes and cytotoxicity would both be expected to reduce the integrity of the retinal endothelium, leading it to become hyperpermeable and favouring fluid and solute accumulation within the retina.

To evaluate factors that might lead to the weakening of the electrical impedance of the human retinal endothelial cell monolayers driven by TNF-α, IL-1β and IL-6, as demonstrated in Chapter 3, an assessment of the impact of these cytokines on the expression of junctional molecules and cell viability was conducted by RT-qPCR, cell immunolabeling, cell quantitation assays and flow cytometric studies. The well-characterized human retinal endothelial cell line that was used for some of the work presented in Chapters 3 and 4,[38] was utilized in these experiments.

5.2. Results

5.2.1. Effect of inflammatory cytokines on expression of junctional molecules by human retinal endothelial cells

To investigate the effect of TNF-α, IL-1β and IL-6 on the expression of junctional molecules,

confluent human retinal endothelial cells were treated with cytokine, or fresh medium alone as a control, for 24 and 48 hours, and ZO-1, VE-cadherin, claudin-5 and β-catenin transcripts were assessed via RT-qPCR. The 24-hour treatment with TNF-α significantly decreased the levels of ZO-1 transcript (p = 0.01) and VE-cadherin transcript (p = 0.04) in comparison with the control; TNF-α reduced VE-cadherin expression by 51%, while ZO-1 was downregulated by 62% (Figure 5.1A). No differences were seen at 48 hours post-treatment (Figure 5.1B), and there were no changes in claudin-5 and β-catenin transcripts at either time point. Interleukin-1β and IL-6 treatments did not induce significant changes in the transcript expression of any of the junctional molecules at 24 or 48 hours after treatment (Figure 5.1 A and B). Since expression of ZO-1 in human retinal endothelial cells had a more marked reduction after TNF-α stimulation than VE-cadherin, ZO-1 was selected for an immunolabelling study.

Cytoimmunofluorescence experiments were undertaken to assess any changes in ZO-1 protein pattern and distribution in confluent cells after stimulation with TNF-α, IL-1β and IL-6 for 24 and 48 hours. Slight differences were observed in the intensity of nuclear and background fluorescence across these experiments. Human retinal endothelial cells from controls were confluent, with a marked ZO-1 fluorescence at the cell border, and mild cytoplasmic and nuclear fluorescence at 24 hours, which became more intense at 48 hours (Figures 5.2, 5.3 and 5.4).

Tumour necrosis factor-α treatment diminished the fluorescent labelling of ZO-1 at the cell border and mildly disrupted the monolayer architecture after 24 hours (Figure 5.2). After 48 hours of TNF-α exposure, reduced labelling persisted at the cell membrane, with a slight increase in the nuclear and cytoplasmic signal. Moreover, a more pronounced architectural disturbance was present at 48 hours post-TNF-α treatment. Interleukin-1β treatment reduced ZO-1 fluorescent labelling at the cell membrane after 24 and 48 hours, and increased labelling was detected in the cytoplasmic compartment and nucleus, while the disruption of the architecture of the monolayer was also noted (Figure 5.3). After 24 hours of IL-6 exposure, no changes in ZO-1 labelling were seen between the treatment and control groups. However, after 48 hours of stimulation, a slight increase in the intensity of ZO-1-associated fluorescence was observed in the cytoplasm and nuclei of cells, with some retinal

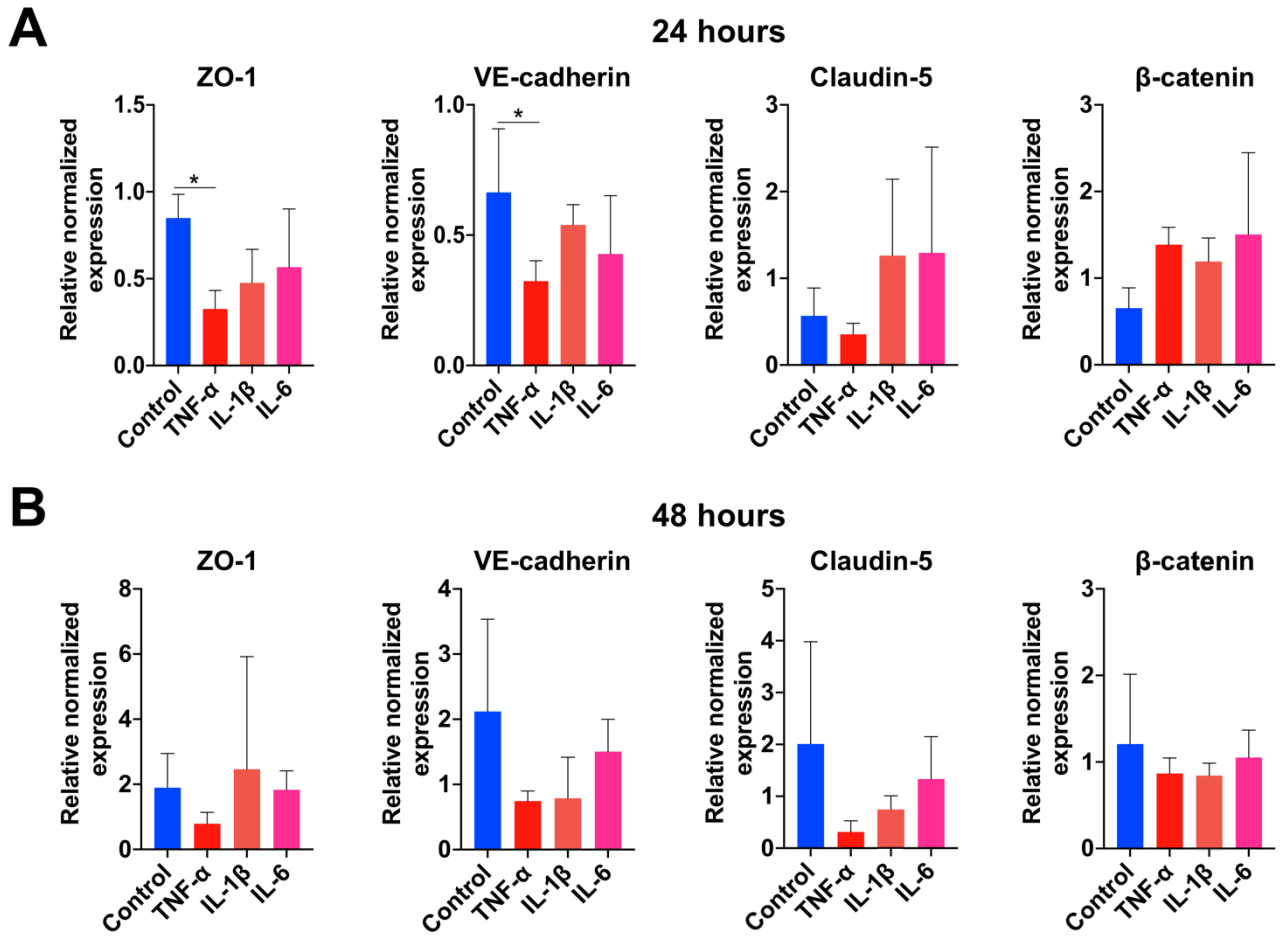


Figure 5.1. Expression of junctional molecule transcripts in activated human retinal endothelial cells

Graphs showing normalized expression of ZO-1, VE-cadherin, claudin-5 and β-catenin, calculated relative to stable reference genes, in human retinal endothelial cells treated with TNF-α (red; 10 ng/mL), IL-1β (light red; 10 ng/mL), IL-6 (pink; 20 ng/mL), or fresh medium control (blue) for 24 hours (A) or 48 hours (B). Bars indicate mean, and error bars indicate standard deviation (n = 4 endothelial cell monolayers per condition). Reference genes were B2M and YWHAZ for 24-hour treatments, and ALAS1 and YWHAZ for 48-hour treatments. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test. *$p < 0.05$.

Figure 5.2. The impact of tumour necrosis factor-α on zona occludens-1 protein expression in human retinal endothelial cells

Fluorescence photomicrographs of confluent human retinal endothelial cells immunolabelled to detect ZO-1 protein 24 and 48 hours after treatment with TNF-α (10 ng/mL) or fresh medium alone. Identically treated cells were immunolabelled in parallel with isotype-matched negative control primary antibody. Alexa Fluor 488 (green) with DAPI nuclear counterstain (blue). Original magnification: 400x.

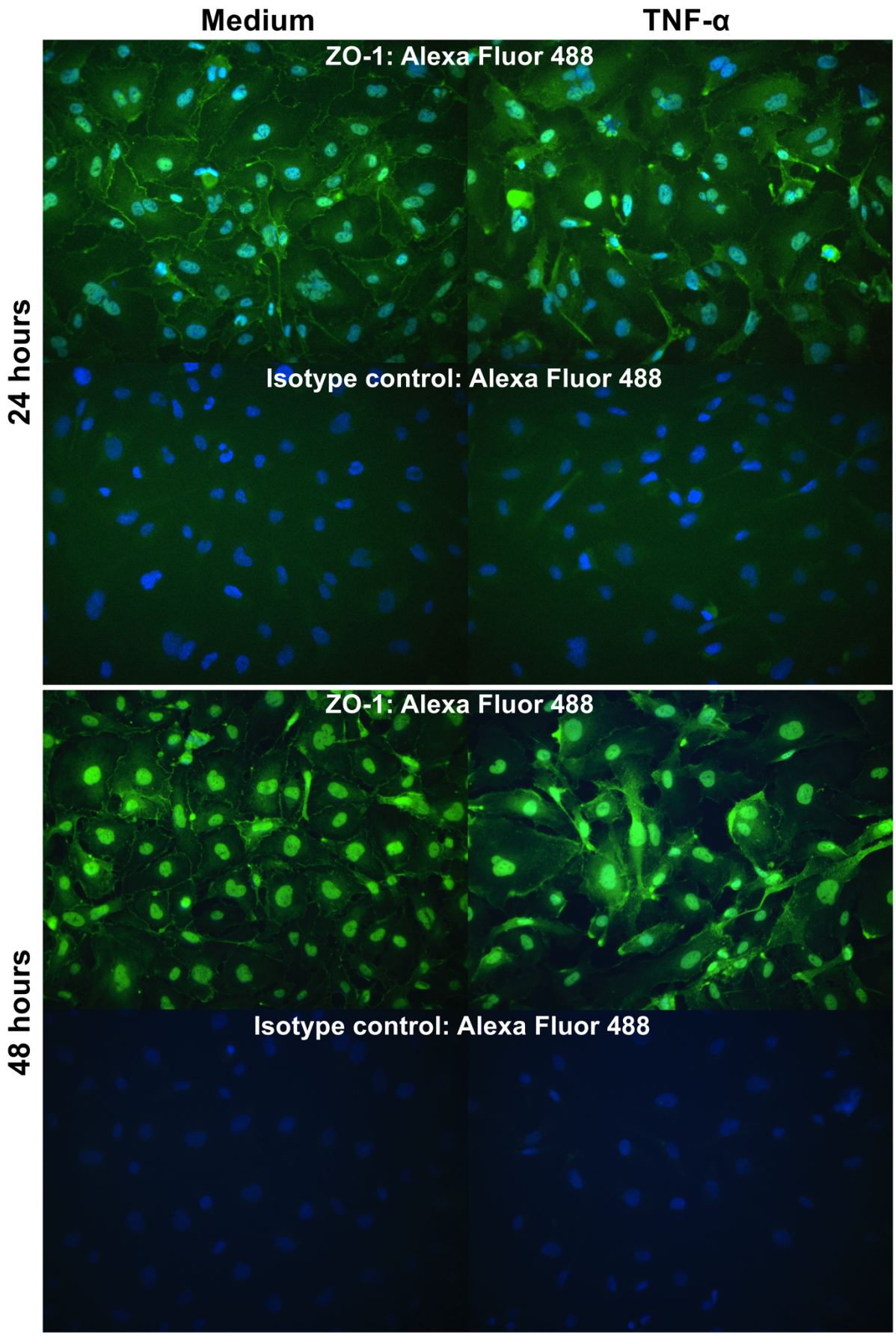

Medium
TNF-α
ZO-1: Alexa Fluor 488
24 hours
Isotype control: Alexa Fluor 488
ZO-1: Alexa Fluor 488
48 hours
Isotype control: Alexa Fluor 488

Figure 5.3. The impact of interleukin-1β on zona occludens-1 protein expression in human retinal endothelial cells

Fluorescence photomicrographs of confluent human retinal endothelial cells immunolabelled to detect ZO-1 protein 24 and 48 hours after treatment with IL-1β (10 ng/mL) or fresh medium alone. Identically treated cells were immunolabelled in parallel with isotype-matched negative control primary antibody. Alexa Fluor 488 (green) with DAPI nuclear counterstain (blue). Original magnification: 400x.

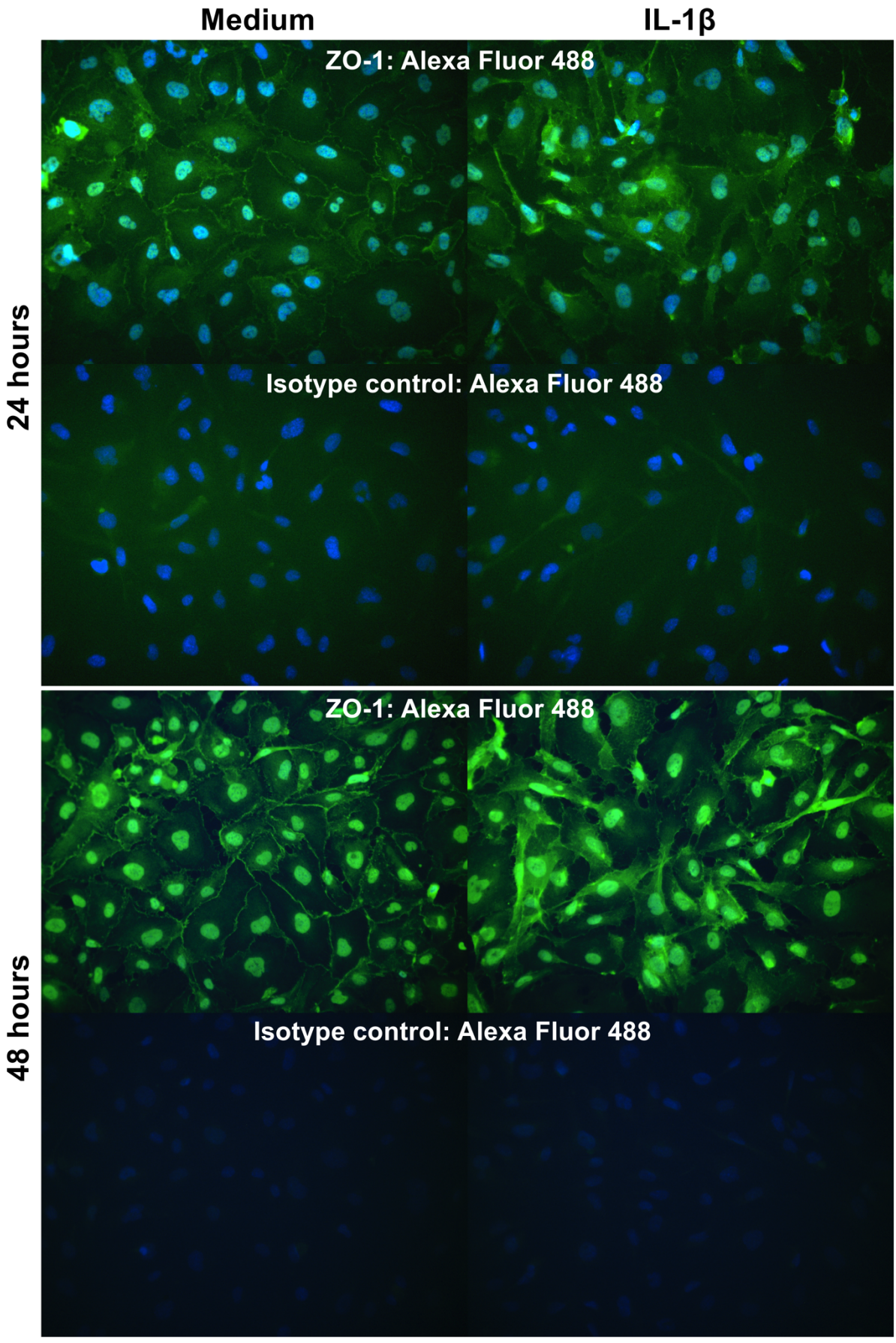
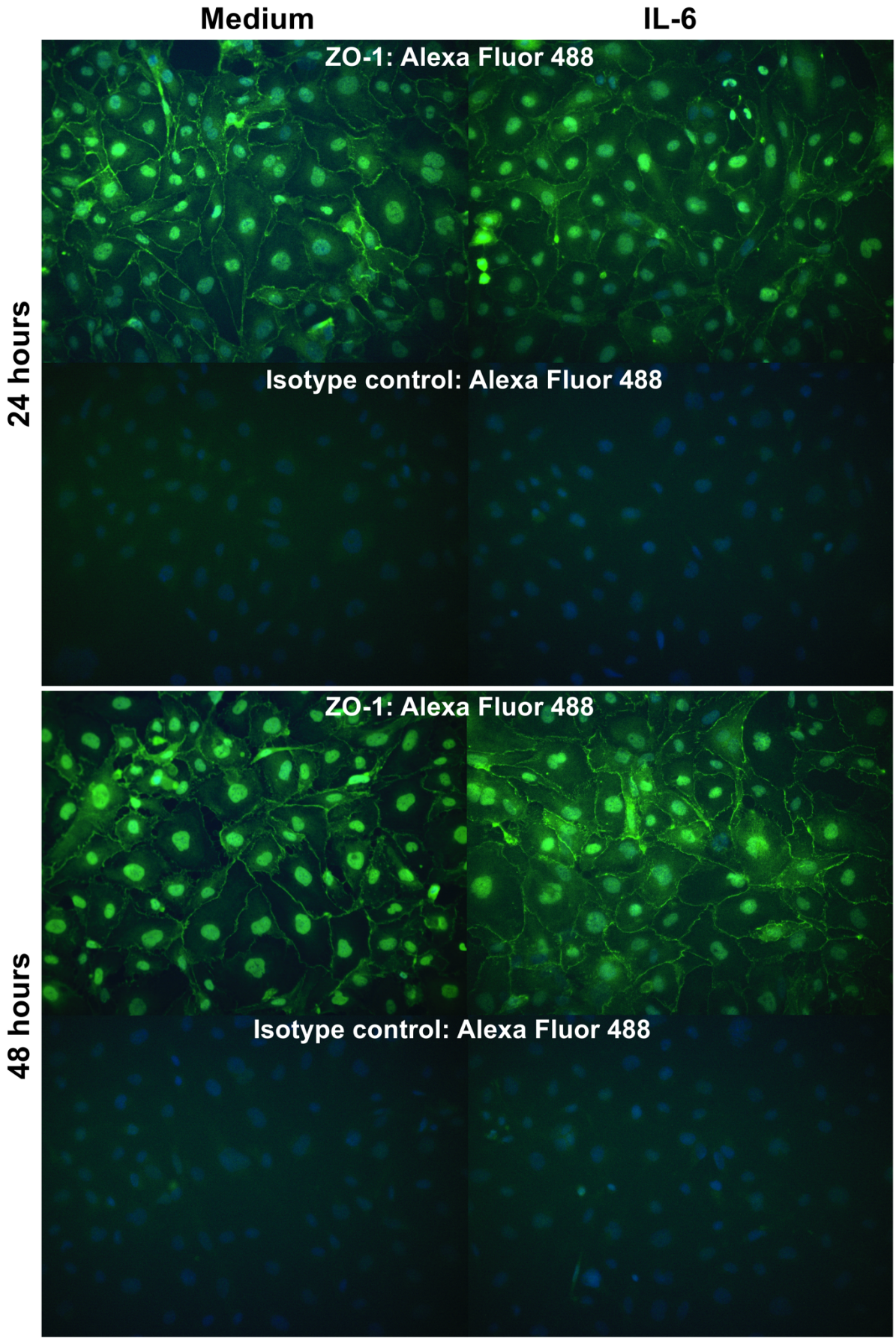

Medium
IL-1β
ZO-1: Alexa Fluor 488
24 hours
Isotype control: Alexa Fluor 488
ZO-1: Alexa Fluor 488
48 hours
Isotype control: Alexa Fluor 488

Figure 5.4. The impact of interleukin-6 on zona occludens-1 protein expression in human retinal endothelial cells

Fluorescence photomicrographs of confluent human retinal endothelial cells immunolabelled to detect ZO-1 protein 24 and 48 hours after treatment with IL-6 (20 ng/mL) or fresh medium alone. Identically treated cells were immunolabelled in parallel with isotype-matched negative control primary antibody. Alexa Fluor 488 (green) with DAPI nuclear counterstain (blue). Original magnification: 400x.


Medium
IL-6
ZO-1: Alexa Fluor 488
24 hours
Isotype control: Alexa Fluor 488
ZO-1: Alexa Fluor 488
48 hours
Isotype control: Alexa Fluor 488

endothelial cells displaying a more elongated shape (Figure 5.4).

In summary, TNF-α was the single cytokine inducing a decrease in ZO-1 and VE-cadherin transcript expression, while TNF-α, IL-1β and, to a lesser degree, IL-6 affected the allocation of ZO-1 protein in confluent human retinal endothelial cells.

5.2.2. Effect of inflammatory cytokines on human retinal endothelial cell survival

To investigate for cytotoxic effects of TNF-α, IL-1β and IL-6 on human retinal endothelial cells, a commercial fluorescence-based DNA-binding assay (CyQUANT NF cell proliferation assay) was used to quantify the number cells grown for 48 hours in multi-well plates (growth area = 0.33 cm^2 per well) with individual cytokines. As well as TNF-α, IL-1β and IL-6, IL-17 was applied to the wells, representing a cytokine that overall did not affect retinal endothelial barrier impedance and therefore would not be expected to alter cell viability. Since seeding number could influence the results of these assays unrelated to cell survival, two different cell densities were plated in wells for the experiments.[734-737]

Over the 48-hour treatment period, TNF-α significantly reduced the number of human retinal endothelial cells present in the wells in comparison to the medium alone control ($p = 0.0004$ at 3,000 cells/well and $p = 0.02$ at 5,000 cells/well) (Figure 5.5A). Likewise, IL-1β decreased cell numbers at both cell densities ($p < 0.0001$) (Figure 5.5B). In contrast, IL-6 and IL-17 did not impact cell quantitation (Figure 5.6 C and D, respectively). To evaluate a dose-response of the effects of TNF-α and IL-1β on cell viability, titrated concentrations of these cytokines were applied to the retinal endothelial cells for 48 hours. The number of retinal endothelial cells decreased across all concentrations of TNF-α and IL-1β treatments. A concentration-dependent effect was observed for TNF-α treated cells, most pronounced at the highest concentration (Figure 5.6A). In retinal endothelial cells treated with IL-1β, the magnitude of the reduction in cell number maintained a relatively stable pattern across all tested concentrations (Figure 5.6B). Cells treated with TNF-α had a maximum drop in cell quantitation of 33%, while IL-1β reduced the number of cells by 27%. Thus, treatment with TNF-α or IL-1β reduced cell number across a range of concentrations, implying cytotoxicity for human retinal endothelial cells, while IL-6 and IL-17 did not influence cell survival.

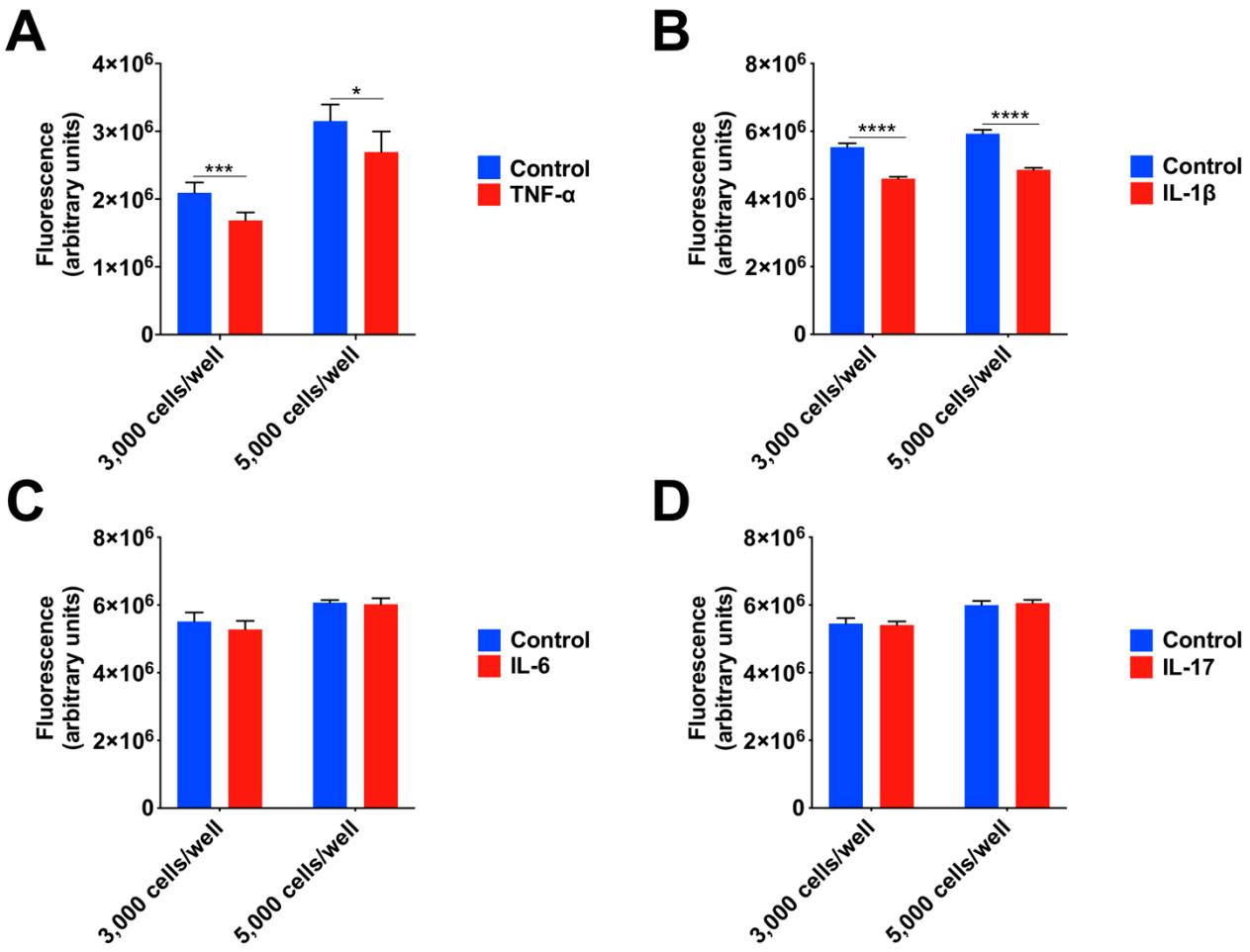


Figure 5.5. Effect of inflammatory cytokines on human retinal endothelial cell numbers

Graphs showing numbers of human retinal endothelial cells quantified in a fluorescence-based, DNA-binding assay after 48-hour treatment with (A) TNF-α (10 ng/mL), (B) IL-1β (10 ng/mL), (C) IL-6 (20 ng/mL) and (D) IL-17 (100 ng/mL), versus fresh medium alone as control. Bars indicate mean, and error bars indicate standard deviation (n = 6 endothelial cell wells per condition). Data were analyzed by unpaired Student's t-test. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$.

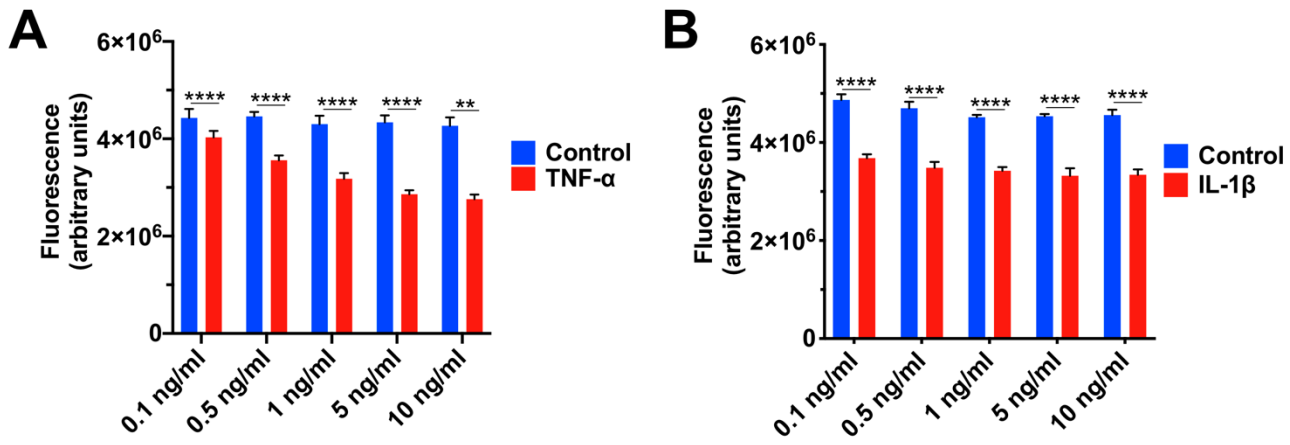


Figure 5.6. Effect of tumour necrosis factor-α and interleukin-1β concentration on human retinal endothelial cell numbers

Graphs showing numbers of human retinal endothelial cells quantified in a fluorescence-based, DNA-binding assay after 48-hour treatment with (A) TNF-α and (B) IL-1β in increasing concentrations, versus fresh medium alone as control. Bars indicate mean, and error bars indicate standard deviation (n = 6 endothelial cell wells per condition). Data were analyzed by unpaired Student's t-test. ** $p < 0.01$; **** $p < 0.0001$.

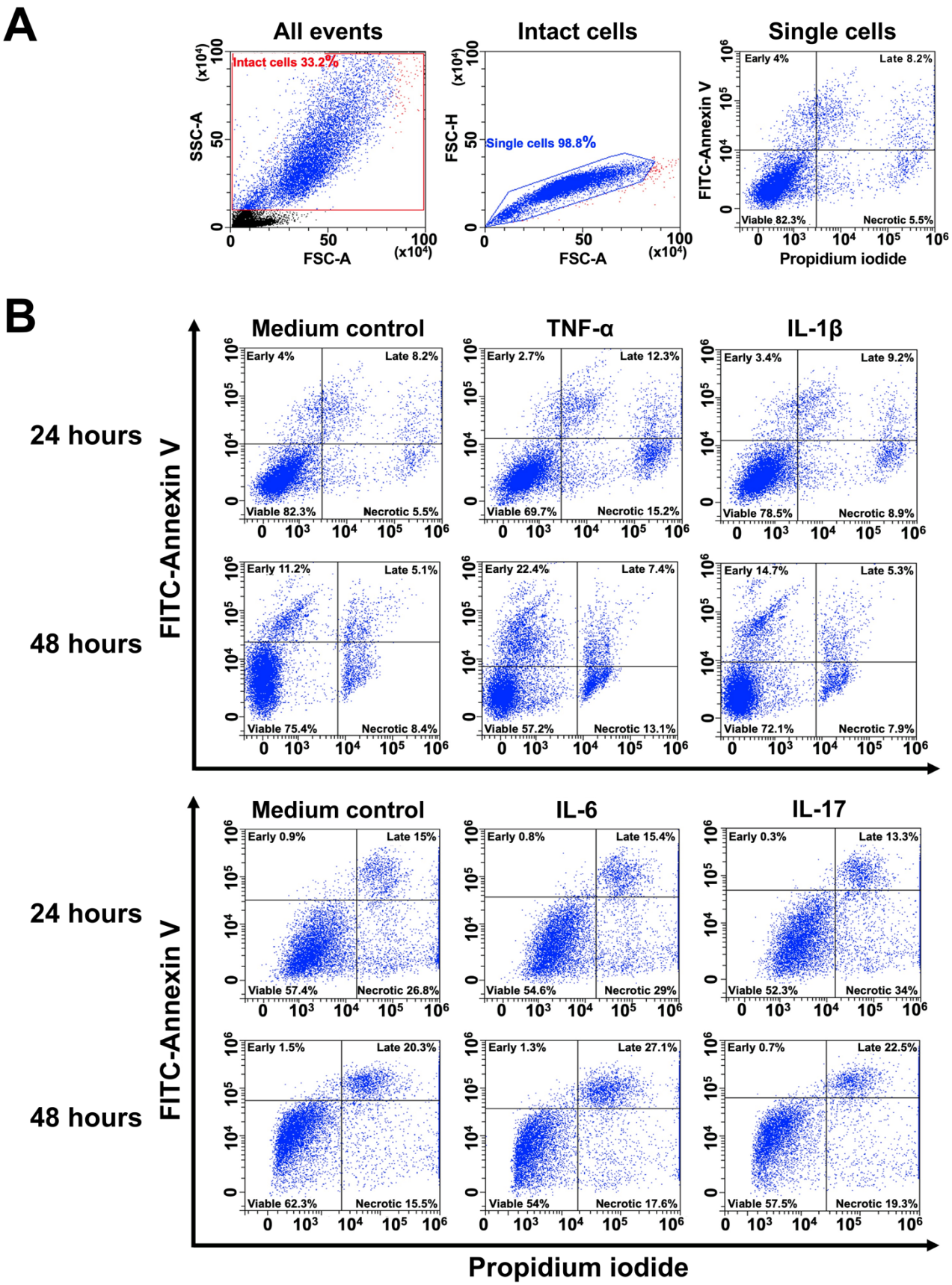
5.2.3. Effect of inflammatory cytokines on apoptosis and necrosis of human retinal endothelial cells

Further investigation of the cytotoxic effects of TNF-α and IL-1β on human retinal endothelial cells involved an assessment of cell viability, apoptosis and necrosis using flow cytometry with FITC-Annexin V and propidium iodide labelling. Annexin V binds to phosphatidylserine, a membrane phospholipid externalized during the apoptotic process.[738, 739] Propidium iodide binds to double-stranded DNA when the cell membrane becomes permeable due to necrosis, but does not bind to viable cells or early apoptotic cells.[740] Late apoptotic cells are double-stained by FITC-Annexin V and propidium iodide. The percentages of viable, apoptotic and necrotic cells were determined using flow cytometry after human retinal endothelial cells were exposed to TNF-α, IL-1β, IL-6 and IL-17, or fresh medium alone, for 24 and 48 hours. Interleukin-6 was included to verify the result obtained in Section 5.2.2, and IL-17 was also again used as a negative control cytokine. Tumour necrosis factor-α- and IL-1β-treated cells had separate controls from cells stimulated with IL-6 and IL-17, and the experiments were independently replicated at least 3 times for each group and time point. The gating strategy aimed to evaluate single cells, selected based on the SSC-A, FSC-A and FSC-H parameters (Figure 5.7).

At 24 hours, the viability of human retinal endothelial cells exposed to TNF-α significantly declined ($p = 0.0003$) and the number of necrotic cells significantly increased ($p = 0.007$), in comparison with controls (Figure 5.8A), resulting in a mean increase of 91% in necrotic cells. At 48 hours, a significant reduction in cell viability of TNF-α-treated cells was observed ($p = 0.003$), while an increase in necrotic cellular percentage did not achieve statistical significance (Figure 5.8C). The mean percentage of reduction in retinal endothelial cell viability after TNF-α treatment for 24 and 48 hours was approximately 15%. Although trending towards a reduction of cell viability and an increase in cellular necrosis rates, IL-1β did not induce significant changes between treated and control retinal endothelial cells at 24 or 48 hours (Figure 5.8 A and C). The exposure of human retinal endothelial cells to IL-6 and IL-17 did not change cell viability or necrosis rates at either time point (Figure 5.8 B and D).

Figure 5.7. Flow cytometric analysis of inflammatory cytokine effects on human retinal endothelial cell viability, apoptosis and necrosis

Human retinal endothelial cells were exposed to TNF-α (10 ng/mL), IL-1β (10 ng/mL), IL-6 (20 ng/mL) and IL-17 (100 ng/mL), or fresh medium alone for 24 and 48 hours, and labelled with FITC-Annexin V and propidium iodide. (A) Representative images demonstrating the gating strategy. Debris and doublets were excluded based on forward and side scatter. (B) Single-cell plots showing gating of viable, early apoptotic, necrotic, and late apoptotic cell populations across different cytokine treatments based on FITC-Annexin V and propidium iodide fluorescence. Representative images of one out of at least 3 independent experiments of 24 and 48-hour time points, using the single-cell gating strategy in different treatment groups. **Abbreviations**: SSC-A = side scatter area; FSC-A = forward scatter area; FSC-H = forward scatter height; early = early apoptotic; late = late apoptotic.


A
All events
Intact cells
Single cells
Intact cells 33.2%
Single cells 98.8%
SSC-A
FSC-H
FSC-A
FITC-Annexin V
Propidium iodide
Early 4%
Late 8.2%
Viable 82.3%
Necrotic 5.5%
B
24 hours
48 hours
Medium control
TNF-α
IL-1β
IL-6
IL-17
Early 2.7%
Late 12.3%
Viable 69.7%
Necrotic 15.2%
Early 3.4%
Late 9.2%
Viable 78.5%
Necrotic 8.9%
Early 11.2%
Late 5.1%
Viable 75.4%
Necrotic 8.4%
Early 22.4%
Late 7.4%
Viable 57.2%
Necrotic 13.1%
Early 14.7%
Late 5.3%
Viable 72.1%
Necrotic 7.9%
Early 0.9%
Late 15%
Viable 57.4%
Necrotic 26.8%
Early 0.8%
Late 15.4%
Viable 54.6%
Necrotic 29%
Early 0.3%
Late 13.3%
Viable 52.3%
Necrotic 34%
Early 1.5%
Late 20.3%
Viable 62.3%
Necrotic 15.5%
Early 1.3%
Late 27.1%
Viable 54%
Necrotic 17.6%
Early 0.7%
Late 22.5%
Viable 57.5%
Necrotic 19.3%

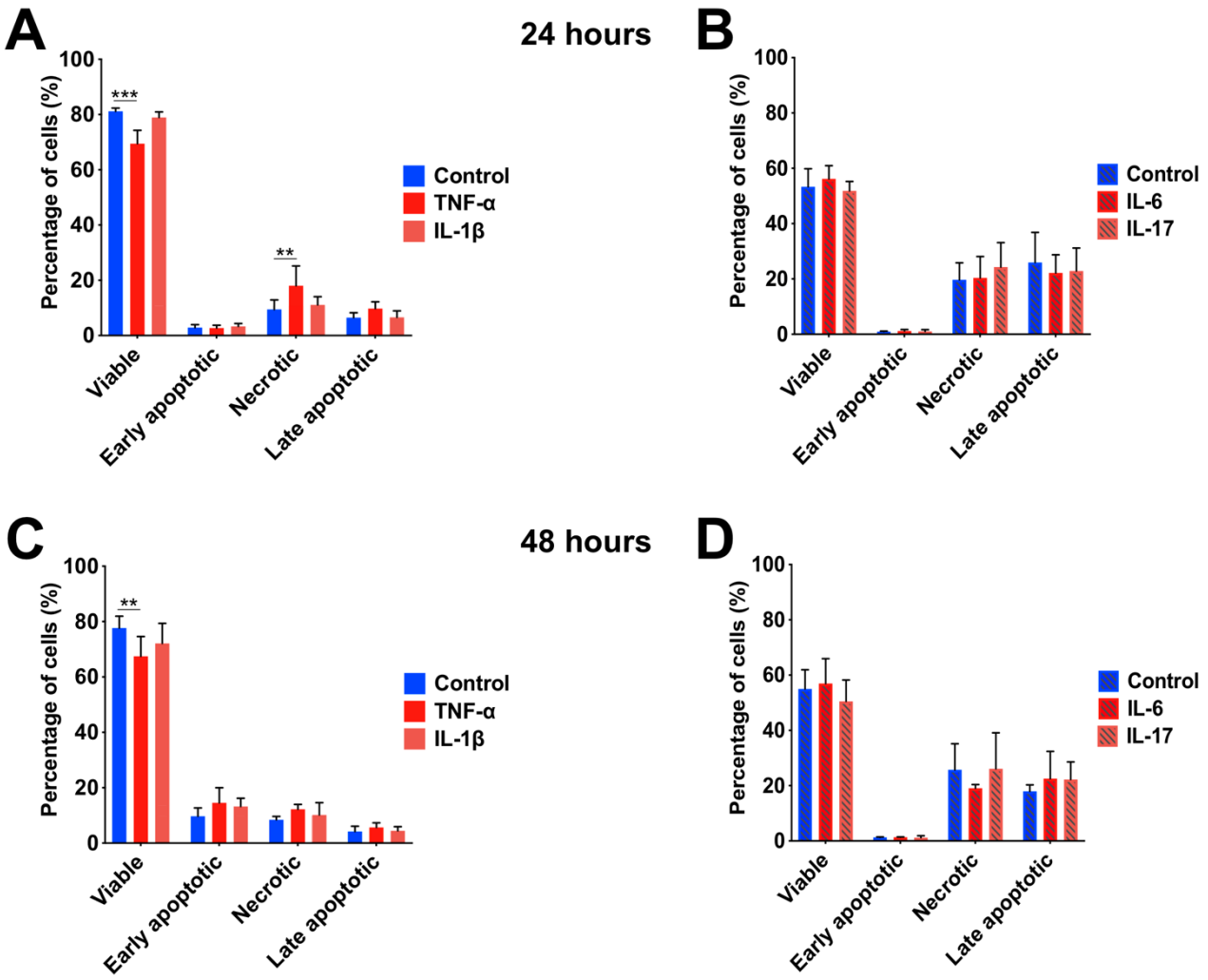


Figure 5.8. Effect of inflammatory cytokines on human retinal endothelial cell viability, apoptosis and necrosis

Graphs depicting the percentage of viable, apoptotic and necrotic cells following treatment with TNF-α (red; 10 ng/mL) and IL-1β (light red; 10 ng/mL) for 24 hours (A) and 48 hours (C), or treatment with IL-6 (red with stripes; 20 ng/mL) and IL-17 (light red with stripes; 100 ng/mL) for 24 hours (B) and 48 hours (D). Fresh medium alone was run in parallel as a control (blue). Bars indicate mean and error bars indicate standard deviation for at least 3 independent experiments. Data were analyzed by two-way ANOVA with post-hoc Tukey's test. $^{**}p < 0.01$; $^{***}p < 0.001$.

The percentage of early apoptotic and late apoptotic cells did not change across all treatments after 24 and 48 hours (Figure 5.8). Although the percentage of early apoptotic cells in the experiment evaluating TNF-α and IL-1β treatments achieved higher percentages at 48 hours, a direct comparison cannot be made against the 24-hour experiment because the experiments were conducted independently. The experiments evaluating apoptosis and necrosis of cells treated with IL-6 and IL-17 for 24 and 48 hours were conducted concurrently. These experiments were independently performed from the experiments assessing TNF- α and IL-1β exposure, and therefore, different rates of cell viability and death were observed. Overall, TNF-α was the only cytokine decreasing cell viability and increasing necrosis of human retinal endothelial cells, in contrast to IL-1β, IL-6 and IL-17.

5.3. Discussion

The experiments presented in this chapter examined the potential mechanisms that are behind the impairment of the human retinal endothelial electrical impedance caused by TNF-α, IL-1β and IL-6, which were described in Chapter 3. Tumour necrosis factor-α and IL-1β disrupted ZO-1 expression patterns, indicating disturbance of junctional complexes, and decreased retinal endothelial cell survival. Interleukin-6 slightly affected ZO-1 configuration, while not affecting cell survival.

Intercellular junctions constitute a paracellular barrier that selectively regulates the transit of water and solutes across the cell monolayer. The expression of tight junction components, ZO-1 and claudin-5, and adherens junction components, VE-cadherin and β-catenin, were analyzed in human retinal endothelial cells upon stimulation with the inflammatory cytokines. At the transcript level, TNF-α downregulated VE-cadherin and ZO-1, while IL-1β and IL-6 did not significantly alter the expression of any analyzed junctional molecules. However, at the protein level, a clear disturbance in the ZO-1 pattern was observed for both TNF-α and IL-1β exposure, and slightly for IL-6.

Tumour necrosis factor-α was the only cytokine affecting junctional molecule mRNA expression, more than halving VE-cadherin and ZO-1 expression after 24 hours, respectively, while changes in ZO-1 immunolabeling were observed at 24 hours and 48 hours following treatment. Normal ZO-1

and VE-cadherin mRNA levels at 48 hours after TNF-α treatment may have reflected a loss of cytokine bioactivity and/or increased ZO-1 and VE-cadherin mRNA synthesis as a compensatory mechanism. Unchanged ZO-1 transcript levels at 48 hours despite an altered ZO-1 distribution pattern may imply ZO-1 internalization, as suggested by the increased ZO-1 fluorescence in the cytoplasm. Interrelationships between VE-cadherin and ZO-1 have been reported, which may raise the possibility that the downregulation of both junctional molecules by TNF-α may be related.[741, 742] Previously, studies using commercial human retinal endothelial cells found reduced ZO-1 expression after TNF-α treatment.[117, 118] Conflicting results on claudin-5[118, 119, 205] and VE-cadherin[119, 122, 123] expression after TNF-α stimulation have been reported in commercial retinal endothelial cells, and TNF-α did not alter β-catenin localization in a preceding publication.[119]

Interleukin-1β did not affect transcriptional levels of junctional molecules; however, IL-1β changed the distribution pattern of the ZO-1 protein. The correlation between transcript levels and protein expression may not be linear and is influenced mainly by mRNA concentration, coding sequence, flanking of untranslated regions, and post-translational modifications.[743, 744] This might explain the difference between ZO-1 transcript and protein expression observed with IL-1β treatment, involving mRNA degradation and turnover, and/or ZO-1 transport to the cell membrane. Because a reduction in the ZO-1 immunofluorescence was observed on the cellular border, but an increased signal was seen in the cytoplasm, the internalization of ZO-1 may have occurred, keeping the mRNA levels unchanged. Previously, in commercial human retinal endothelial cells, transcript levels of ZO-1 were downregulated by IL-1β in less than 24 hours after exposure,[117] with no changes in protein levels,[138] while VE-cadherin protein expression and fragmented labelling were reduced.[124] Neither claudin-5 nor β-catenin expression have been previously evaluated in human retinal endothelial cells treated with IL-1β.[116, 745]

Interleukin-6 did not affect the differential expression of junctional molecules at the transcript level in retinal endothelial cells, with no disruption of ZO-1 immunolabeling on the cell border, but a subtle punctate enhancement of ZO-1 intensity in the cytoplasm and nuclei was observed at 48 hours post-treatment. There is conflicting evidence on ZO-1 expression after IL-6 stimulation of retinal

endothelial cells, where downregulation of ZO-1 mRNA and reduction in marginal ZO-1 labelling was reported after 48 hours of IL-6 stimulation,[125] but higher concentrations of IL-6 did not impact ZO-1 immunolabelling in commercial human retinal endothelial cells.[284] Also, VE-cadherin and claudin-5 mRNA levels did not change after IL-6 treatment,[125] while up to now there has been no published work reporting on β-catenin expression following IL-6 exposure in human retinal endothelial cells.

Tumour necrosis factor-α has been extensively implicated in apoptosis and necrosis in different diseases.[746, 747] However, TNF-α also suppresses cell death, and promotes cell survival and proliferation.[748] Here, it was observed that TNF-α affected the viability of human retinal endothelial cells in a dose-dependent fashion, also increasing necrosis after 24 hours of stimulation. Previous studies of TNF-α effects on the viability of commercial human retinal endothelial cells have produced conflicting results,[206, 283, 354] but these involved metabolic activity assays which have limitations.[749] Zhang et al.[208] reported that apoptosis was induced within one hour of treatment with TNF-α. The different findings reported in this chapter might be explained on the basis of differences in the analyzed time points or by distinctive characteristics between the cells prepared in the home laboratory versus the commercial cells used in other studies.

Interleukin-1β steadily reduced cell survival across different concentrations and did not significantly induce necrosis and apoptosis in human retinal endothelial cells. Previous studies using commercially acquired human retinal endothelial cells showed that IL-1β did not reduce cell viability during similar or longer incubation periods.[138, 206, 283] In agreement with the lack of effect of IL-1β on apoptosis, Yun et al.[138] did not show changes in apoptosis rates in commercial human retinal endothelial cells; however, opposing results on apoptosis were reported in non-human retinal endothelial cells after IL-1β stimulation.[116, 254]

Neither IL-6 nor IL-17 impacted the survival of human retinal endothelial cells. Increased cell viability was observed after stimulation with IL-6 at lower concentrations for longer periods in a commercial human retinal endothelial cell line.[206, 283] However, consistent with the results presented here, there were no signs of IL-6 cytotoxicity across a wide range of concentrations in bovine vascular endothelial cells.[750] Interestingly, augmented apoptosis rates were observed when human retinal

endothelial cells were simultaneously stimulated by IL-6 and sIL-6R, suggesting that trans-signalling may be the pathway for triggering apoptosis,[285] whereas this work focused on classical signalling as mediated via mIL-6R, shown in Chapter 4 to be expressed on retinal endothelial cells. Contrary to the observations of the results obtained in this work, IL-17 induced apoptosis and necrosis in commercial human retinal endothelial cells over a short incubation period.[314] It is possible that effects on cell viability are short-lived and not evident after 24 hours, but the absence of IL-17-associated cytotoxicity is also consistent with the lack of any disturbance of electrical impedance, as reported in Chapter 3.

The biological homeostasis of multi-cellular organisms requires a balance between cell proliferation and cell death, which occurs mainly through apoptosis and necrosis. Other forms of cell death have been reported – such as pyroptosis, ferroptosis, necroptosis, and autophagy – which were also described in retinal endothelial cells.[751-756] Cytokines are known to promote cell death, with some being associated with particular types of cell death.[757-759] For example, TNFR are especially involved in the extrinsic pathway of apoptosis, pyroptosis and necroptosis,[751] while IL-1β has been associated with pyroptosis.[751, 760] It is possible that the investigated cytokines induce different types of cell death by processes other than necrosis and apoptosis.

One interesting technical observation was made in the course of the immunofluorescence studies: ZO-1 labelling was observed in the nuclei of all cells, including controls, and it was enhanced after cytokine treatment. Cytokine-stimulated cells also had an increase in the cytoplasmic ZO-1. Cytoplasmic and nuclear localization of ZO-1 has been reported in other cell types, associated with reduced cell-cell contact extension or maturity, onset of cell death, epithelial-mesenchymal transition, enhanced secretion of inflammatory mediators and T-cell recruitment, and cell migration.[761-764] Therefore, in disturbing intercellular contact, TNF-α, IL-1β, and to a lesser extent, IL-6, may have prompted an accumulation of nuclear and cytoplasmic ZO-1. Cytotoxicity of TNF-α and IL-1β may also have contributed to this accumulation.

The immunolabeling study undertaken here was limited to the well-described junctional molecule, ZO-1. This work now could be extended to VE-cadherin, and even claudin-5 and β-catenin that were

not altered at the transcript level, since protein assessment might have demonstrated otherwise. An investigation of cytokine-induced transcellular transport, which was shown to be provoked by TNF-α in extra-ocular endothelial cells,[765-767] might also be informative of mechanisms that contribute to the impaired electrical impedance of human retinal endothelial cell monolayers in the presence of TNF-α, IL-1β and IL-6. Another future direction for this work could be a study of other types of cell death in the retinal endothelium after exposure to inflammatory cytokines.

Among the six inflammatory cytokines examined in Chapter 3, the work of this chapter focused on the three cytokines that were responsible for impairing the human retinal endothelial cell monolayer barriers: TNF-α, IL-1β and IL-6. The findings presented here indicated that IL-1β and TNF-α were the most active on human retinal endothelial cells, implying that these are major cytokines to be considered when studying the mechanisms of inflammation in the retinal endothelium. Some earlier publications have reported on the effects of cytokines on aspects related to those addressed in this chapter; however, other authors used commercially acquired human retinal endothelial cells and descriptions of the cell assessments were limited. The findings of this chapter imply that TNF-α and IL-1β, are principal cytokines involved in the disruption of human retinal endothelial barrier function, and that they are likely to contribute to retinal vascular leakage and retinal capillary loss in non-infectious uveitis.

CHAPTER 6: EXPRESSION OF LONG NON-CODING RNAS IN ACTIVATED HUMAN RETINAL ENDOTHELIAL CELLS

6.1 Introduction

The retinal vascular endothelium is the major component of the inner blood-retinal barrier.[19] Dysfunction of the retinal endothelium is linked to the breakdown of the barrier across a spectrum of retinal diseases; in particular, this is a prominent feature of non-infectious posterior uveitis, which is amongst the foremost causes of vision loss.[1] The inflammatory cytokines, TNF-α and IL-1β, have been strongly implicated in the pathogenesis of non-infectious posterior uveitis,[96] and TNF-α blockade is an effective treatment for some patients with this condition.[89]

Long non-coding RNAs participate in a myriad of molecular regulatory processes and have been associated with diverse medical conditions.[461, 462, 768] There has been recent interest in modulating lncRNAs for the treatment of retinal diseases,[769] and numerous articles have described the expression of lncRNAs by human retinal endothelial cells in experimental studies of diabetic eye disease,[480, 492, 500, 514, 516] but not non-infectious uveitis. The work presented in this chapter was undertaken to identify candidates lncRNAs that might be explored as drug targets in non-infectious posterior uveitis.

6.2 Results

6.2.1 Selection of candidate long non-coding RNAs

Candidate lncRNAs were selected through a literature search conducted using the NCBI PubMed database of the United States National Library of Medicine, using the search terms, 'long noncoding RNA' or 'long non-coding RNA', and 'inflammation' and 'endothelial', and an English-language restriction (Figure 6.1). The literature search returned 112 articles, which were reviewed in full. Ninety-three of these 112 articles identified a total of 45 endothelial lncRNAs that had been implicated in inflammation. To focus the selection of lncRNAs, results were cross-referenced with an RNA-seq dataset for the human retinal endothelial cell that had been previously generated in-house.[483] Interrogation of the human retinal endothelial transcriptome for the 45 lncRNAs identified 11 lncRNAs for further study (Table 6.1).

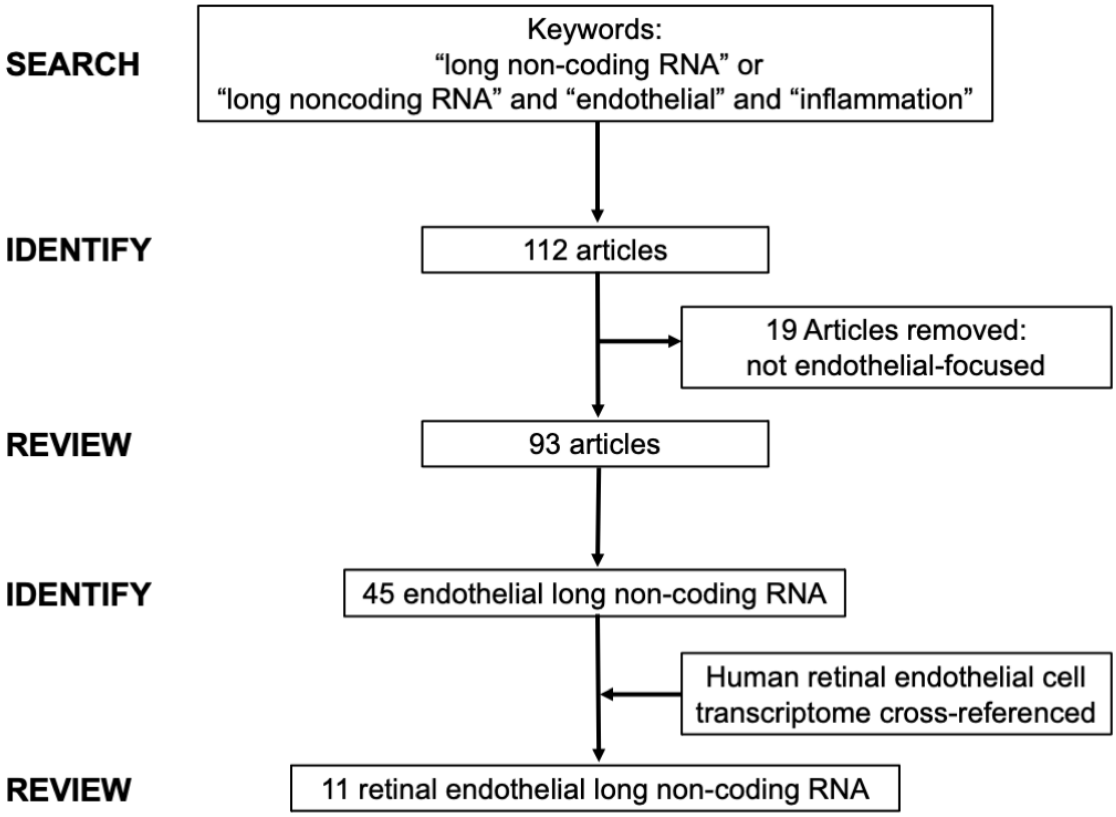


Figure 6.1. Identification of long non-coding RNA candidates in human retinal endothelial cells

A literature search was conducted in the NCBI PubMed database of the United States National Library of Medicine on April 2, 2021. The human retinal endothelial cell transcriptome was sourced from a published RNA-seq dataset.

Table 6.1 Description of long non-coding RNAs identified in human retinal endothelial cells and implicated in inflammation

Abbreviation	Long non-coding RNA	Chromosome	Length (bp)	Accession number
GAS5	Growth arrest specific 5	1q25.1	702	NR_002578.3
KCNQ1OT1	KCNQ1 opposite strand/antisense transcript 1	11p15.5	91671	NR_002728.3
LINC00294	Long intergenic non-protein coding RNA 00294	11p13	3322	NR_015451
MALAT1	Metastasis associated lung adenocarcinoma transcript 1	11q13.1	12819	NR_002819.4
MEG3	Maternally expressed 3	14q32.3	1595	NR_002766.2
MIR155HG	MIR155 host gene	21q21.3	1500	NR_001458.3
NEAT1	Nuclear paraspeckle assembly transcript 1	11q13.1	3756	NR_131012.1
NORAD	Non-coding RNA activated by DNA damage	20q11.23	5378	NR_027451
OIP5-AS1	OIP5 antisense RNA 1	15q15.1	1269	NR_026757.2
TUG1	Taurine up-regulated 1	22q12.2	7542	NR_110492.1
SENCR	Smooth muscle and endothelial cell enriched migration/differentiation-associated lncRNA	11q24.3	1319	NR_038908

Abbreviations: bp = base pair; KCNQ1 = potassium voltage-gated channel subfamily Q member 1; MIR155 = microRNA-155; OIP5 = Opa interacting protein 5; lncRNA = long non-coding RNA.

6.2.2 Expression of long non-coding RNAs in human retinal endothelial cells

As visualized in Figure 6.2, the expression of all 11 candidate lncRNAs was confirmed by RT-PCR of total RNA extracted from five primary retinal endothelial cell isolates from human cadaveric eye pairs (four females and one male; 50 to 62 years at death), as well as the retinal endothelial cell line. Since the expression of lncRNAs in the cell line was similar to that seen in the primary cell isolates, the cell line was used in subsequent experiments to assess levels of the lncRNAs following treatment with TNF-α or IL-1β.

6.2.3 Differential expression of long non-coding RNAs in activated human retinal endothelial cells

Cell monolayers were treated with 10 ng/mL TNF-α or IL-1β in medium, or fresh medium alone (no cytokine control) for intervals of 4, 12, 24 and 48 hours. This working concentration was based on previous work conducted by the home laboratory,[640, 657] and others[770] that showed biological activity of the cytokine at that concentration. Changes in human retinal endothelial cell expression of the 11 lncRNAs were assessed by RT-qPCR at pre-specified time points up to 48 hours following treatment with TNF-α or IL-1β.

First, activation of the retinal endothelium by each inflammatory cytokine at the selected working concentration was confirmed by assessing the induction of ICAM-1, which is a key endothelial cell adhesion molecule that regulates leukocyte migration during inflammation.[771] As shown in Figure 6.3, retinal endothelial cell ICAM-1 was significantly upregulated by TNF-α ($p < 0.05$) and IL-1β ($p \leq 0.01$) across all time points.

The expression kinetics of the 11 lncRNAs in the activated endothelial cells is presented in Figure 6.4 and Figure 6.5. Treatment with TNF-α (Figure 6.4) significantly upregulated KCNQ1OT1, LINC00294, MEG3, NORAD and SENCR at 4 hours, SENCR and TUG1 at 24 hours, and MIR155HG at all time points. Treatment with IL-1β (Figure 6.5) significantly upregulated MEG3 and SENCR at 4 and 24 hours. After the 24-hour treatment with IL-1β, LINC00294, NORAD, OIP5-AS1 and TUG1 all increased significantly, and MIR155HG was increased significantly at 4-, 24- and 48-hour

Figure 6.2. Expression of long non-coding RNAs in human retinal endothelial cells

Images showing long non-coding RNAs and reference gene amplicons run on 2% agarose gel. L = DNA ladder (500 base pairs indicated by the red cross); 1–5 = primary human retinal endothelial cell isolates from individual donors; 6 = human retinal endothelial cell line; NT = no cDNA template control. Expected product sizes (indicated by red arrows): GAS5 = 127 bp; KCNQ1OT1 = 567 bp; LINC00294 = 225 bp; MALAT1 = 396 bp; MEG3 = 187 bp; MIR155HG = 149 bp; NEAT1 = 137 bp; NORAD = 157 bp; OIP5-AS1 = 198 bp; SENCR = 240 bp; TUG1 = 140 bp; YWHAZ = 94 bp. Controls loaded with no cDNA template did not amplify.

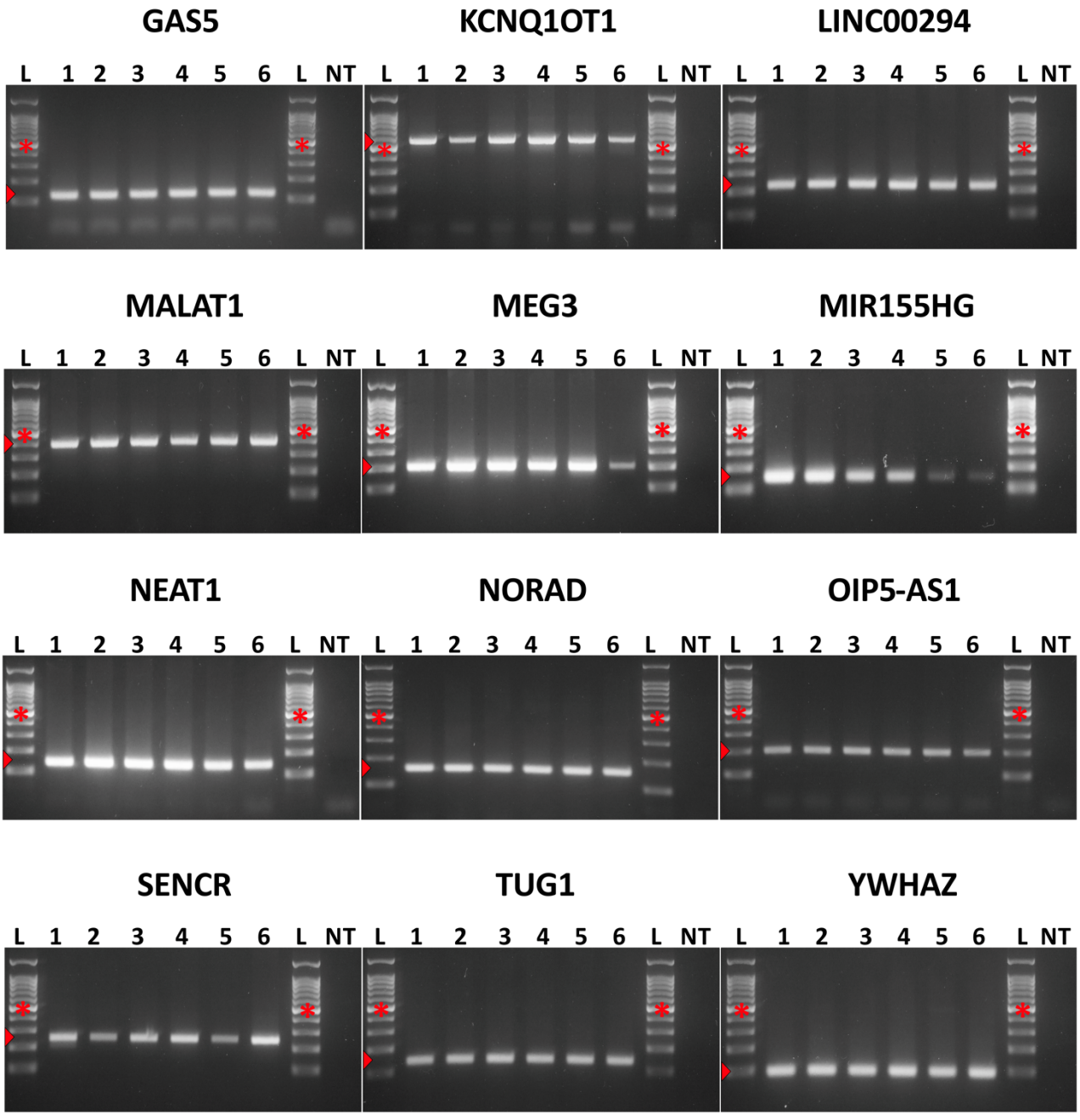

GAS5
KCNQ1OT1
LINC00294
MALAT1
MEG3
MIR155HG
NEAT1
NORAD
OIP5-AS1
SENCR
TUG1
YWHAZ
L 1 2 3 4 5 6 L NT

Figure 6.3. Expression of intercellular adhesion molecule-1 in human retinal endothelial cells treated with inflammatory cytokines

Graphs showing relative normalized expression of ICAM-1 transcript by human retinal endothelial cells at 4, 12, 24 and 48 hours following treatment with TNF-α (A) or IL-1β (B) versus medium alone control. Reference genes: RPLP0 and PPIA. Bars represent mean normalized expression, and error bars indicate standard deviation (n = 3-4 cultures/condition). Black columns: controls; grey columns: TNF-α (A) or IL-1β (B) treatment. Data were analyzed by unpaired Student's t-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

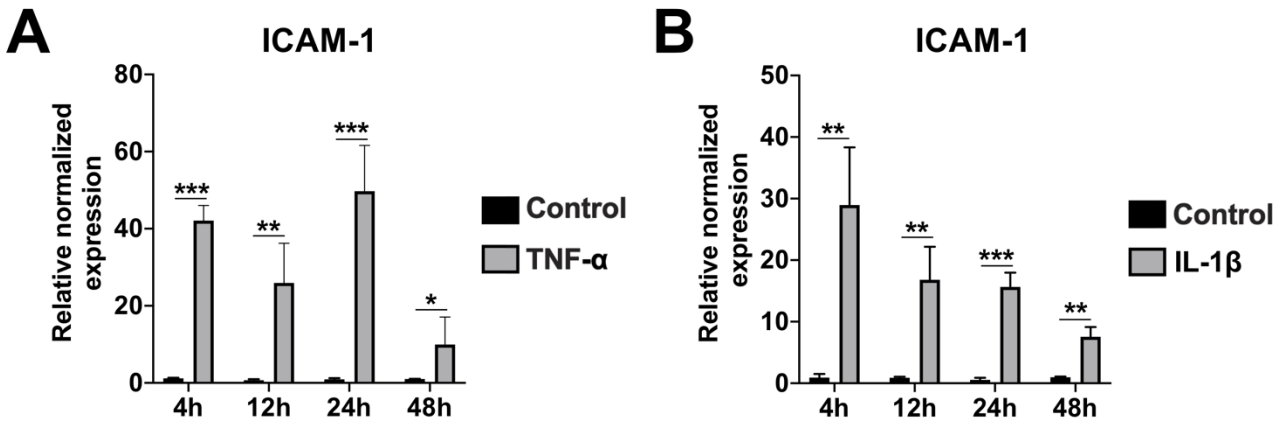

A
ICAM-1
Relative normalized expression
80
60
40
20
0
***
**
***
*
4h
12h
24h
48h
Control
TNF-α
B
ICAM-1
Relative normalized expression
50
40
30
20
10
0
**
**
***
**
4h
12h
24h
48h
Control
IL-1β

Figure 6.4. Expression of long non-coding RNAs in human retinal endothelial cells treated with tumour necrosis factor-α

Graphs showing relative normalized expression of long non-coding RNAs by human retinal endothelial cells at 4, 12, 24 and 48 hours following treatment with TNF-α versus medium alone control. Reference genes: RPLP0 and PPIA. Bars represent mean relative normalized expression, and error bars indicate standard deviation (n = 3-4 cultures/condition). Black columns: controls; grey columns: TNF-α. Data were analyzed by unpaired Student's t-test *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

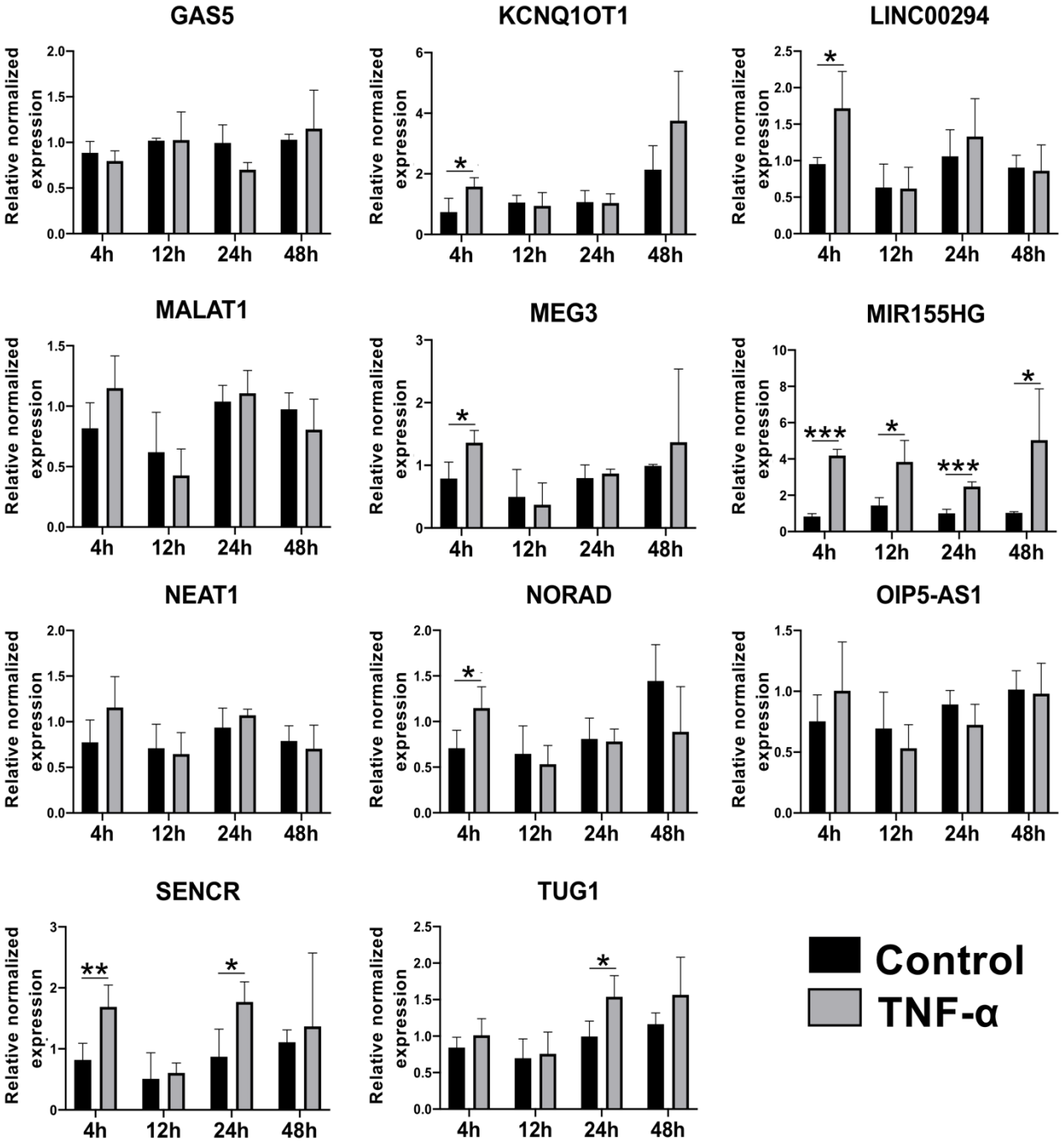
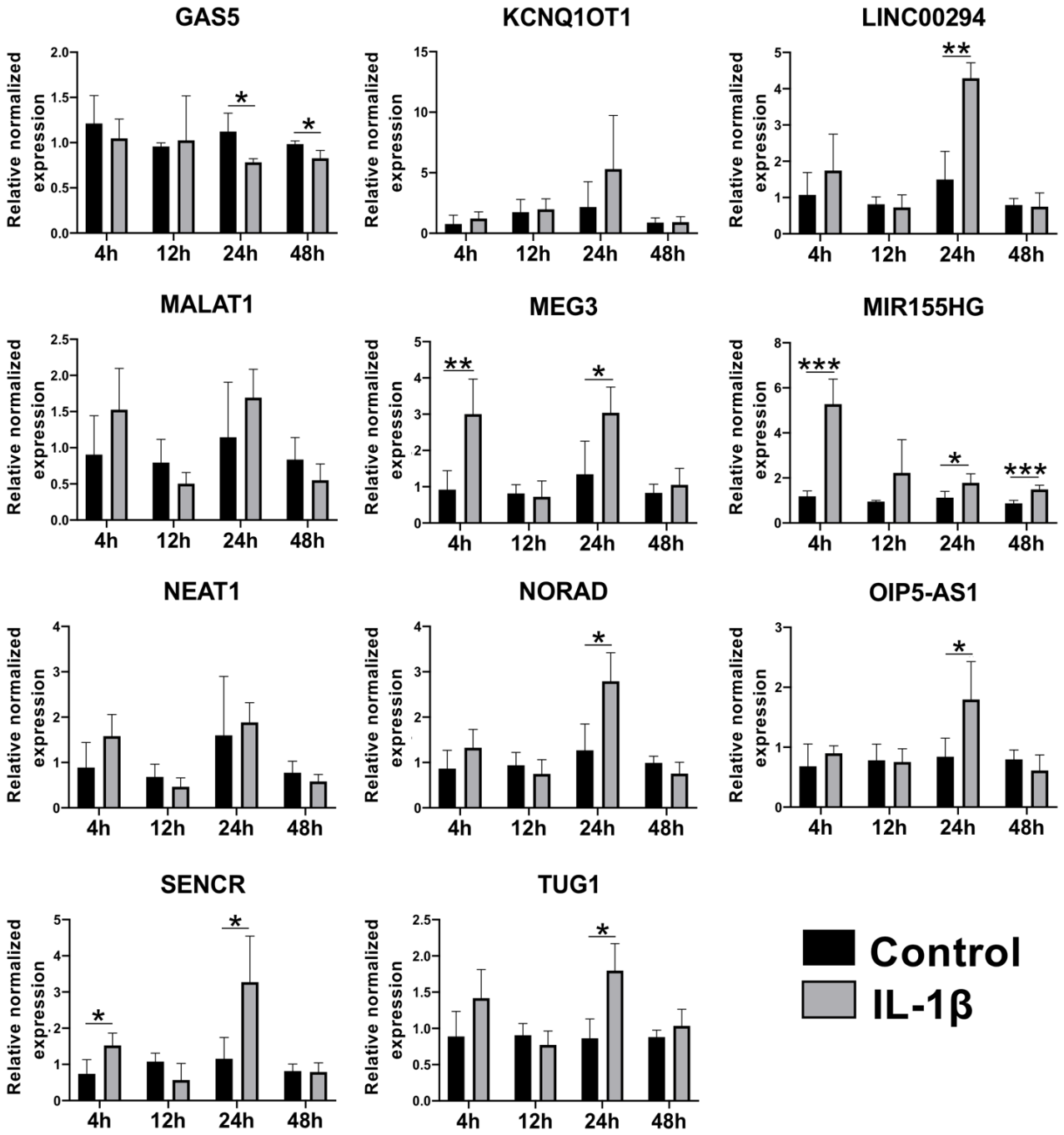

GAS5
KCNQ1OT1
LINC00294
MALAT1
MEG3
MIR155HG
NEAT1
NORAD
OIP5-AS1
SENCR
TUG1
Relative normalized expression
4h
12h
24h
48h
Control
TNF-α

Figure 6.5. Expression of long non-coding RNAs in human retinal endothelial cells treated with interleukin-1β

Graphs showing relative normalized expression of long non-coding RNAs by human retinal endothelial cells at 4, 12, 24 and 48 hours following treatment with IL-1β versus medium alone control. Reference genes: RPLP0 and PPIA. Bars represent mean relative normalized expression, and error bars indicate standard deviation (n = 3-4 cultures/condition). Black columns: controls; grey columns: IL-1β. Data were analyzed by unpaired Student's t-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.


GAS5
KCNQ1OT1
LINC00294
MALAT1
MEG3
MIR155HG
NEAT1
NORAD
OIP5-AS1
SENCR
TUG1
Relative normalized expression
4h
12h
24h
48h
Control
IL-1β

treatment intervals. However, IL-1β exposure led to significant downregulation of GAS5 at both 24 and 48 hours.

Considering the 11 lncRNAs as a group, TNF-α upregulated the expression of 7 lncRNAs, most often at 4 hours (LINC00294, MEG3, MIR155HG, SENCR, NORAD and TUG1), while IL-1β consistently altered the expression of 8 lncRNAs at 24 hours (GAS5, LINC00294, MEG3, MIR155HG, NORAD, OIP5-AS1, SENCR and TUG1). The majority of the lncRNAs (LINC00294, MEG3, MIR155HG, NORAD, SENCR and TUG1) were increased at one time point by both TNF-α and IL-1β. In addition, TNF-α increased KCNQ1OT1, while IL-1β induced OIP5-AS1 and downregulated GAS5. Neither cytokine impacted the expression of MALAT1 or NEAT1. The expression of MIR155HG was elevated throughout the 48 hours of treatment with TNF-α and IL-1β, although statistical significance was not reached at 12 hours for IL-1β activation.

6.3 Discussion

Eleven lncRNAs were identified in human retinal endothelial cells, and the differential expression of nine of these was demonstrated following activation of the cells with TNF-α or IL-1β. The pattern of differential expression varied between the cytokines, with TNF-α inducing changes relatively early, and IL-1β inducing changes relatively late. Most lncRNAs did not change across all time points studied, with the exception of MIR155HG, which was consistently upregulated by TNF-α and IL-1β across the 48-hour period of stimulation. One question arising from these findings is how might these cytokines regulate the lncRNAs? Both TNF-α and IL-1β can act to induce NF-kB and NF-IL6, which in turn can regulate the expression of specific lncRNAs.[772]

MIR155HG encodes miR-155, a pleiotropic miRNA involved in hematopoiesis, immune regulation and inflammation.[773] miR-155 has been noted previously to be upregulated by TNF-α and IL-1β in human retinal pigment epithelial cells,[774] where its expression is necessary for the development of EAU.[775] The results presented in this chapter suggest that MIR155HG is also involved in endothelial activation during intraocular inflammation. Consistent with the observations of this chapter, Savage

et al.[480] observed TNF-α induction of MIR155HG in human retinal endothelial cells in a study focusing on mechanisms of diabetic retinopathy that involved different experimental parameters. However, although miR-155 has been associated with the development of inflammation in other cells,[776-778] whether the induction of MIR155HG by TNF-α and IL-1β in human retinal endothelium promotes or provides a block on activation is yet to be determined. Indeed, MIR155HG also encodes P155, a micropeptide that modulates antigen presentation and reduces the severity of experimental autoimmune encephalomyelitis.[779]

Of the other differentially expressed lncRNAs that were identified, only two have been previously studied in human retinal endothelial cells: KCNQ1OT1[492] and MEG3.[500] MEG3 was induced by both TNF-α and IL-1β. Interestingly, overexpression of MEG3 reduces inflammatory cytokine production by human retinal endothelial cells exposed to high levels of glucose,[500] suggesting that this lncRNA may play an autoregulatory role in endothelial cell activation during inflammation. Expression of NORAD was also increased in retinal endothelial cells after treatment with TNF-α and IL-1β. This lncRNA has been implicated in the inflammatory response in a mouse model of systemic vascular disease, acting by a regulatory mechanism that involves sponging the microRNA, miR-495-3p.[780] OIP5-AS1, which was upregulated by IL-1β, has been shown to promote the secretion of inflammatory cytokines by human umbilical vein endothelial cells.[781] These observations support a pro-inflammatory effect of retinal endothelial cell NORAD and OIP5-AS1 during non-infectious posterior uveitis. Both cytokines upregulated SENCR, which has been shown to play an important role in stabilizing adherens junctions and therefore membrane integrity in human umbilical vein endothelial cells subjected to laminar shear stress.[782] Relatively little is known of the function of LINC00294.

TUG1 was upregulated in human retinal endothelial cells by both IL-1β and TNF-α after 24 hours. The role of TUG1 in inflammatory eye disease is unknown, but it has been implicated in the differentiation of the murine photoreceptors,[783] and also has been suggested to play a role in the development of human cataract.[784] Further, one variant is associated with susceptibility to, severity

of, and therapeutic response to VEGF blockade in patients with diabetic retinopathy.[785] GAS5 operates as a tumour suppressor,[786] and is downregulated in many human cancers, including uveal melanoma.[787] Reports of expression in human endothelial cells or cells of the retina are limited. In human umbilical vein endothelial cells, silencing of GAS5 enhanced cell viability, proliferation and tube formation.[788] The increased expression of IL-1β and TNF-α noted after exposure of human retinal pigment epithelial cells to high glucose levels was found to be ameliorated by GAS5 overexpression.[531] In these experiments, human retinal endothelial cells activated by IL-1β exhibited decreased GAS5 expression, a finding which suggests the promotion of angiogenic and inflammatory processes.

Intriguingly, the expression of MALAT1 did not change significantly in human retinal endothelial cells treated with IL-1β or TNF-α. In retinal endothelial cells exposed to high glucose, MALAT1 upregulation has been associated with inflammation, vascular permeability and angiogenesis.[497, 498, 514, 516] Further, Savage et al.[480] have reported that human retinal endothelial cells stimulated by TNF-α upregulate MALAT1.[480] The difference between their observations and the results presented in this chapter might relate to the lower concentrations of TNF-α used in their experiments. That was appropriate as their study focused on diabetic retinopathy, a condition in which intraocular levels of TNF-α are lower than in non-infectious uveitis.[166, 789] It is likely that specific lncRNAs are involved in the pathology of uveitis versus diabetic retinopathy. NEAT1 is another example of a lncRNA that has been upregulated in human retinal endothelial cells under high glucose conditions, but was not induced in the experiments presented in this chapter.[503]

This discovery research sought to identify lncRNAs potentially involved in human retinal endothelial cell activation. Future investigations should include experiments that test gain or loss of function, as well as studies of specific lncRNA-RNA, -DNA and -protein interactions.[790, 791] Long non-coding RNAs may act as competing endogenous RNAs, and thus bioinformatics-directed delineation of the lncRNA-miRNA-mRNA network that is activated in the retinal endothelium during inflammation is also highly relevant.[792] Other studies might test how secondary and tertiary structures of retinal

endothelial cell lncRNAs impact their interactions with nucleic acids and proteins, and assess ribosome profiling, which may indicate coding properties of lncRNAs.[791] One of the obstacles to conducting *in vivo* studies of lncRNA function is the limited conservation across species, and the development of humanized animal models may address this issue.[793] This *in vitro* work was conducted with human retinal endothelial cells, optimising the relevance to human disease mechanisms.

Long non-coding RNAs have been identified as potential therapeutic targets in other diseases.[769] This *in vitro* study was undertaken to identify human retinal endothelial cell lncRNAs that might be the basis of new therapeutics for non-infectious posterior uveitis. Future work should explore the effects of blocking or augmenting MIR155HG, and possibly other lncRNAs, such as NORAD and OIP5-AS1, on specific molecular pathways involved in the activation of the retinal endothelium.

The work of this chapter formed the basis of a manuscript published in Ocular Immunology and Inflammation: Barros Ferreira L, Ashander LM, Appukuttan B, Ma Y, Williams KA, Smith JR. Expression of long non-coding RNAs in activated human retinal vascular endothelial cells. 2022:1-6.

CHAPTER 7: GENERAL DISCUSSION

One of the greatest challenges when treating a patient with non-infectious posterior uveitis is leakage from the retinal vasculature. This mainly affects the macular region, but also other parts of the retina, often decreasing visual acuity. Retinal endothelial dysfunction is a central process in the pathogenesis of uveitis, linked to the release of inflammatory cytokines. Thus far, most of what has been written about the role of inflammatory cytokines in retinal endothelial dysfunction and uveitis has stemmed from animal studies, and *in vitro* work with extra-ocular and non-human retinal endothelial cells or commercially acquired human retinal endothelial cells. Additionally, previously published studies have focused on a limited number of cytokines. There has been no sustained effort to examine the direct influence of multiple cytokines on primary human retinal endothelial cells has been published.

Uncertainties persist on how some cytokines influence retinal endothelial barrier function, and whether a cytokine receptor, IL-6R, is expressed in human retinal endothelial cells. Additionally, although the expression of lncRNAs in the human retinal endothelium has been previously explored,[292, 479-513] whether lncRNAs are involved in uveitis is still to be determined. The work of this thesis was undertaken to achieve a more comprehensive understanding of the underlying mechanisms of retinal endothelial dysfunction in uveitis, aiming to improve the clinical management of patients, as it is unclear which therapeutic agent is optimal for tackling this complication.

First, the barrier function of human retinal endothelial cells was examined following stimulation by a number of inflammatory cytokines, individually and in combination (Chapter 3). The results demonstrated that TNF-α, IL-1β and IL-6 significantly decreased the electrical impedance of the human retinal endothelial cell monolayer, with TNF-α and IL-1β showing immediate and substantial effects, while IL-6 exhibited a lesser effect. In contrast, IL-8, IL-17 and CCL2 did not significantly affect the barrier function. However, in combination with TNF-α or IL-1β, IL-6 did not further decrease transcellular electrical impedance.

These data suggested that a functional IL-6R might be expressed by human retinal endothelial cells, and this hint was subsequently confirmed: expression of IL-6R protein was demonstrated in primary human retinal endothelial cells from multiple isolates and in a cognate cell line (Chapter 4). The finding overthrows the prevailing, widespread belief that retinal endothelial cells do not express the IL-6R protein.[284, 285, 288] Furthermore, the IL-6R was found to be downregulated in human retinal endothelial cells that had been activated by IL-1β, TNF-α, or LPS. The finding with respect to LPS emphasizes the importance of using stringent cell culture techniques, to preclude contamination with bacterial products when appraising IL-6R expression in human vascular endothelial cells. Moreover, the confirmation of ETS1 as a transcription factor involved in IL-6R expression *in vitro,* as predicted *in silico*, points towards a potential target for dampening the inflammatory axis of IL-6.

The likely mechanisms of how TNF-α, IL-1β and IL-6 decreased the transcellular electrical impedance of a human retinal endothelial cell monolayer, as observed in Chapter 3, was then addressed (Chapter 5). Tumour necrosis factor-α and IL-1β altered junctional molecule expression in human retinal endothelial cells, decreasing the protein localization at the cell margins and increasing it in the interior of cells, suggesting internalization. Contrastingly, IL-6 had little demonstrable effect on junctional protein distribution. In addition, TNF-α and IL-1β decreased the survival of human retinal endothelial cells, suggesting cytotoxicity. A deeper look into cell viability, evaluating both apoptosis and necrosis, showed that only TNF-α increased the percentage of necrotic cells, while IL-1β and IL-6, plus IL-17 (chosen as a negative control), altered neither necrosis nor apoptosis.

The results presented in Chapters 3 and 5 indicated major effects of TNF-α and IL-1β on human retinal endothelial cell monolayer integrity. Therefore, the impact of these cytokines on the regulation of lncRNA expression was assessed. Cytokines may influence the expression of lncRNAs, which are known to participate in retinal endothelial dysfunction[461, 462, 794] and are a "hot" topic in the field of molecular biology. Although lncRNAs have been described in the human retinal endothelial cells[292, 479-513] they have not hitherto been studied in the context of uveitis. Hence, in Chapter 6, the

expression of a number of lncRNAs was confirmed – many for the first time – in primary human retinal endothelial cells and similarly in the cell line. The cell line was then used to evaluate the differential expression of lncRNAs following retinal endothelial cell activation by TNF-α and IL-1β. A key lncRNA appears to be MIR155HG, which was consistently upregulated by both TNF-α and IL-1β. MIR155HG encodes miR-155, a miRNA recognised to be involved in intraocular inflammation, and which could be a potential future target for the treatment of uveitis.[775-779]

A schematic representation of the key findings reported in this thesis is presented in Figure 7.1. Selected inflammatory cytokines were tested on the human retinal endothelial cell monolayer, and three were found to impair the electrical impedance of the endothelial barrier: TNF-α, IL-1β and IL-6 (Figure 7.1A). The expression of the IL-6R protein was definitively demonstrated in human retinal endothelial cells, suggesting that IL-6 reduced the electrical impedance acting via the classical signalling pathway (Figure 7.1B). Also, IL-6R was downregulated by inflammatory stimuli, and the transcription factor, ETS1, was found to regulate IL-6R expression. Tumour necrosis factor-α and IL-1β affected the electrical impedance of the endothelial cell monolayer by affecting junctional protein distribution and inducing cytotoxicity (Figure 7.1C). However, there are likely other mechanisms behind the impairment of the barrier function provoked by IL-6. Tumour necrosis factor-α and IL-1β also upregulated lncRNAs in human retinal endothelial cells, particularly suggesting an important role of MIR155HG in uveitis (Figure 7.1D).

Chapters 3 and 5 showed that TNF-α and IL-1β were most strongly implicated in reducing the electrical impedance of the retinal endothelial cell monolayer, and also in changing the pattern of junctional protein distribution and in inducing cell death. The question of which activity is primarily responsible for the loss of retinal endothelial barrier function. It is likely that the cytotoxicity of these cytokines was the main contributor to the impairment of the retinal endothelial barrier function, since a parallel analysis between the percentage decrease in electrical impedance and cell proliferation at 48 hours for TNF-α and IL-1β indicated a similar reduction, by approximately one-third.

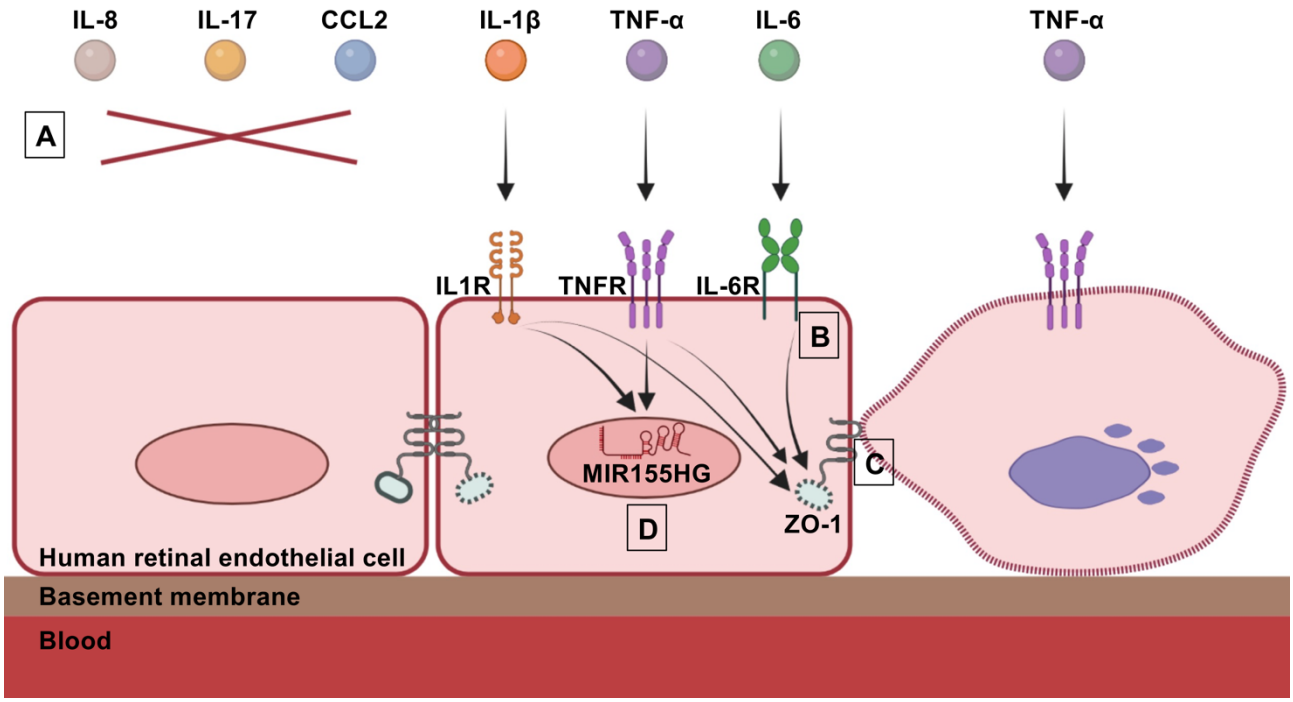


Figure 7.1. Summary of the work presented in this thesis, which investigated effects of inflammatory cytokines on human retinal endothelial cells

This cartoon depicts the main findings described in this thesis (created at BioRender.com). (A) Chapter 3. TNF-α, IL-1β and IL-6 affected the human retinal endothelial barrier, while IL-8, IL-17 and CCL2 had no effect. (B) Chapter 4. The expression of the IL-6 receptor was reported in human retinal endothelial cells. (C) Chapter 5. TNF-α, IL-1β and, to a lesser extent, IL-6, altered the ZO-1 expression in human retinal endothelial cells, while TNF-α induced cell necrosis. (D) Chapter 6. TNF-α and IL-1β altered the expression of a specific subset of long non-coding RNAs in human retinal endothelial cells, consistently upregulating MIR155HG. **Abbreviations:** IL = interleukin; CCL2 = C-C motif chemokine ligand 2; TNF-α = tumour necrosis factor-α; IL1R = IL-1 receptor; TNFR = TNF receptor; IL-6R = IL-6 receptor; ZO-1 = zona occludens-1; MIR155HG = microRNA 155 host gene.

Unexpectedly, IL-6 did not exacerbate the reduction of the electrical impedance of the human retinal endothelial cell monolayer when applied in combination with TNF-α or IL-1β (Chapter 3). A plausible explanation for these findings is that TNF-α and IL-1β stimulation reduced IL-6R expression (Chapter 4). A possible mechanism for the downregulation of the IL-6R by TNF-α and IL-1β was also identified (Chapter 6): these cytokines upregulated MIR155HG which encodes miR-155. In turn, miR-155 is known to target ETS1[778] in human Th cells, so that diminished ETS1 levels might have downregulated IL-6R expression in human retinal endothelial cells. As a consequence, IL-6 did not further decrease the impairment of retinal endothelial barrier function when combined with TNF-α or IL-1β. The influence of different and multiple cytokines on the expression of cytokine receptors is important when considering patients with uveitis, who have varying intraocular levels of different cytokines, even within a single uveitis subtype.[151, 157, 160, 163, 164, 182] Such variability of the inflammatory milieu in uveitis indicates that the management of this condition might be improved by personalized medicine techniques.[185]

Approaches to personalized medicine in uveitis is certainly possible. Enhanced assay technology that enables the assessment of multiple biological markers in intraocular fluids has contributed to decoding cytokine signatures of certain uveitis aetiologies. This has provided potential treatment targets and conceivably soon may be a powerful tool in solving challenging diagnoses, analysing therapy response, and unveiling prognosis of patients with uveitis. Curnow et al.[160] precisely distinguished idiopathic uveitis from non-inflammatory eye conditions when performing cluster analysis of a subset of cytokines to differentiate between the two conditions. Velez et al.[185] explored the vitreous protein components of patients with infectious and non-infectious uveitis, and conducted an unbiased clustered analysis of the markers, which accurately led to the diagnosis of one patient with progressive uveitis of unknown aetiology to autoimmune retinopathy, further confirmed by specific antibody testing. Moreover, increased serum levels of IL-6 were linked to a decreased anatomical improvement of macular oedema, whereas higher levels of circulating T-regulatory cells were predictors of continued anatomical resolution of macular oedema, at a one-year follow-up of

patients with uveitic macular oedema.[189]

This work described in this thesis was conducted using retinal endothelial cells, which are the principal cell type in the maintenance of the barrier properties of the inner blood-retinal barrier. Hence, other cellular components which are present *in vivo*, such as pericytes and Müller cells, were not evaluated. The robust effect of TNF-α, IL-1β and, to a lesser extent, IL-6 on human retinal endothelial dysfunction, implies that these are the main cytokines responsible for the impairment of the inner blood-retinal barrier in uveitis. The lack of effect of IL-8, IL-17 and CCL2 on the breakdown of the human retinal endothelial cell monolayer and of IL-17 on cytotoxicity does not mean that these cytokines do not play a role in the pathogenesis of uveitis, but that they may act in conjunction with other inflammatory mediators and/or in different steps of the inflammatory cascade, such as in leukocyte migration.

The thesis work employed *in silico* methods and several *in vitro* techniques of cellular and molecular biology. A limitation of the work was that it was conducted entirely *in vitro*. When cultured, cells tend to de-differentiate and lose their phenotypic characteristics over time.[795] However, the use of primary human retinal endothelial cells isolated from cadaveric eyes, which were cultured up to the fourth passage, constituted a prime resource in this research. When higher numbers of cells were required for experimentation, a cell line locally expanded from primary cells was used. Despite the possible interference of the cell immortalization process on cellular genetic integrity and physiology, these cells had previously been shown to maintain the morphological and molecular signatures of primary human retinal endothelial cells.[38] Research using animal models are excellent for comprehending disease mechanisms;[148, 796] however, animal models are not flawless, especially when conducting immunologic investigations, given the differences between the immune systems of rodents and humans.[797, 798] Moreover, as an example, *in vivo* experimentation with intraocular cytokine injection may result in a transient increase of the inner blood-retinal barrier permeability due to compensatory antagonistic mechanisms or inactivation of the agent, while in cell culture the cytokine is applied once over the endothelial cell monolayer and not removed, thus theoretically continuously impacting

the barrier function of the monolayer and mimicking the persistent inflammatory state, such as in uveitis.

The cytotoxic effects of TNF-α and IL-1β reported in this thesis are clinically evident in uveitis when analysing the terminal retinal vasculature of the macula. Using optical coherence tomography-angiography to assess the retinal microcirculation, patients with uveitis were shown to have lower parafoveal capillary vascular density and enlarged foveal avascular zones in comparison with healthy controls, regardless of macular oedema.[799, 800] In another study, uveitic eyes with macular oedema showed a significant reduction in the vascular density in the deep capillary plexus when compared to uveitic eyes without macular oedema.[799] Despite the macular anatomical improvement and the reduction in vascular leakage observed in fluorescein angiograms of patients with uveitis under corticosteroid therapy, eyes with a longer duration of uveitic macular oedema have a diminished therapeutic response.[801] The cytotoxic effects of TNF-α, and possibly IL-1β, may be associated with terminal capillary loss in chronic macular oedema, leading to a decreased vascular density of the parafoveal plexuses, foveal avascular zone enlargement, and worse visual prognosis. Thus, anti-TNF-α treatment may reduce capillary loss, likely when implemented early in the course of the disease, preventing this anatomical change that correlates with lower visual function.[802, 803]

The data presented in this thesis support the positive effects of systemic anti-TNF-α for the treatment of uveitic macular oedema, since it was demonstrated that TNF-α is potently implicated in the breakdown of retinal endothelial barrier function. However, anti-TNF-α therapy is not always effective for the treatment of non-infectious uveitis,[89, 90, 223, 804-806] and one of the bases for that is the development of anti-drug antibodies.[227, 228, 807] Moreover, anti-TNF-α therapy has paradoxically been associated with the onset or enhancement of uveitis, particularly with the use of etanercept, which is currently not used for the treatment of non-infectious uveitis.[225] There are reports of enhancement or development of uveitis with infliximab or adalimumab,[225, 226, 808, 809] mostly when used intravitreally.[232, 238-240] The reported mechanisms of anti-TNF-α cytotoxicity include the activation of the complement system, antibody-dependent cell-mediated cytotoxicity and induction of apoptosis

and cell cycle arrest.[810] The systemic use of anti-TNF-α agents may not always reach effective intraocular concentrations of the drug, but since ocular toxicity has been reported with intravitreal use, a reformulation of the therapeutic for intraocular use may be necessary to reduce the potential for local complications. As TNF-α was demonstrated to disrupt ZO-1 protein allocation and to be cytotoxic to retinal endothelial cells, a possible cause of unresponsiveness to TNF-α blockers could be the timing of treatment initiation, implying that earlier introduction of these agents would improve their effectiveness.

Based on the data presented in Chapters 3 and 5, it could be argued that IL-1β blockade would improve vascular leakage in uveitis. Interleukin-1β blockers have been shown to improve inflammation in non-infectious uveitis.[259-264] However, with the failure of the systemic anti-IL-1β monoclonal antibody, gevokizumab, in meeting the primary endpoint of delaying time to first acute ocular exacerbation of uveitis in patients with Behçet's disease,[265] the enthusiasm for targeting IL-1β has waned. Despite the negative primary endpoint result, gevokizumab reduced the incidence of macular oedema, retinal vasculitis and worsening of visual acuity, suggesting that the drug was active in preserving the retinal endothelial barrier function. As the drug was given subcutaneously, it is possible that the ocular levels would not have been enough to induce a stronger effect and achieve the primary endpoint of the study. An important consideration is whether intravitreal blockade of IL-1β might prevent vascular leakage in humans, but this has not been attempted to date.

Confirmation of the expression of functional IL-6R protein in human retinal endothelial cells has therapeutic implications. Efforts have been made to develop a selective blocker of sIL-6R, due to the hypothesis that trans-signalling is responsible for the pro-inflammatory activities of IL-6, while classical signalling would promote anti-inflammatory activity.[278] A fused recombinant protein containing part of the sgp130 – which selectively blocks sIL-6R – is under phase II clinical investigation for the treatment of ulcerative colitis.[811] However, given the data presented, this potential new antibody is predicted to fail in the treatment of vascular leakage and macular oedema secondary to uveitis, as the effect of IL-6 on the retinal endothelial barrier will continue to occur via

classical signalling. An anti-IL-6R humanized antibody, satralizumab, which blocks both mIL-6 and sIL-6R, was shown to reduce the risk of relapses in neuromyelitis optica spectrum disorder when administered systemically;[812] however, the drug has not been investigated for the treatment of uveitis. The development of an anti-IL-6 monoclonal antibody for the treatment of diabetic and uveitic macular oedema is in the pipeline (Roche, Basel, Switzerland) and is expected to be released from the year 2025 onwards – a promising therapeutic since it will be administered intravitreally.[813, 814]

The list of the multiple retinal endothelial lncRNAs involved in inflammation (Chapter 6) paves the way for future investigations researching specific miRNAs interactions and downstream pathways. The discovery of important lncRNAs regulating endothelial dysfunction in inflammation may potentially be converted into drug targets for the treatment of uveitis. Different RNA-based treatments have been developed – antisense oligonucleotides anti-miRNAs, small siRNAs, short hairpin RNAs, miRNA mimics, miRNA sponge, therapeutic circular RNAs and CRISP-Cas9.[815] In ophthalmology, there is currently one antisense oligonucleotide drug approved against cytomegalovirus retinitis, but none for the treatment of non-infectious uveitis.[815]

When treating non-infectious uveitis by the intravitreal route, the drug must diffuse initially through the vitreous to the inner blood-retinal barrier in order to act on retinal endothelial cells, while when delivering a drug systemically, the agent would have to cross the tight intercellular connections of the vascular endothelium to reach the other components of the inner blood-retinal barrier and neural retina. The physiological limitation of solute transport across the inner blood-retinal barrier is a challenge to overcome when considering drug delivery, because even when the retinal endothelium is dysfunctional, it may still have some barrier function. The blood-retinal barrier possesses different types of membrane transporters that could serve as drug carriers.[816, 817] The study of nutrient transport across the barrier may suggest therapeutic agents with similar molecular properties, which could move more easily across the blood-retinal barrier.[817] Modulators of cellular tight junctions are potential tools to facilitate drug access across the barrier.[818] The combination of the systemic and intravitreal delivery of a drug may be the optimized approach to tackle vascular leakage in uveitis.

The involvement of multiple Th lineages in the pathogenesis of non-infectious uveitis may also explain why targeting a single cytokine pathway may not result in complete remission of the disease; therefore, the blockade of multiple inflammatory mediators may potentially improve the management of the disease.[92, 94, 819]

The management of vascular leakage in non-infectious uveitis continues to defy clinicians. Grasping the disease processes at a molecular level enables the emergence of treatment alternatives with appropriate targets. Across the chapters of this thesis and via multiple methodologies, TNF-α, IL-1β, and IL-6 to a lesser degree, were revealed to be central mediators of retinal endothelial vascular leakage, whereas IL-8, IL-17 and CCL2 were not implicated in the disruption of the retinal endothelial barrier. These findings point to TNF-α, IL-1β, and IL-6 as important drug targets for the management of retinal endothelial dysfunction in non-infectious uveitis. This work brings together a body of new knowledge that advances the identification of the central cytokines involved in human retinal endothelial dysfunction in uveitis and their mechanisms of action. The research unveils the presence of a key cytokine receptor in the human retinal endothelium and a transcription factor involved in its regulation, and reports several lncRNAs which could potentially be future drug targets to treat intraocular inflammation and prevent blindness.

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APPENDIX

Table. List of transcription factors predicted to regulate the IL-6R promoter

Transcription factors are ranked on the basis of binding site count predicted by JASPAR 2020.

Abbreviation	Transcription factor	Number of binding sites
TFAP2A	Transcription factor AP-2 alpha	15
SPI1	Spi-1 proto-oncogene	12
ETS1	ETS proto-oncogene 1, transcription factor	10
TFAP2E	Transcription factor AP-2 epsilon	10
NR2C2(var.2)	Nuclear receptor subfamily 2 group C member 2 var.2	9
RHOXF1	Rhox homeobox family member 1	8
GATA2	GATA binding protein 2	7
NFIX	Nuclear factor I X	7
TFAP2C	Transcription factor AP-2 gamma	6
THAP1	THAP domain containing 1	6
KLF15	Krüppel-like factor 15	5
SOX18	SRY-box 18	5
SP1	Sp1 transcription factor	5
CEBPB	CCAAT enhancer binding protein beta	4
FOS::JUN	Fos and Jun: AP-1 transcription factor subunits	4
HIC2	HIC ZBTB transcriptional repressor 2	4
KLF2	Krüppel-like factor 2	4
KLF5	Krüppel-like factor 5	4
NFIC	Nuclear factor I C	4
SP3	Sp3 transcription factor	4
TCF3	Transcription factor 3	4

Abbreviation	Transcription factor	Number of binding sites
TFAP2A(var.2)	Transcription factor AP-2 alpha var.2	4
ZEB1	Zinc finger E-box binding homeobox 1	4
ETV4	ETS variant 4	3
FOSL1	FOS like 1, AP-1 transcription factor subunit	3
JUNB	JunB proto-oncogene, AP-1 transcription factor subunit	3
MEIS1	Meis homeobox 1	3
SP2	Transcription factor Sp2	3
TFAP2B	Transcription factor AP-2 beta	3
BATF::JUN	Basic leucine zipper transcription factor ATF-like::Jun proto-oncogene, AP-1 transcription factor subunit	2
CEBPA	CCAAT enhancer binding protein alpha	2
CEBPD	CCAAT enhancer binding protein delta	2
CEBPE	CCAAT enhancer binding protein epsilon	2
EGR1	Early growth response 1	2
FOSB::JUNB	FosB proto-oncogene, AP-1 transcription factor subunit::JunB proto-oncogene, AP-1 transcription factor subunit	2
FOSL2::JUNB	FOS like 2, AP-1 transcription factor subunit::JunB proto-oncogene, AP-1 transcription factor subunit	2
FOSL2::JUND	FOS like 2, AP-1 transcription factor subunit::JunD proto-oncogene, AP-1 transcription factor subunit	2
FOXD2	Forkhead box D2	2
FOXO4	Forkhead box O4	2
FOXO6	Forkhead box protein O6	2
GSX2	GS Homeobox 2	2
HOXA1	Homeobox A1	2
HOXA2	Homeobox A2	2

Abbreviation	Transcription factor	Number of binding sites
HOXA5	Homeobox A5	2
HOXB2	Homeobox B2	2
HOXB5	Homeobox B5	2
JUND	JunD proto-oncogene, AP-1 transcription factor subunit	2
KLF4	Krüppel-like factor 4	2
KLF6	Krüppel-like factor 6	2
LHX9	LIM/homeobox protein Lhx9	2
MAZ	MYC associated zinc finger protein	2
MZF1	Myeloid zinc finger 1	2
NFKB2	Nuclear factor kappa B subunit 2	2
NKX2-8	Homeobox protein Nkx-2.8	2
OTX2	Homeobox protein OTX2	2
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region	2
SNAI1	Snail family transcriptional repressor 1	2
SNAI2	Snail family transcriptional repressor 2	2
SOX10	Box transcription factor 10	2
SP9	SP9 transcription factor	2
SPIB	SPIB transcription factor	2
TCF4	Transcription factor 4	2
TFAP2A(var.3)	Transcription factor AP-2 alfa var.3	2
TFAP2B(var.3)	Transcription Factor AP-2 beta var.3	2
TFAP2C(var.3)	Transcription Factor AP-2 gamma var.3	2
TFE3	Transcription factor binding to IGHM enhancer 3	2
YY1	YY1 transcription factor	2

Abbreviation	Transcription factor	Number of binding sites
ZBTB14	Zinc finger and BTB domain containing 14	2
ZNF354C	Zinc finger protein 354C	2
BARX1	Homeobox protein bar H-like 1	1
BATF	Basic leucine zipper transcription factor ATF-like	1
BATF3	Basic leucine zipper transcription factor ATF-3	1
BSX	Brain specific homeobox	1
CEBPG	CCAAT enhancer binding protein gamma	1
E2F1	E2F transcription factor 1	1
E2F4	E2F transcription factor 4	1
E2F6	E2F transcription factor 6	1
EBF1	EBF transcription factor 1	1
EHF	ETS homologous Factor	1
ELF5	E74 Like ETS transcription factor 5	1
ELK1	ETS transcription factor ELK1	1
ELK4	ETS transcription factor ELK4	1
EMX1	Empty spiracles homeobox 1	1
EN2	Engrailed homeobox 2	1
ETS2	ETS proto-oncogene 2, transcription factor	1
EVX2	Even-skipped homeobox 2	1
FIGLA	Folliculogenesis specific bHLH transcription factor	1
FLI1	Fli-1 proto-oncogene, ETS transcription factor	1
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	1
FOS::JUNB	Fos proto-oncogene, AP-1 transcription factor subunit::JunB proto-oncogene, AP-1 transcription factor subunit	1

Abbreviation	Transcription factor	Number of binding sites
FOS::JUND	Fos proto-oncogene, AP-1 transcription factor subunit::JunD proto-oncogene, AP-1 transcription factor subunit	1
FOSL1::JUN	FOS like 1, AP-1 transcription factor subunit::Jun proto-oncogene, AP-1 transcription factor subunit	1
FOSL1::JUNB	FOS like 1, AP-1 transcription factor subunit::JunB proto-oncogene, AP-1 transcription factor subunit	1
FOSL1::JUND	FOS like 1, AP-1 transcription factor subunit::JunD proto-oncogene, AP-1 transcription factor subunit	1
FOSL2	FOS like 2, AP-1 transcription factor subunit	1
FOSL2::JUN	FOS like 2, AP-1 transcription factor subunit::Jun proto-oncogene, AP-1 transcription factor subunit	1
FOXC1	Forkhead box C1	1
FOXL1	Forkhead box L1	1
FOXO3	Forkhead box O3	1
FOXP3	Forkhead box P3	1
GSC	Goosecoid homeobox	1
GSX1	GS homeobox 1	1
HOXA4	Homeobox A4	1
HOXA7	Homeobox A7	1
HOXB3	Homeobox B3	1
HOXB4	Homeobox A4	1
HOXB6	Homeobox B6	1
HOXB8	Homeobox B8	1
HOXC4	Homeobox C4	1
HOXD3	Homeobox D3	1
HOXD4	Homeobox D4	1
HOXD8	Homeobox D8	1

Abbreviation	Transcription factor	Number of binding sites
IRF7	Interferon regulatory factor 7	1
JUN(var.2)	Jun proto-oncogene var.2	1
KLF10	Krüppel-like factor 10	1
KLF11	Krüppel-like factor 11	1
KLF16	Krüppel-like factor 16	1
KLF3	Krüppel-like factor 3	1
LBX2	Ladybird homeobox 2	1
LHX1	LIM homeobox 1	1
MSX1	Msh homeobox 1	1
MXI1	MAX Interactor 1, dimerization protein	1
NFATC2	Nuclear factor of activated T-cells 2 interacting protein	1
NHLH1	Nescient helix-loop-helix 1	1
NKX6-3	NK6 homeobox 3	1
NOTO	Notochord homeobox	1
NR2C1	Nuclear receptor subfamily 2 group C member 1	1
NRF1	Nuclear respiratory factor 1	1
OTX1	Orthodenticle homeobox 1	1
PDX1	Pancreatic and duodenal homeobox 1	1
PITX1	Paired like homeodomain 1	1
PITX2	Paired like homeodomain 2	1
PITX3	Paired like homeodomain 3	1
RFX7	Regulatory factor X7	1
RORB	RAR related orphan receptor B	1
RORC	RAR Related orphan receptor C	1

Abbreviation	Transcription factor	Number of binding sites
RUNX2	RUNX family transcription factor 2	1
RUNX3	RUNX family transcription factor 3	1
SREBF1	Sterol regulatory element binding transcription factor 1	1
SREBF2	Sterol regulatory element binding transcription factor 2	1
TBX4	T-box transcription factor 4	1
TCF12(var.2)	Transcription factor 12 var.2	1
TFEB	Transcription factor EB	1
TFEC	Transcription factor EC	1
USF1	Upstream transcription factor 1	1
VENTX	VENT Homeobox	1
ZBTB26	Zinc finger and BTB domain containing 26	1
ZNF148	Zinc finger protein 148	1
ZNF263	Zinc finger protein 263	1
ZNF341	Zinc finger protein 341	1
ZNF75D	Zinc finger protein 75D	1