Manipulation of the Transcription of Intercellular Adhesion Molecule 1 in Human Retinal Endothelial Cells

By

Alwin Tan Chun Rong

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ABSTRACT

Uveitis is the term used to describe intraocular inflammation. The disease is defined according to its location inside the eye, and can be infectious or non-infectious in nature. Patients may present with eye redness, floaters and/or blurred sight, and the vision is often impaired. Non-infectious uveitis is associated with dysregulation of the immune response, leading to autoinflammation or autoimmunity. Current treatment of non-infectious uveitis includes the use of corticosteroids and immunomodulatory drugs, such as methotrexate and adalimumab. These drugs can be effective in managing the inflammation, but they often have adverse effects. The work described in this thesis centres around a novel therapeutic approach to treat non-infectious posterior uveitis.

During non-infectious posterior uveitis, leucocytes migrate across the retinal endothelium into the posterior segment of the eye. This process is tightly regulated by adhesion molecules, which are expressed on the surface of the endothelium. In particular, intercellular adhesion molecule 1 (ICAM-1), being the most well-studied member of the immunoglobulin superfamily cell adhesion molecules, is known to interact with integrins on leucocytes to direct leucocyte trafficking. ICAM-1 expression is primarily controlled at the level of gene transcription. Its expression increases following stimulation by inflammatory cytokines that include tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β).

This thesis presents experimental work that explores ICAM-1 expression by human retinal endothelial cells, and investigates the manipulation of *ICAM1* gene transcription in these cells as the basis of a new treatment for non-infectious posterior uveitis. In addition to the full-length ICAM-1 transcript, a novel ICAM-1 transcript variant was identified in human retinal endothelial cells. Inflammatory cytokines, TNF-α and IL-1β, upregulated full-length and novel ICAM-1 transcript expression in primary human retinal endothelial cell isolates. A significant increase in ICAM-1 protein expression on cell isolates was also observed following TNF-α and IL-1β stimulation.

A range of transcription factors (TFs) were predicted to regulate *ICAM1* gene transcription by *in silico* methods, and Ets-1 was chosen as the candidate TF for targeting in human retinal endothelial cells. Ets-1 is encoded by a proto-oncogene. It is the best studied of the Ets TF family, and most often acts as a transcriptional activator. Thus, it was hypothesised that Ets-1 was a transcriptional activator of the *ICAM1* gene in human retinal endothelial cells, and it was expected that ICAM-1 expression by these cells would be reduced when Ets-1 activity was inhibited.

To investigate the effect of Ets-1 blockade on ICAM-1 expression in human retinal endothelial cells, Ets-1 transcript was targeted with small interfering (si)RNA in a cell line derived from primary cells. The Ets-1 transcript was expressed in the cell line, as in primary human retinal endothelial cells, and there was a significant reduction in transcript expression following siRNA transfection. Unexpectedly, ICAM-1 transcript and protein expression increased following Ets-1 siRNA transfection and treatment with cytokines, TNF-α and IL-1β, compared to the fresh medium control. These findings show for the first time that blocking Ets-1 can increase the expression of ICAM-1.

The overall goal of this thesis was to progress a new therapeutic approach for non-infectious posterior uveitis, involving manipulation of the transcription of the *ICAM1* gene in retinal endothelial cells. The work has demonstrated that ICAM-1 protein expression is increased in the face of Ets-1 blockade. However, delivering an Ets-1 activator as a treatment for non-infectious posterior uveitis is unlikely to gain traction, given the potential for Ets-1 to drive tumour development. This study highlights Ets-1 as an important regulator of ICAM-1 expression in retinal endothelial cells.

DECLARATION

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

Alwin Tan Chun Rong

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LIST OF ABBREVIATIONS

2-ME: 2-mercaptoethanol

5mC: 5-methylcytosine

AdMLP: Adenovirus major late promoter

AHR: Aryl hydrocarbon receptor

AMD: Age-related macular degeneration

AP-1: Activator protein-1

ATF3: Activating transcription factor 3

BAB: Blood-aqueous barrier

BACH1: BTB domain and CNC homolog 1

BCL6: B-cell lymphoma 6

BET: Bromodomain and extraterminal domain

bHLH: Basic helix-loop-helix

Blimp1: B lymphocyte-induced maturation protein 1

BPTF: Bromodomain PHD finger

BRB: Blood-retinal barrier

BSA: Bovine serum albumin

CAMs: Cellular adhesion molecules

CEBPA: CCAAT enhancer binding protein alpha

CEBPB: CCAAT enhancer binding protein beta

CEBPD: CCAAT enhancer binding protein delta

ChEA3: ChIP-X enrichment analysis 3

ChIP: Chromatin immunoprecipitation

ChIP-seq: ChIP sequencing

COE1: Collier/Olf-1/EBF1

CREB: cAMP response element-binding protein

CTCF: CCCTC-binding factor

CUTL1: CCAAT Displacement protein

DAPI: 4', 6-diamidino-2-phenylindole

DBD: DNA binding domain

DCE: Downstream core element

DMO: Diabetic macular oedema

DMSO: Dimethyl sulphoxide

DNA: Deoxyribonucleic acid

Dnmts: DNA methyltransferases

DPE: Downstream promoter element

DTT: Dithiothreitol

EAU: Experimental autoimmune uveitis

EBF: Early B-cell factor 1

EBS: ETS-binding site

EGR1: Early growth response 1

ELF1: E74 like ETS transcription factor 1

ELICA: Enzyme-linked immune-culture assay

ELK1: ETS like-1 protein

ESE-1: Epithelium-specific ETS-like transcription factor 1

ETS1: ETS Proto-Oncogene 1

ETS2: ETS Proto-Oncogene 2

FBS: Fetal bovine serum

FDA: United States Food and Drug Administration

FLI1: Friend leukemia integration 1

FOXM1: Forkhead box protein M1

FOXP3: Forkhead box P3

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GATA3: GATA-binding factor 3

GATA6: GATA-binding factor 6

GCR2: Glycolytic genes transcriptional activator 2

GSH: Glutathione

HATs: Histone acetyltransferases

HCC: Hepatocellular carcinoma

HDACs: Histone deacetylases

HGF: Hepatocyte growth factor

HIF1: Hypoxia-inducible factor 1

HIF1A: Hypoxia-inducible factor 1 alpha

ICAM-1: Intercellular adhesion molecule 1

ICAMs: Intercellular adhesion molecules

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IKZF1: Ikaros zinc finger 1

IL-1β: Interleukin-1 beta

IRE: Iron-responsive element

IRF1: Interferon regulatory factor 1

IRF8: Interferon regulatory factor 8

IUSG: International uveitis study group

JAK: Janus kinase

KLF2: Kruppel-like factor 2

KLF4: Kruppel-like factor 4

KLF5: Kruppel-like factor 5

KLF10: Kruppel-like factor 10

LFA-1: Lymphocyte function-associated antigen 1

LYL1: Lymphoblastic leukemia derived sequence 1

Mac-1: Macrophage-1 antigen

Maf: V-maf musculoaponeurotic fibrosarcoma

MAP: Mitogen-activated protein

MAX: Myc-associated factor X

MDM2: Murine double minute 2

MMP: Matrix metalloproteinases

mRNA: Messenger RNA

MTE: Motif ten element

MYC: Master regulator of cell cycle entry and proliferative metabolism

NCBI: United States National Library of Medicine National Centre for Biotechnology

NF-AT1: Nuclear factor of activated T-cells

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B-cells

NFE2L2: Nuclear factor erythroid 2 like 2

NR1H3: Nuclear receptor subfamily 1 group H member 3

NR3C1: Nuclear receptor subfamily 3 group C member 1

OGT: O-GlcNAc transferase

P-13: 2-desoxy-4β-propylcarbamate-pulchellin

Pax2: Paired box 2

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PDGF: Platelet-derived growth factor

PDR1: Pectin degradation regulator 1

PFA: Paraformaldehyde

PIC: Pre-initiation complex

PKC: Protein kinase C

PKCα: Protein kinase C alpha

PPARD: Peroxisome proliferator activated receptor delta

PPARG: Peroxisome proliferator activated receptor gamma

PU.1: Purine rich box-1

qPCR: Quantitative polymerase chain reaction

RACE: Rapid amplification of cDNA ends

RARA: Retinoic acid receptor alpha

RELA: REL-associated protein

RISC: RNA-induced silencing complex

RNA: Ribonucleic acid

RNA Pols: RNA polymerases

RNAP: RNA polymerase

RNA-seq: RNA sequencing

RPc: Closed promoter complex

RPLP0: Ribosomal protein lateral stalk subunit P0

RPo: Open promoter complex

rRNA: Ribosomal RNA

RT: Reverse transcription

RUNX1: Runt-related transcription factor 1

RUNX2: Runt-related transcription factor 2

SELEX: Systematic evolution of ligands by exponential enrichment

shRNA: Short hairpin RNA

sICAM-1: Soluble intercellular adhesion molecule 1

siRNA: small interfering RNA

SOX17: SRY-Box transcription factor 17

Sp-1: Specificity protein 1

Spi-1: SFFV proviral integration site -1

STAT: Signal transducer and activator of transcription

STAT1: Signal transducer and activator of transcription 1

STAT3: Signal transducer and activator of transcription 3

STAT4: Signal transducer and activator of transcription 4

STAT5: Signal transducer and activator of transcription 5

STAT6: Signal transducer and activator of transcription 6

SUN: Standardization of uveitis nomenclature

TACE: TNF-α converting enzyme

TAE: Tris-acetate-EDTA

TAFs: TBP- associated factors

TAL1: T-cell acute lymphocytic leukemia 1

TBP: TATA box-binding protein

TCF7: T-cell factor 7

TF: Transcription factor

TNF-α: Tumour necrosis factor-alpha

tRNA: Transfer RNA

TSS: Transcription start site

VCAM-1: Vascular cell adhesion molecule 1

VDR: Vitamin D receptor

VEGF: Vascular endothelial growth factor

VLA-4: Very late antigen 4

VWF: Von-Willebrand factor

PREFACE

The material found in this Master of Science thesis is my own work, with the exception of:

- Isolation of primary human retinal endothelial cells (Chapters 3 and 5). This was performed by Dr. Yuefang Ma.
- Human retinal endothelial cell RNA sequencing data set used for the *in silico* analyses (Chapter 4). This was generated by Professor Justine Smith's and Professor David Lynn's teams at Flinders University and the South Australian Health & Medical Research Institute.

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CHAPTER 1: INTRODUCTION

1.1 General Introduction

Uveitis describes a range of intraocular inflammatory diseases (Krishna et al., 2017) that are major causes of blindness in developed and developing countries (Rothova et al., 1996, Durrani et al., 2004, de Smet et al., 2011). There is need for better treatments for non-infectious posterior uveitis in particular, as this form of uveitis involves the retina and thus commonly threatens the vision. Current first-line therapies with corticosteroids, such as prednisolone, and conventional immunosuppressive drugs, such as methotrexate are ineffective for many patients with non-infectious posterior uveitis and are often associated with side effects (Jabs et al., 2000). Adalimumab, a human anti-tumour necrosis factor-alpha (TNF-α) antibody, was approved in 2016 for treatment of non-infectious uveitis involving the posterior segment of the eye, following clinical trials that showed substantial improvements in visual function (Sheppard et al., 2017). However, this biologic drug is not suitable for every form of uveitis, and complications include allergic reactions and serious infections, as summarised by LaMattina and Goldstein (LaMattina and Goldstein, 2017).

Inflammatory conditions are defined by the accumulation of leucocytes at the disease site. The process of leucocyte migration into a tissue is tightly controlled by adhesion molecules and chemokines expressed on the vascular endothelium of that tissue (Engelhardt and Wolburg, 2004). During inflammation, expression of endothelial adhesion molecule and chemokines increases in response to inflammatory cytokines that include TNF-α and interleukin (IL)-1β (Bharadwaj et al., 2013a, Xie et al., 2014). Both of these master inflammatory cytokines are implicated in the development of non-infectious posterior uveitis, which is driven by a Th1 or Th17 helper T-cell response (Luger et al., 2008). Migration of T-cells and other leucocytes across the retinal vascular endothelium in uveitis is controlled in large part by the intercellular adhesion molecule (ICAM)-1 (Bharadwaj et al., 2013b, Bharadwaj et al., 2017, Paulsen et al., 2015). Hence, this endothelial adhesion molecule presents a potential therapeutic target for the treatment of non-infectious posterior

uveitis. However, a challenge remains, as drugging the endothelial adhesion molecules that mediate the onset of the disease, might at the same time impede physiological tissue surveillance, and increase the patient's risk of infection or tumour malignancy (Roland et al., 2007, Ren et al., 2011, Hua, 2013).

Intercellular adhesion molecule 1, an immunoglobulin (Ig) superfamily member, presents a target for transcriptional modulation, given its expression is regulated at the level of gene transcription (Pate et al., 2010). Despite transcription initially being described as undruggable (Yan and Higgins, 2013), in recent years there have been attempts to modulate transcriptional activity in cancer treatment, such as with the use of BET inhibitors in advanced solid tumours and myelodysplastic syndrome (Anand et al., 2013). Furthermore, in a proof-of concept paper, Ashander and colleagues demonstrated that in blocking the ICAM-1 transcription factor (TF), NF-kB1, constitutive ICAM-1 expression levels were maintained (Ashander et al., 2016), strengthening the argument that drugging *ICAM1* gene transcription may potentially be translated into a clinical treatment for non-infectious posterior uveitis. The research in this project investigates whether TF blockade leads to a reduction in ICAM-1 expression in human retinal endothelial cells stimulated with inflammatory cytokines.

1.2 Gene transcription

1.2.1 DNA structure

The human genome contains all the genes required for the phenotype and functioning of the many different populations of cells throughout the body. Much cell-specific gene expression is regulated at the level of transcription (Gorkin et al., 2014). In eukaryotic cells, DNA encodes all the essential genetic information. The classic double helical DNA structure was first described by James Watson and Francis Crick in 1953 (Watson and Crick, 1953). Two strands of polynucleotides form this amazing helical structure. The simple monomeric form, known as a nucleotide, consists of four chemical bases, adenine (A), guanine (G), thymine (T) and cytosine (C), plus a deoxyribose sugar and a phosphate group. Two DNA strands run in opposite directions, in which the bases are held strongly by hydrogen bonds, formed by the base pairing of the nitrogenous bases, pyrimidines (thymine and cytosine) and purines (adenine and guanine). The complementary sequence of the base

pairs include adenine with thymine, held by two hydrogen bonds, and guanine with cytosine, held by three hydrogen bonds. The DNA is coiled tightly, with nucleotides wrapped by histones, forming structures known as chromosomes that are enclosed within the crowded site of the interphase nucleus. In humans, there are 23 pairs of chromosomes. The process of gene transcription requires DNA access, made possible by helicase activity. Helicases unwind the double stranded DNA in an ATP-dependent manner, which is also required for several other processes, such as DNA replication, DNA repair, telomere maintenance and chromosome segregation (Brosh and Bohr, 2007, Dillingham, 2011, Bernstein et al., 2010).

DNA can be chemically modified to alter gene regulation in a process known as DNA methylation. Studies supporting DNA methylation in regulating gene expression and cell differentiation did not appear until the 1980s (Holliday and Pugh, 1975, Compere and Palmiter, 1981). By today, this epigenetic mechanism of DNA methylation has been well established. DNA methylation involvement in gene regulation is through the recruitment of proteins that repress gene expression or the inhibition of transcription factor binding to DNA (Moore et al., 2013). This process is catalysed by DNA methyltransferases (Dnmts), which add a methyl group to the fifth carbon of a cytosine residue, forming 5-methylcytosine (5mC) (Moore et al., 2013). Husquin and colleagues demonstrated that DNA methylation significantly correlates to the transcription of 811 basal genes and 230 immune stimulated genes, suggesting DNA methylation involvement in immune gene regulation (Husquin et al., 2018). In their study, they identified several regulators of DNA methylation in *trans*, plus a regulatory hub near the *CTCF* gene accounting for the changes in DNA methylation of various ancestry origin. In mammalian cells, CCCTC-binding factor (CTCF) is a TF responsible for maintaining cell viability, and it functions as a regulator of the genome architecture (Gambetta and Furlong, 2018). In particular, CTCF acts as an insulator, whereby it creates chromatin loops between CTCF bounded sites, creating a barrier inhibiting the contact of chromosomal regions within and outside the loop (Narendra et al., 2015, Hanssen et al., 2017, Nora et al., 2017).

1.2.2 Transcription regulation

The process of gene transcription is highly regulated and involved in biological processes such as cell differentiation and function. Transcription is the process in which DNA is transcribed into RNA, including messenger RNA (mRNA). In eukaryotes, this process requires three RNA polymerases (Pols). These enzymes are RNA Pol I, Pol II, and Pol III, each of which are involved in transcribing a different set of genes and producing various types of RNA (Roeder and Rutter, 1969). Among these three Pols, eukaryotic Pol II, a 12-subunit DNA dependent RNA polymerase, is the key enzyme for initiating transcription and synthesizing mRNA, which then acts as the template for protein synthesis (Sainsbury et al., 2015). Pol I and Pol III have different functions, producing ribosomal RNA (rRNA) and transfer RNA (tRNA), respectively (Hantsche and Cramer, 2016). There are three phases to transcription regulation: initiation, elongation, and termination.

Initiation of RNA synthesis requires the identification of the promoter DNA sequence, usually located upstream of a gene. In eukaryotes, RNA Pol II dependent transcriptional activity involves six general TFs – TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH – at the DNA promoter site (Burley and Roeder, 1996). Transcription factors are proteins which bind to specific regulatory regions of the DNA, that include promoters and enhancers, and are involved in the primary control of targeted gene expression. Transcription factors contain 2 regions: a DNA-binding domain and an activator domain. These TFs assemble at the start site of the DNA promoter, with TFIID being the first basal TF bound to the core promoter, assembling the preinitiation complex (PIC) (Burley and Roeder, 1996). The DNA double helix is first unwound by helicase activity, leaving approximately 15 base pairs of promoter DNA exposed, forming a “transcription bubble” (Liu et al., 2013). The PIC then undergoes a change from a “closed” to “open” promoter complex (Liu et al., 2013). This isomerization process from the closed promoter complex (RPc) to the open promoter complex (RPo) is poorly understood, but is believed to recruit multiple intermediate states of RNA polymerase (RNAP) and DNA (Hantsche and Cramer, 2016). In the last few years, cryo-electron microscopy has provided new insights into the molecular details of eukaryotic transcription initiation, which includes detailed

structures, such as the regulatory elements, improving our understanding of the mechanism regulating gene expression (Nogales, 2016). A cryoelectron microscopy study by Glyde and colleagues established the structure of an activator-bound intermediate complex that exists between RPc and RPo (Glyde et al., 2017). Similarly, the study proposed that DNA melting initiated in the RPc led to the conformational changes in RNAP, with intermediates that were quite distinct from RPo and RPc (Glyde et al., 2017).

RNA synthesis follows the initiation of transcription. This highly regulated transcription machinery requires core promoter elements, such as a TATA element and a pyrimidine-rich initiator element (Nikolov and Burley, 1997). Hence, the interaction between regulatory elements, such as enhancers and promoters, plays a vital role in transcriptional regulation activity. A termination signal is encoded in sequence located at the end of the gene, and the termination phase of transcription includes mRNA 3'-end processing (Davis and Shi, 2014). Following recognition of the termination signal, the RNA is cut from the moving RNA Pol II, and a stretch of adenines is added, in the process known as "polyadenylation".

1.2.2.1 Structure of the eukaryotic promoter

The process of transcription first requires identification of a promoter site for binding of RNA Pol. The prokaryotic promoter contains two motifs, located at 10 and 35 base pairs upstream from the transcription start site (TSS) (Harley and Reynolds, 1987). In eukaryotes, the promoter is structurally more complex and contains several motifs that include a TATA box, BRE, INR box, GC box and CCAAT-box (Bucher, 1990). To assemble a functional PIC, core promoters are generally sufficient. In humans, only 5-10% of core promoters have been associated with the presence of a TATA element based on genome-wide analyses, but motif ten element (MTE), downstream promoter element (DPE) and downstream core element (DCE) are usually absent (Lenhard et al., 2012). There are two classes of metazoan promoters: broad TSS promoters with precise nucleosome positioning, high in CpG content, and focused TSS promoters, consisting of an INR and a TATA box, often with low GC content (Lenhard et al., 2012).

1.2.2.2 Core promoter elements

Within the eukaryotic promoter site, the TATA box is an AT-rich sequence that is located 25 to 30 base pairs upstream of TSS, and is specific to TBP binding (Hantsche and Cramer, 2016). It can function independently or in combination with transcription initiator (Inr) element binding regulatory factors. Transcription is regulated with the binding of transcriptional activators to the sequence located upstream between 50 and 200 base pairs of the TSS (Nikolov and Burley, 1997). Distal enhancer elements located far from the initiation site present as another DNA target for factors mediating Pol II activity (Nikolov and Burley, 1997). Besides that, the TATA box-binding protein (TBP) and other TBP-associated factors (TAFs) are found at TFIID (Sainsbury et al., 2015). The TFIID has its effect at the promoter site, binding tightly to the TATA element and mediating RNA production (Nikolov and Burley, 1997). Human TFIID binding to DNA was first showcased using the adenovirus major late promoter (AdMLP) (Sawadogo and Roeder, 1985). This showed clearly that the TBP subunit of TFIID was involved mainly in sequence-specific interactions between human TFIID and the TATA element (Burley and Roeder, 1996). Essential genes that encode for mammalian TFIID contain the TBP complex along with the 13 highly conserved TAF proteins (Dynlacht et al., 1991, Meisterernst et al., 1991).

The recruitment of TFIID for each pol II promoter is believed to occur in four different processes: (1) interactions with the mediator coactivator subunits that attract and align TFIID at the core promoter (Johnson et al., 2002); (2) binding of human TFIID to chromatin binding domains (TAF1-BrDs and TAF3-PHD) that follows the acetylation and/or methylation of histone tails modifying the promoter nucleosome (Bhuiyan and Timmers, 2019); (3) a complex of TAF1, TAF2, TAF4, and the BC core assembled by TFIID at the promoter site (Bhuiyan and Timmers, 2019, Patel et al., 2018); and (4) activator-TAF interactions with enhancer-promoter looping that recruit TFIID to the core promoter (Levine et al., 2014).

1.2.2.3 Activators and repressors

The binding of transcriptional activators to upstream activating sequences may alter the process of gene-specific transcription through the recruitment of chromatin remodelling and modifying complexes and also the transcriptional apparatus (Lee and Young, 2000). Hence, activators and repressors play an essential role in the regulation of gene expression (Lee and Young, 2000).

1.2.2.4 Transcriptional activators

An activator acts to coordinate the transcription of multiple genes in the genome. In addition, multiple activators may also act to regulate a single gene and hence provide a combinatorial control over that gene. The two domains presented on an activator include those that bind specific DNA sequences (Triezenberg, 1995) and others that recruit and activate the transcription apparatus (Ptashne and Gann, 1997).

Chromatin-modifying complex, such as SAGA and Swi/Snf, are recruited to the promoter site by transcriptional activators (Natarajan et al., 1999, Neely et al., 1999, Yudkovsky et al., 1999). The importance of chromatin remodelling is highlighted by the fact that histone acetylases represent coactivators in transcription regulation. This is evident following the activation of the *HO* gene in yeast, in which the transcriptional activator Swi5p recruits both Swi/Snf and SAGA histone acetylase activity before the indirect recruitment of a second activator, SBF (Cosma et al., 1999). Subsequently, SBF binds to sequence nearest to the *HO*'s TATA box and recruits the transcription initiation apparatus (Cosma et al., 1999).

The importance of the transcription apparatus machinery for transcription involving RNA Pol II has been demonstrated *in vitro* (Hampsey, 1998). Activators recruit the transcription apparatus in one or multiple stages, as shown in studies of purification of transcription complexes containing RNA Pol II, general TFs, and other proteins involved in stimulating transcriptional activators (Koleske and Young, 1994, Ossipow et al., 1995). In yeast, TBP binding is dependent on Srb4, a Pol II holoenzyme component (Kuras and Struhl, 1999). This functional Srb4 containing Pol II is crucial to achieve

stable binding of the TBP to the promoter, leading to the formation of the initiation apparatus (Kuras and Struhl, 1999). This also shows that the transcription initiation apparatus can be assembled in a single step.

1.2.2.5 Transcriptional repressors

While activators are important in the regulation of cell activity, negative regulation is of equal importance with its conservation through evolution. The two forms of repression are general and gene specific.

General transcriptional repressors regulate transcription through interactions with the TBP. Mot1-mediated transcriptional repression is linked to its ability to disrupt the TBP-DNA complexes in an ATP-dependent manner, causing the dissociation of TBP from the DNA (Auble et al., 1997, Muldrow et al., 1999, Wade and Jaehning, 1996). Another general negative regulator, NC2, is involved in regulation of class II and III gene expression (Goppelt et al., 1996). It is able to bind to the TBP domain on promoter DNA, preventing RNA Pol II binding to TFIIB subunit, thereby blocking the assembly of the initiation complex (Goppelt et al., 1996).

Gene-specific repressors may bind to activators or compete for the activator binding sites. An example is Hsp90, which blocks Hsf1 trimer formation by binding to the heat shock transcription activator, Hsf1, inhibiting the heat shock element binding (Zou et al., 1998). Repressor proteins may compete with activators, such as CREB, binding to the same regions on the DNA. The *ACR1* gene encodes the ATF/CREB transcriptional repressor, which contains a specific bZIP domain (Vincent and Struhl, 1992). This domain is important for the binding of specific DNA to the ATF/CREB site (Vincent and Struhl, 1992). By deleting ACR1, transcription is increased via the ATF/CREB sites (Vincent and Struhl, 1992). Gal80 protein binds to a segment of the Gal4 activation domain, and thus exhibits negative regulation (Sil et al., 1999).

1.2.3 Long-range regulation

Two basic principles of metazoan enhancer activity in transcriptional regulation are as follows: (1) enhancers may mediate the expression of distant target genes in a linear distance; and (2) the gene most influenced by an enhancer is not necessarily the closest by linear distance, as illustrated in several studies (Feng and Li, 2017, Lancho and Herranz, 2018, Moisan et al., 2018). In mammalian cells, higher order chromatin is closely linked to long-distance gene regulation, which switches on cell development and cell fate (Gorkin et al., 2014). There is supporting evidence of long-range regulation related to the close proximity of enhancers to the promoters of their target genes *in vivo* (de Laat and Duboule, 2013, Moisan et al., 2018). This allows interactions between protein complexes bound at enhancers and those bound at the promoters, mediating the transcription of specific genes.

1.3 Drugging transcription

1.3.1 Transcription factors as therapeutic targets

The common biologic treatment targets for various diseases include receptors, protein kinases and proteins involved in downstream signal transduction. Transcription factors were for a long time determined as “undruggable” targets (Yan and Higgins, 2013). There have been challenges, due to the absence of three-dimensional structures for many TFs, in particular the protein-protein binding domains, and the limited state of assay technology to investigate their mode of action (Fontaine et al., 2015). However, TFs are central in the regulation of gene transcription, contributing to the development of several diseases, including diabetes mellitus, neurodegenerative diseases, and cancer (Song et al., 2019, Jin et al., 2019, Huilgol et al., 2019). In fact, breakdown in gene regulatory systems is an important cause of disease, whereby dysfunctional TFs contribute to one-third of human developmental disorders (Vaquerizas et al., 2009). Thus, knowledge of the complex networks involved in transcriptional regulation should suggest new therapeutics.

Since the launch of projects for documenting genomic functional elements, like the ENCODE consortium in 2003 (Consortium, 2012) and the FANTOM5 project (Kawaji et al., 2017), which share information related to identifying, locating, and sequencing functional elements, our understanding

of the genome structure and function has vastly improved. A recent area of research has been in exploring methods of modulating TF activity, particularly in the field of oncology (Hagenbuchner and Ausserlechner, 2016, Lambert et al., 2018a). Methods of modulating TF activity may include blocking the DNA binding domain (DBD) of the TF; targeting chromatin remodelling and epigenetic reader proteins; and inhibiting the protein-protein interactions.

1.3.2 Modulating transcription

One approach to modulating transcription is by small compounds that bind to the essential pockets in the DBD of the TF, preventing target promoter recognition and blocking the DNA interaction. The main challenge in this area is identifying compounds that can compete with the target DNA sequence in a highly specific and targeted manner. One example of this strategy involves targeting of Signal Transducer and Activator of Transcription 3 (STAT3). Using an *in silico* virtual drug screening approach based on crystal structures, Huang and colleagues demonstrated that the compound, inS3-54, effectively interacted with the STAT3 DBD to block target gene expression (Huang et al., 2014). An improved version of the compound with less off-target effect, inS3-54A18, not only bound directly to the DBD and inhibited DNA binding by STAT3 *in situ* and *in vitro*, but also blocked both basal and IL-6-stimulated expression of target genes controlled by STAT3 (Huang et al., 2016). The ability of this molecule to block activation of STAT3 target genes was shown to impede tumour growth and metastasis, showing the effectiveness of this approach in modulating disease.

The second approach to modulating transcription is based on altering the accessibility of the target gene DNA for the TF of interest, by modulating epigenetic factors that in turn alter DNA methylation and/or remodel the chromatin in the cell. DNA methylation is the most studied epigenetic modification and is associated with transcriptional repression. Chromatin remodelling is regulated by several classes of histone modifiers, known as writers, readers, and erasers (Hagenbuchner and Ausserlechner, 2016). Chromatin writers are enzyme complexes that lay down a histone code by modifying key amino acids on histone tails, and include histone acetyltransferases (HATs), histone kinases and methyltransferases. Eraser proteins are able to remove redundant histone tail

modifications, and include histone deacetylases (HDACs), phosphatases and demethylases. Finally, reader proteins have binding domains which recognise specific histone tail modifications, thereby interpreting the histone code and facilitating either open chromatin (euchromatin) or closed chromatin (heterochromatin).

In the development of cancer, there are complex changes in DNA methylation and chromatin structures, both of which are already therapeutic targets. Therapeutic compounds used clinically, such as azacitidine and decitabine, are DNA methylation inhibitors, and were developed to target specific epigenetic changes in cancer cells (Christman, 2002). These compounds induce transcription of tumour suppressor genes, thereby reducing oncogenic potential of the cancer cells (Giri and Aittokallio, 2019). The HDACs have also been identified as pharmacological targets, with HDAC inhibitory agents, such as vorinostat and belinostat, showing excellent anti-cancer activity in the treatment of leukaemia and other haematological malignancies (Lakshmaiah et al., 2014, Mihaila, 2017). These agents act generally on acetylated residues and upregulate expression of tumour suppressor proteins. One issue is that these HDAC inhibitors are often non-selective to a specific HDAC isoenzyme, leading to off-target deregulation and resulting in adverse effects in patients (Clawson, 2016).

The final area of focus for modulating transcription is targeting protein-protein interactions. Transcription factors form complexes, and the formation of these homo- or heterodimers are key to recognising specific DNA sequences. Since gene transcription is regulated through interactions between TFs and their subsequent recruitment to the promoter site, these protein-protein interaction surfaces are potential drug targets (Hagenbuchner and Ausserlechner, 2016). This was initially demonstrated for p53 (Momand et al., 1992), a TF that influences cell cycle progression and cell death (Muller and Vousden, 2013). In cancer, an increase in expression of the E3-ubiquitin ligase, MDM2, results in the proteasomal degradation of p53 via a p53-MDM2 interaction (Devine and Dai, 2013). Hence, inhibiting the p53-MDM2 interaction is hypothesized to lead to an increase in cellular p53 levels, thereby restoring the important protective mechanisms mediated by p53 (Nag et al., 2013).

This p53-MDM2 interaction has been heavily studied, and new therapeutics that act as p53-MDM2 complex inhibitors are in trials for treatment of acute and chronic myeloid leukaemia, and some solid tumours (Fang et al., 2020).

1.3.3 Application of transcription factor blockade

Multiple *in vitro* and *in vivo* studies have been conducted in the cancer field showing the potential for TFs to be drugged. Most of the studies have been conducted using cell lines and targeting the DBD. An example demonstrating this is the identification of a compound, BRD32048, as a top candidate that targets the DBD of the TF, ETS variant 1 (ETV1), in prostate cancer and Ewing sarcoma (Oh et al., 2012, Pop et al., 2014). BDR32048 is able to bind directly to ETV1, inhibiting its transcriptional activity on the matrix metalloproteinases 1 (MMP1) promoter. Inhibition of ETV1 leads to a reduction in ETV-1-mediated invasion by prostate cancer cells and melanoma cells.

Recent studies by Grimley and colleagues have highlighted that the DBD of Pax2 may be targeted with a small molecule (Grimley et al., 2017). The Paired box (Pax) gene family codes TFs that regulate embryonic development and the differentiation of various cell lineages. The compound, EG1, which was discovered from screening of potential candidates that blocked Pax2 activity, shows promise as a cell-specific anti-cancer agent. EG1 prevents the proliferation of Pax2-positive renal and ovarian cancer cell lines.

Another inhibitor, $I_{HSF}115$, was designed to target the DBD of the TF, heat shock transcription factor 1 (HSF1) (Vilaboa et al., 2017). Heat shock transcription factor 1 is the master regulator of the mammalian heat shock response, important in the regulation of cellular proteostasis under conditions of heat or stress (Hartl et al., 2011). Several proteins are known to bind HSF1 or the HSF1 complex, most notably ATF1. During stress, ATF1/CREB recruits HSF1 to the target promoter (Takii et al., 2015). Unfortunately, $I_{HSF}115$ did not inhibit the binding activity of HSF1 to the DNA; however, it did inhibit the TF machinery involved in assembly of the ATF1 complex in HeLa cells, a human cervical cancer cell line (Vilaboa et al., 2017).

In summary, blocking a TF does present challenges, due to the lack of specificity and potential off-target effects, and mostly the approach has been employed in cancer treatment. However, if an inhibitor designed to bind to the DBD of a TF is highly specific and does not interfere with other TFs regulating the same pathway, its application would be beneficial and should be translatable to treating other types of disease.

1.4 The Eye

1.4.1 Anatomy and physiology of the eye

The eye is a globe that measures approximately 25 mm in diameter (Purves et al., 2001). It is separated into two segments, the anterior segment and the posterior segment. The anterior segment contains the cornea, conjunctiva, iris, ciliary body, lens and is filled with aqueous humour. Other ocular structures such as the retina, choroid and sclera, make up the vitreous humour-filled posterior segment of the eye. The eye has outer, middle and inner layers. The outer layer is made up of white fibrous tissue, known as the sclera, and a transparent disc at the front of the eye, known as the cornea. The middle layer consists of three structures: the choroid, the ciliary body and the iris. The choroid contains a meshwork of blood vessels, providing blood supply to the outer retina. The ciliary body is contiguous with the choroid and includes the ciliary processes, which secrete aqueous humour, and the ciliary muscle, which controls the refractive power of the lens, innervated by the parasympathetic fibres of the third cranial nerve. The iris separates the anterior and posterior chambers, and creates “the colour of the eye” with a central opening, the pupil, allowing light to enter into the retina. The retina makes up the innermost layer of the posterior eye wall. It is the most complex tissue, comprising photoreceptors and other neurons, which are involved in transmitting visual signals to the brain, and supporting cells, as well as a vascular network. A schematic of the eye structure can be found in **Figure 1.1**.

Light crosses the cornea, lens and two liquid environments before reaching the retina. These liquid environments are the aqueous and the vitreous. The anterior chamber is an aqueous-filled space between the cornea and the lens. The aqueous fluid that is secreted by the ciliary processes flows

from the posterior chamber through the pupil, into the anterior chamber. The aqueous fluid also provides nutrients to the cornea and to a lesser extent, the lens. The posterior segment of the eye is filled with the transparent and viscous vitreous fluid, which provides shape to the eye.

1.4.2 The blood-ocular barriers

The blood-retinal barrier (BRB) and the blood-aqueous barrier (BAB) are tissue barriers that protect the eye from a range of molecular and cellular insults. The blood-ocular barrier is made up of two components: The BRB is the posterior segment barrier, consisting of the retinal pigment epithelium and the retinal endothelium, plus several other cell populations, including pericytes, Müller cells and neurons. It plays an important role in separating the retina and the vitreous environment from the systemic circulation, preventing passage of molecules from the choroid. The BAB forms the anterior segment barrier and is made up of endothelial cells of blood vessels in the iris and the non-pigmented ciliary epithelium.

These blood-ocular barriers control the movement of fluid and solutes from the bloodstream into the eye (Barar et al., 2016). Any drug that is intended for action inside the eye must have physical-chemical characteristics that allow it to cross; more often, local delivery systems are used to move a drug into the eye (Dubald et al., 2018).

1.4.2.1 Ocular drug delivery

In treating posterior segment diseases, the three main delivery methods include systemic, intravitreal and periocular or suprachoroidal (Kang-Mieler et al., 2014). These methods are shown in **Table 1.1**. Eye drops are generally not considered an option, but newer formulations developed using nanocarriers have increased the permeation through tissue barriers, offering the possibility that small molecules may be delivered to the posterior segment (Del Amo et al., 2017). Multiple reviews have been published lately, highlighting the current techniques and advancement in posterior eye drug delivery (Kang-Mieler et al., 2014, Jiang et al., 2018, Behar-Cohen, 2019, Himawan et al., 2019, Varela-Fernandez et al., 2020).

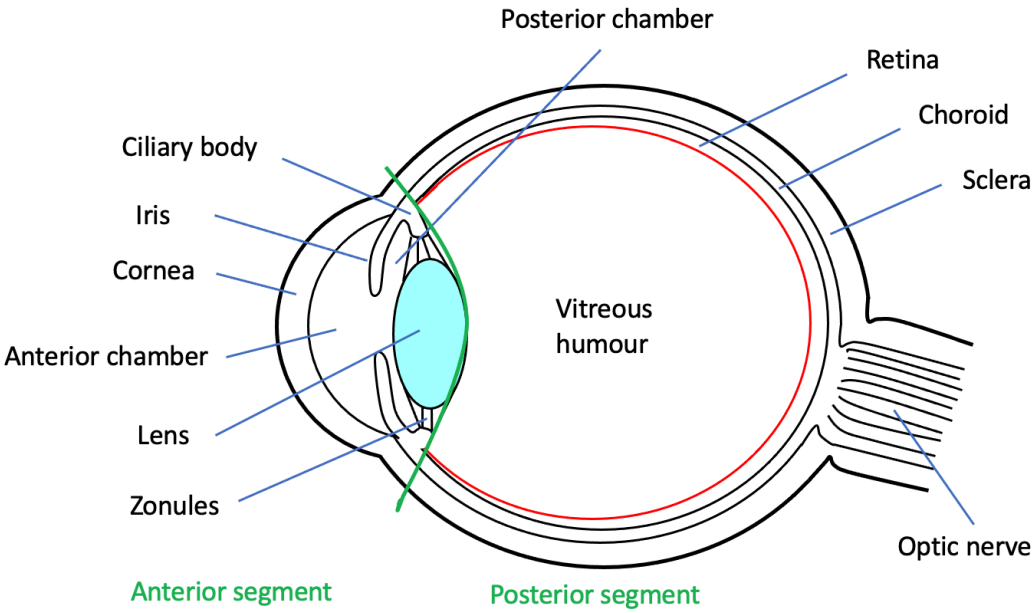

Figure 1.1 Cartoon of the anatomy of the eye shown in cross-section

Intravitreal injections and implants are the most popular method today, as they provide the most direct approach of delivering a therapeutic inside the eye (Yavuz and Kompella, 2017). However, the inner limiting membrane remains a barrier for macromolecule diffusion (Jackson et al., 2003). Advances in nanotechnology and biomaterials have led to the development of biodegradable micro- and nanoparticles, thermo-responsive hydrogels, permeation-enhancing liposomes and emulsions, all of which allow for greater efficiency in therapeutic delivery (Kang-Mieler et al., 2014). A recent study investigated the difference following the administration of triamcinolone acetonide via periocular injection to intravitreal dexamethasone implants for intraocular inflammation (Errera et al., 2019). From the study, the results were conclusive that intraocular administration was more effective. This result was validated in another study that demonstrated an intraocular triamcinolone injection or dexamethasone implant were far more effective for inflammatory macular oedema than a periocular injection of triamcinolone (Thorne et al., 2019).

Periocular injections are also used to treat posterior segment diseases. The routes of administration include peribulbar and retrobulbar (Gaudana et al., 2010). The drug reaches the posterior segment transsclerally and via the systemic circulation. A related method for posterior segment drug delivery is suprachoroidal injection. Injection into the suprachoroidal space can lead to high bioavailability of the drug, and is best suited for use of sustained release drugs. This is due to the high blood flow in the chorio-capillaries that flushes away both micro and macromolecules (Kim et al., 2014). Microneedle technology has improved drug delivery into the suprachoroidal space in a controlled manner, getting the agent closer to the targeted tissue. For example, this has allowed lower dosages of triamcinolone acetonide to be used, while maintaining the same efficacy, but with fewer side effects in the treatment of diabetic macular oedema, retinal vein occlusion, and uveitis (Habot-Wilner et al., 2019, Chiang et al., 2018).

Table 1.1 Advantages and disadvantages of routes for delivering drugs to the posterior segment of the eye (modified from (Yavuz and Kompella, 2017))

Route	Advantages	Disadvantages	Application in treatment of disease
Systemic	• Non-invasive • Self-administration possible • Patient convenience	• Low ocular bioavailability • Blood-aqueous and blood-retinal barriers • Systemic toxicity and side effects	Scleritis, intermediate uveitis, posterior uveitis
Intravitreal	• Effective retinal delivery • Sustained delivery • Bypasses blood-retinal barrier	• Invasive • Patient inconvenience	AMD, DMO, intermediate uveitis, posterior uveitis
Periocular/ suprachoroidal	• Possible depot site • Delivery to both posterior and anterior segments	• Invasive • Patient inconvenience • Clearance by circulation • Blood-retinal barrier for retinal delivery • Risk of haemorrhage	DMO, AMD, intermediate uveitis, posterior uveitis

Abbreviations: AMD = age-related macular degeneration, DMO = diabetic macular oedema

1.5 Uveitis

Uveitis is a term used to describe a range of different types of intraocular inflammation (Krishna et al., 2017). This disease is one of the top five causes of blindness in developed countries (Rothova et al., 1996, Durrani et al., 2004). In Australia, the uveitis incidence is reported at 21.54 per 100,000 person-years with a period prevalence of 36.27 per 100,000 persons, according to a recent cross-sectional study (Hart et al., 2019). Uveitis may occur bilaterally or unilaterally in the eye, and vision loss is often attributed to macular pathology (Rothova et al., 1996). The disease is classified originally according to primary anatomical location of the inflammation inside the eye (Deschenes et al., 2008). Currently, the most commonly accepted classification scheme for the disease comes from the Standardization of Uveitis Nomenclature (SUN) Working Group (Jabs et al., 2005). This is the "gold standard" for uveitis classification in the peer-reviewed literature. Uveitis is categorised into four different types: anterior uveitis, intermediate uveitis, posterior uveitis, and panuveitis. (Smith and Rosenbaum, 2000, Krishna et al., 2017). Anterior uveitis affects the iris and the pars plicata (Smith and Rosenbaum, 2000). Posterior uveitis affects either the retina or the underlying choroid, and sometimes both structures (Krishna et al., 2017). Among the three types of uveitis, intermediate uveitis is the least common form, involving the pars plana and often also the peripheral retina. Panuveitis is the term used when the inflammation occurs across multiple regions of the eye (Krishna et al., 2017).

Ocular inflammation may be infectious or non-infectious in nature. Infectious uveitis is commonly observed in developing and tropical countries, where infectious causes are linked to more than half of the cases, including toxoplasmosis, tuberculosis and onchocerciasis (London et al., 2010). Non-infectious uveitis has been shown to be more common in other countries, comprising approximately 81% of cases in a report from western Europe (Barisani-Asenbauer et al., 2012). This form of uveitis may be associated with systemic conditions arising from the dysregulation of the immune system. The systemic diseases commonly linked with non-infectious uveitis include spondyloarthopathy, multiple sclerosis, inflammatory bowel disease and sarcoidosis. The current first-line therapy in

treating non-infectious uveitis involves the use of corticosteroids (e.g. prednisolone), either through topical application for anterior uveitis, or periocular application or orally for intermediate uveitis and posterior uveitis (Jabs et al., 2000). However, a short course of corticosteroid is often not effective in preventing relapses, and there may be side effects from systemic use, including weight gain, accelerated cardiovascular disease and bone pathology. Thus, other broad-acting conventional immunomodulatory drugs (e.g. methotrexate or azathioprine) may be administered systemically to control the intraocular inflammation (Julian et al., 2013). Foster and colleagues have presented a detailed summary of the current practice in the management of uveitis (Foster et al., 2016).

1.5.1 Cellular and molecular mechanisms of uveitis

The focus of this thesis is non-infectious uveitis, and posterior uveitis in particular. Non-infectious uveitis develops from an inappropriate immune response, caused by autoimmunity or autoinflammation. Animal models have been developed to better understand the mechanisms involved in non-infectious uveitis. However, there is yet to be an animal model that is fully representative of the human disease. The most commonly used model is experimental autoimmune uveitis (EAU), which can be categorised into induced (immunisation) and spontaneous (related to genetic modifications in mice) (Caspi, 2010). The EAU model is triggered when animals are immunised with one of several retinal photoreceptors antigens, producing a T-cell-directed immune response against the antigen (Caspi et al., 1988). Nonetheless, there are other experimental models of uveitis, such as HLA-A29 transgenic mice (Szpak et al., 2001) and recurrent equine uveitis (Gilger et al., 1999). There are also other rodent models representative of autoinflammatory uveitis, in which muramyl dipeptide (Rosenzweig et al., 2009) and lipopolysaccharide (Rosenbaum et al., 2008) are injected systemically or intraocularly.

Studies in animal models have demonstrated that T-cells play an important role in the mediation of non-infectious uveitis. $CD4^+$ T-cells are crucial in controlling EAU development and by blocking the activity of these cells, EAU is prevented (Atalla et al., 1990). The cytokine, interferon (IFN)-γ, is produced by $CD4^+$ Th1 effector cells, while another cytokine, IL-17, is produced by Th17 cells; both

are involved in directing inflammation. However, based on data from animal models of uveitis, it is now clear that the dominant T-cell effector response varies between Th17 or Th1 according to the antigen presentation (Luger et al., 2008). In addition, there is also increasing evidence which suggest that Th1 and Th17 cells both may play a pathogenic role (Damsker et al., 2010).

Smith and colleagues have discussed the mechanisms centred around B-cell involvement in non-infectious uveitis (Smith et al., 2016). Reports have suggested that B-cell blockade may be of therapeutic benefit in patients with non-infectious uveitis, given B-cells have been shown to be involved in promoting intraocular inflammation via presentation of antigen to T-cells, producing several inflammatory cytokines and supporting T-cell survival (Smith et al., 2016). Macrophages are the main contributor to tissue destruction in EAU (Merida et al., 2015). Also, an article recently published by Okunuki and colleagues has demonstrated that microglia, which are resident immune cells in the retina, play a significant role in the initiation of retinal autoimmune responses in a murine model of EAU (Okunuki et al., 2019). By reducing the number of microglia, the migration of leucocytes into the retina is reduced. Thus, the absence of microglia was suggested to prevent the development of autoimmune uveitis (Okunuki et al., 2019).

A common feature exhibited in posterior uveitis is the breakdown of the blood-retinal barrier arising from the activation of the inflammatory cascade. Up-regulation of adhesion molecules on the retinal endothelium and also the release of chemokines move leucocytes to the site of inflammation (Krishna et al., 2017). A recent review highlighted the important roles cytokines have in non-infectious uveitis (Weinstein and Pepple, 2018)

1.5.2 Leucocyte transendothelial migration cascade

Leucocyte migration into the posterior eye across the BRB is dependent on various factors, including the leucocyte subset, the specific site and the microenvironment initiated by the inflammatory stimulus (Crane and Liversidge, 2008, Muller, 2013). Experimental autoimmune uveoretinitis has been used to demonstrate how the compromised BRB allows the migration of lymphocytes and

monocytes into the retina, leading to the tissue damage (Dick et al., 1996). Leucocyte migration into the inflamed retina is controlled by inflammatory cytokines, such as TNF-α and IL-1β, which upregulate expression of adhesion molecules and chemokines on the retinal endothelium (Crane and Liversidge, 2008).

The movement of leucocytes across the endothelium is facilitated in paracellular (move between) or transcellular (through the cell) fashion (Engelhardt and Wolburg, 2004). Binding between endothelial adhesion molecules and receptors on the leucocyte surface allows the endothelium to mediate leucocyte migration in stages. These stages include: rolling; firm adhesion; spreading and crawling; and diapedesis (Lawson and Wolf, 2009). This process is illustrated in **Figure 1.2** and explained in detail in **Table 1.2**. Studies suggest leucocyte migration across retinal endothelial cells follows mainly a paracellular route (Greenwood et al., 1994). Furthermore, in the retinal vascular endothelium, histopathological studies have shown endothelial cell activation leads to post-capillary venules conversion, causing high endothelial cell morphology (McMenamin et al., 1992). The involvement of adhesion molecules in leucocyte-induced breakdown of the BRB has been investigated with the use of scanning laser ophthalmoscopy in rodent EAU models (Crane and Liversidge, 2008). Leucocyte trafficking can be visualised in real-time via fluorescent labelled T helper cell subsets or monocytes, allowing better understanding of the molecules involved in the process (Xu et al., 2005).

During the process of inflammation, interaction between integrins and the Ig superfamily of adhesion molecules are vital in mediating leucocyte migration (Ulbrich et al., 2003). The adhesion molecule, ICAM-1, plays an essential role in lymphocyte and monocyte migration across the retinal endothelium, and this migration activity includes both lymphocyte function-associated antigen 1 (LFA-1)-dependent and LFA-1-independent mechanisms (Greenwood and Calder, 1993, Greenwood et al., 1995, Mesri et al., 1996, Bharadwaj et al., 2013b, Bharadwaj et al., 2017, Crane and Liversidge, 2008). ICAM-1 is minimally expressed at basal level in retinal endothelial cells, but its expression is

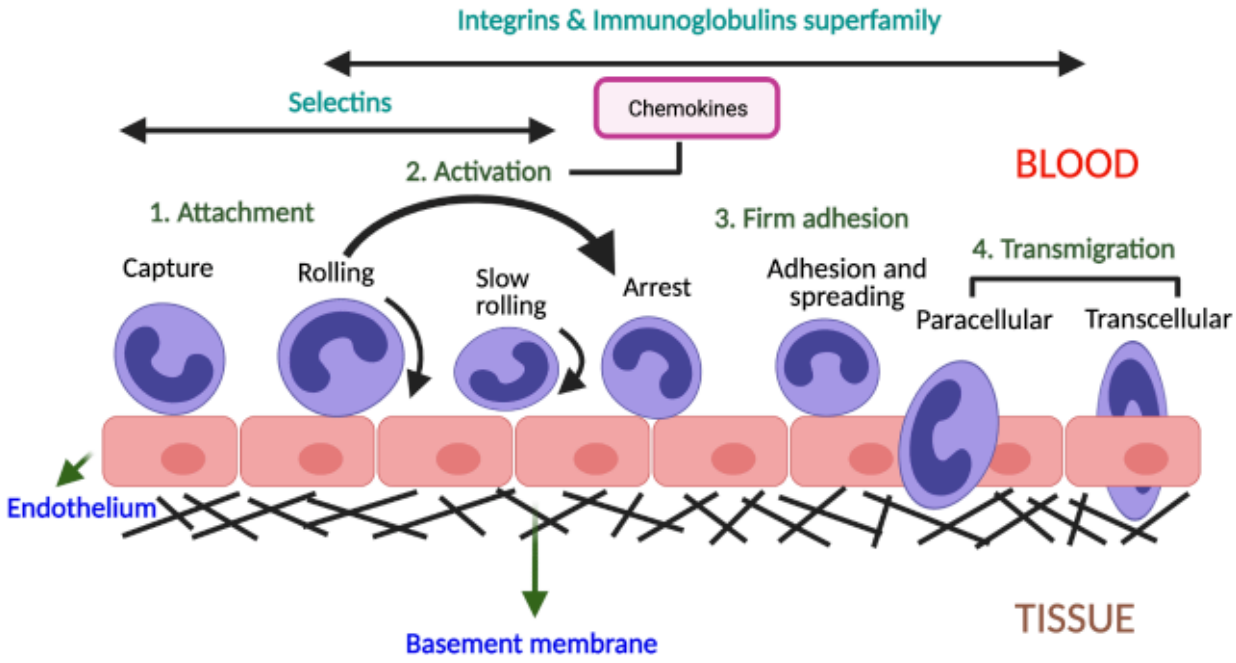

Figure 1.2 Schematic depicting transendothelial migration of leucocytes from the bloodstream into the tissue (Created with BioRender.com)

Table 1.2 Summary of leucocyte transendothelial migration cascade (modified from (Lawson and Wolf, 2009))

Steps	Processes
1: Attachment	Leucocytes roll along the endothelium, interacting with selectins via glycoprotein ligands.
2: Activation	Chemokines activate integrins on leucocytes, alter their conformation, thus increasing adhesion to endothelial ligands, LFA-1 and Mac-1.
3: Firm adhesion	Leucocytes travel along the endothelium, adhering firmly to endothelium via receptor-ligand interactions that include ICAM-1/LFA-1, ICAM-1/Mac-1 and VCAM-1/VLA-4.
4: Transmigration	Leucocytes move into the subendothelial space. Activation of signalling molecules triggers decreased expression of junctional adhesion molecules and endothelial cell contraction.

Abbreviations: LFA-1 = lymphocyte function-associated antigen 1, Mac-1 = macrophage-1 antigen, ICAM-1 = intercellular adhesion molecule 1, VCAM-1 = vascular cell adhesion molecule 1, VLA-4: very late antigen 4

induced by TNF-α and IL-1β (Bharadwaj et al., 2013a, Xie et al., 2014). The role of ICAM-1 in leucocyte migration into the retina may not be restricted by its expression on the retinal endothelium. An *in vitro* study conducted in mice showed that ICAM-1 expressed on the retinal pigment epithelium may facilitate T lymphocyte movement from the choroidal circulation into the retina (Dewispelaere et al., 2015).

1.5.3 Biologic treatment of uveitis

Apart from the standard therapy using corticosteroids and conventional immunomodulatory drugs, newer treatments for non-infectious uveitis are the biologic drugs, such as infliximab and adalimumab. Among these agents, adalimumab has been approved for the treatment of uveitis by the United States Food and Drug Administration, the European Medicines Agency and the Australian Therapeutic Goods Administration within the past 5 years. Adalimumab is a human monoclonal antibody that targets TNF-α, and is used in treating rheumatoid arthritis and also in the treatment of several other inflammatory diseases, including Crohn's disease, ulcerative colitis, ankylosing spondylitis, juvenile idiopathic arthritis, and hidradenitis suppurativa (Duica et al., 2018). Prior to its approval for non-infectious uveitis, several randomised controlled trials were conducted, investigating the effects of adalimumab for intermediate uveitis, posterior uveitis and panuveitis (Jaffe et al., 2016, Nguyen et al., 2016). Sheppard and colleagues also demonstrated the significant improvement in patient-reported visual functioning following the use of adalimumab (Sheppard et al., 2017). Recently, Ferreira and colleagues has provided a summary on the latest biologics used in treatment of non-infectious uveitis (Ferreira et al., 2021). Despite their high effectiveness in managing the inflammation, adverse effects in patients continue to be a challenge.

In light of the strong interest in biologic drugs for uveitis, targeting the retinal endothelial adhesion molecules that control leucocyte trafficking during the inflammation is an appealing therapeutic approach for non-infectious uveitis. However, a biologic drug needs to allow physiological processes to continue, while simultaneously targeting the disease mediator. Another option is to target the induction of a molecule that occurs during uveitis. A proof of concept study, published in 2016 by

Ashander and colleagues, investigated the effect of transcriptional blockade on TNF-α-induced versus constitutive expression of ICAM-1 by human retinal endothelial cells, and also on the interaction between leucocytes and human retinal endothelium (Ashander et al., 2016). In the study, NF-κB blockade decreased induced ICAM-1 expression, but not basal expression of ICAM-1, suggesting that the transcription of this key endothelial adhesion molecule has the potential to be a drug target for the treatment of non-infectious posterior uveitis.

1.6 Intercellular adhesion molecule 1 (ICAM-1)

Intercellular adhesion molecules (ICAMs) are members of the Ig superfamily, and the specific ligands for integrins, such as LFA-1 (CD11a/CD18) (Staunton et al., 1990) and Mac-1 (CD11b/CD18) (Smith et al., 1989). There are five ICAMs, all of which span the cell membrane and have a short cytoplasmic tail, containing two or more common Ig domains (Hubbard and Rothlein, 2000). Each ICAM has a different integrin binding affinity. ICAM binding to the extracellular domain of an integrin induces conformational changes, and this leads to signal transmission from this domain to the cytoplasm, which is important for the regulation of downstream gene expression (Qin et al., 2004, Hogg et al., 2011, Verma and Kelleher, 2014). ICAM-1 is the most studied of the five ICAM molecules. At basal conditions or non-inflammatory states, ICAM-1 is expressed at a relatively low level on endothelial cells (Almenar-Queralt et al., 1995, Scholz et al., 1996). An increase in ICAM-1 expression is generally indicative of an inflammatory response, and this elevated expression on endothelium especially, may continue for a period of time (Scholz et al., 1996). LFA-1, a member of β_2 family of integrins, is expressed on cell surface of leucocytes. It binds with greater affinity to its main ligand, ICAM-1, in comparison to ICAM-2 and ICAM-3 (de Fougerolles and Springer, 1992). Studies by Bharadwaj and colleagues showed ICAM-1 mediates the migration of Th1 and Th17 cells, and B-cells across the retinal vascular endothelium (Bharadwaj et al., 2013b).

1.6.1 Molecular description of ICAM-1

The human *ICAM1* gene is located on the short arm of chromosome 19, containing 7 exons, and totalling 15.5 kilobase (kb) (Gu et al., 2012). A full-length ICAM-1 transcript variant, encoding the main ICAM-1 isoform, is 2967 base pairs (bp) in length (Howe et al., 2021). The protein produced from this transcript is made up of 532 amino acids. Molecular weight varies across cell type and environment, and ranges from 80 to 114 kDa, related to level of glycosylation (Mukhopadhyay et al., 2016). The ICAM-1 protein contains five Ig domains, a transmembrane domain, and a short cytoplasmic tail with multiple threonine residues (Staunton et al., 1988). It can form a homodimer, which increases its affinity for integrin binding. Apart from being a ligand for LFA-1 and MAC-1 binding, ICAM-1 also binds to hyaluronan, fibrinogen, PfEMP1 (a Plasmodium falciparum RBC membrane protein) and major group of rhinoviruses (Diamond et al., 1991, McCourt et al., 1994, Languino et al., 1995, Ockenhouse et al., 1992, Smith et al., 2000, Tse et al., 2004). As well as the membrane-bound protein, ICAM-1 also exists as a soluble form (sICAM-1).

Similar to many other molecules, the ICAM-1 transcript may be alternatively spliced (Ergun et al., 2013). Alternative splicing is a common mechanism that occurs post transcriptionally to regulate gene expression. This mechanism is believed to occur in more than 90% of multiexon genes, in which certain exons of a sequence are skipped, leading to the formation of multiple forms of mature mRNA (Wang et al., 2015b). In mice, alternative splicing of ICAM-1 transcript gives rise to at least seven membrane-bound proteins and one soluble form of ICAM-1 (Giorelli et al., 2002, King et al., 1995, Ochietti et al., 2002, Robledo et al., 2003, van Den Engel et al., 2000, Wakatsuki et al., 1995). However, the number of human ICAM-1 transcript and protein isoforms is not well-established. To date, the full-length variant of membrane-bound ICAM-1 has been well-characterised and studied in various cell lines and primary cells (Shin et al., 2018, Zhang et al., 2017, Yu et al., 2015, Lee et al., 2015, Zhang et al., 2014). A second ICAM-1 transcript variant, ICAM-1-202, was predicted to exist from a large-scale cDNA sequencing project of human synovial membrane tissue (Wakamatsu et al., 2009). Other human ICAM-1 transcript variants have been modelled *in silico*, such as ICAM-1-204

(Howe et al., 2021). ICAM-1-202 lacks exon 2, exon 3 and some sections of exon 4; and ICAM-1-204 contains only exons 1, 2 and 3.

As a type I transmembrane protein, the membrane-bound form of ICAM-1 anchors to the lipid membrane with a stop-transfer anchor sequence. In contrast, soluble ICAM-1 (sICAM-1) contains an extracellular domain, but has no transmembrane or cytoplasmic region. There are two processes that lead to the production of sICAM-1. First, sICAM-1 can be generated from an mRNA transcript. This expression is cell-specific and driven by the presence of specific cytokines, e.g. IFNβ-1a (Giorelli et al., 2002). Second, proteolytic activity of matrix metalloproteinases (MMPs), cathepsin G, TNF-α converting enzyme (TACE) and neutrophil elastase cleaves the extracellular portion of membrane-bound ICAM-1, releasing sICAM-1 (Robledo et al., 2003, Champagne et al., 1998, Grenier and Bodet, 2008, Tsakadze et al., 2004). Membrane-bound ICAM-1 is the focus of this project, and therefore sICAM-1 will not be discussed further.

Ramos and colleagues have published a comprehensive review, discussing existing knowledge surrounding ICAM-1 isoforms in ICAM-1-deficient mice models (Ramos et al., 2014). Among the membrane-bound versions of ICAM-1, the number of Ig domains ranges from two to five. In addition, there is a spliced variant, in which truncation is present in the fifth Ig domain, but it is lacking 24 amino acids (Mizgerd et al., 2002). However, this variant is described as being expressed at low levels post-lipopolysaccharide challenge in organs such as the kidney, spleen, and lung (Mizgerd et al., 2002). The mechanisms by which each ICAM-1 isoform participates in inflammation vary. Other factors that contribute to the roles in inflammation include the different isoforms expressed in the tissue, and changes in this group of isoforms as inflammation progresses.

1.6.2 Expression and regulation of ICAM-1

ICAM-1, which is also known as Cluster of Differentiation 54 (CD54) is expressed on endothelial cells, platelets, fibroblasts, glial cells, leucocytes, keratinocytes and epithelial cells (Lee and Benveniste, 1999, Roebuck and Finnegan, 1999). Lipski and colleagues found ICAM-1 expressed in

many parts of the eye in EAU, such as the ciliary body and the retina, with the highest level detected in retinal pigment epithelium (Lipski et al., 2014). The molecule is involved in multiple functions, such as activation of lymphocytes, leucocyte migration and other aspects of the immune response (Lawson and Wolf, 2009). Multiple TFs, such as STAT3, NF-kappaB1, NF-kappaB, STAT1, STAT1α, STAT1β and δCREB, may upregulate *ICAM1* gene expression (Roebuck and Finnegan, 1999). An increase in ICAM-1 protein has been well-documented following stimulation of various cells with inflammatory cytokines, including TNF-α and IL-1β (Hubbard and Rothlein, 2000).

In the past 10 years, there have been multiple studies conducted that have investigated the regulation of ICAM-1 expression across various cell types, and most of them are focused on endothelial cells. Some of the high-quality studies performed include the identification of PKCα-p38-SP-1 pathway involvement in endothelial cells, in which ICAM-1 upregulation from the co-culture of human umbilical vein endothelial cells and human melanoma cell line (Lul205) can be blocked using siRNA specific for protein kinase Cα (PKCα) (Zhang et al., 2014). Another study by Zhang and colleagues showed that Sp-1 binds to the ICAM-1 promoter and plays a role in hyperglycaemia-induced ICAM-1 upregulation (Zhang et al., 2017). The mechanism by which hyperglycaemia regulates ICAM-1 expression was studied in human umbilical vein endothelial cells and also rat retinal capillary endothelial cells. Both ICAM-1 targeted siRNA and the O-GlcNAc transferase (OGT) inhibitor led to a reduction in ICAM-1 expression.

It is well-established that nuclear import of nuclear factor-κB (NF-κB) has an important role in ICAM-1 gene transcription. Hayashi and colleagues showed a new mechanism for myocardin transcription factor (MRTF-A) and MRTF-B in vascular endothelial cells (Hayashi et al., 2015). They highlighted that TFs, MRTF-A and MRTF-B, inhibited ICAM-1 mRNA expression through forming a complex with NF-κB p65 in the nucleus. In another study by Kojima and colleagues, ICAM-1 mRNA and protein expression were inhibited using the flavonoid, butein, in human umbilical vein endothelial cells (Kojima et al., 2015). Butein inhibited NF-κB and activator protein-1 (AP-1)

activation induced by TNF-α and phorbol 12-myristate 13-acetate, mediated through the inhibition of IκBα phosphorylation by IκB kinase activity.

Two transcription factors, FOXO1 and Epithelium-Specific Ets-Like Transcription Factor 1 (ESE-1), have also been shown to bind the ICAM-1 promoter, regulating ICAM-1 transcriptional expression (Dong et al., 2015, Yu et al., 2015). FOXO1 is required for dendritic cell activation of lymphocytes *in vivo*, and its activity is regulated through the mitogen-activated protein kinase (MAPK) pathway. In experiments carried out by Dong and colleagues, the transfection of ICAM-1 expressing plasmid reversed the negative effect of FOXO deletion on dendritic cell phagocytosis and chemotaxis (Dong et al., 2017). In addition, the study also showed FOXO to be important for promoting dendritic cell activity via downstream target genes for stimulating T and B lymphocytes and activating antibody defence against bacteria. Yu and colleagues investigated the effect of ESE-1 knockout in lung epithelial cell lines, and found that ICAM-1 expression was consequently reduced, implicating ESE-1 in ICAM-1 transcription in airway inflammation (Yu et al., 2015). Apart from that, another transcription factor ETS-1, belonging to the family of ETS transcription factor also may regulate ICAM-1 expression. There are known Ets binding sites located on the *ICAM1* gene promoter (de Launoit et al., 1998). Zhou and colleagues showed an increased ICAM-1 expression following overexpression of ETS-1 in bronchial carcinoma cell lines (Zhou et al., 2018).

1.6.3 ICAM-1 as a biologic drug target

ICAM-1 has attracted much attention as a biomarker in diagnosing a wide variety of conditions, such as diabetic nephropathy (Hellemons et al., 2012), chronic pain (Luchting et al., 2017) and colorectal carcinoma (Schellerer et al., 2019). Moreover, ICAM-1 has been identified as a target for biological therapy, given it is the main regulator of leucocyte recruitment in inflammation. Pharmacological targeting of ICAM-1 has been extensively explored in treatment of inflammatory conditions, including neuroinflammatory diseases (Turowski et al., 2005) and diabetes mellitus (Gu et al., 2012). However, such therapeutics may pose challenges, as blocking ICAM-1 completely may lead to unwanted side effects, such as infection and even malignancy (Roland et al., 2007). Leucocytes are

functionally important in immunosurveillance, and they must be able to move into extravascular spaces and tissues to destroy pathogens and cancer cells.

Drugging gene transcription is an approach that has been much discussed for treating disease. It is clear from the oncology field that TFs can be selectively targeted for treatment (see Section 1.3). Given that ICAM-1 is regulated at the level of gene transcription by various TFs, drugging ICAM-1 transcription may be a viable approach to treat non-infectious uveitis. A proof-of-concept study that involved drugging the *ICAM1* gene transcription of ICAM-1 has been described (see Section 1.5.3). The main rationale in targeting gene transcription instead of ICAM-1 protein is that some leucocyte activity is crucial for immune responses to pathogens and cancer cells. Thus, maintaining a certain basal level of ICAM-1 expression, allowing normal physiological functions, is appealing.

1.7 Aims of the thesis

Uveitis is a group of conditions characterised by intraocular inflammation. This disease affects many people worldwide, and in severe cases may lead to blindness. Symptoms and signs vary according to the location of inflammation within the eye. Patients may experience a range of problems, from eye redness and pain, to blurred vision and floaters. The current treatment options for non-infectious uveitis include corticosteroids, and immunomodulatory drugs, such as methotrexate and adalimumab. These drugs are not effective for all patients, and they can have serious side effects.

In this project, the centre of focus is the form of uveitis known as non-infectious posterior uveitis. The exact cause of non-infectious posterior uveitis is not fully understood, but there is dysregulation of the immune response, leading to autoinflammation or autoimmunity. In non-infectious posterior uveitis, accumulation of leucocytes in the posterior eye results from retinal transendothelial migration of the leucocytes. This process is highly regulated by adhesion molecules and chemokines. In particular, the adhesion molecule, ICAM-1, has a pivotal role in directing leucocyte migration. ICAM-1 expression is primarily controlled at the level of gene transcription. Expression increases

following stimulation by inflammatory cytokines. Thus, ICAM-1, and in particular its transcription, is an appealing target for uveitis therapeutics.

The work that is presented in this thesis explores ICAM-1 expression by human retinal endothelial cells, and investigates the manipulation of ICAM-1 transcription in these cells. The studies have been designed with the long-term goal of drugging of ICAM-1 transcription in human retinal endothelium for the treatment of non-infectious posterior uveitis. The three specific aims of the thesis are:

1. To characterise ICAM-1 transcript and protein expression in human retinal endothelial cells.
2. To identify transcription factors that regulate ICAM-1 expression in human retinal endothelial cells.
3. To investigate the effect on human retinal endothelial cell ICAM-1 transcript and protein expression of silencing the transcription factor, Ets-1.

CHAPTER 2

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CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

Chemicals and other reagents used in the research presented in this thesis are listed in Tables 2.1 to 2.5, with manufacturers, catalogue number, and if appropriate, working concentrations. Chemicals were of analytical grade. Distilled water (Thermo Fisher Scientific – Gibco) was used for the preparation of cell culture medium, and deionised water was used in preparation of other solutions. Nuclease-free water was used for RT-PCR and RT-qPCR reactions.

Table 2.1 Chemicals used in experimental procedures of this research project

Reagents	**Manufacturer**	**Location**	**Catalogue number**
2-mercaptoethanol (2-ME)	Merck-Sigma Aldrich	St. Louis, MO	M3148-25ML
4', 6-diamidino-2-phenylindole (DAPI)	Merck-Sigma Aldrich	St. Louis, MO	D9542-5MG
Bovine serum albumin (BSA)	Merck-Sigma Aldrich	St. Louis, MO	A2058-5G
Dimethyl sulphoxide	Merck-Sigma Aldrich	St. Louis, MO	67-68-5
Instant skim milk powder	Coles	Victoria, Australia	A0406-0002
Nuclease free water (H_2O)	Merck-Sigma Aldrich	St. Louis, MO	W4502-1L
Paraformaldehyde (PFA)	Merck-Sigma Aldrich	St. Louis, MO	P6148-500G
Agarose	Thermo Fisher Scientific	Vilnius, Lithuania	00630733

Table 2.2 Cell culture reagents

Reagents	**Manufacturer**	**Location**	**Catalogue number**
10% Fetal bovine serum (FBS)	Thermo Fisher Scientific-Gibco	Foster City, CA	10099-141
Collagenase type II	Thermo Fisher Scientific-Gibco	Paisley, United Kingdom	17101-015
EGM-2 Endothelial SingleQuots™ kit	Lonza-Clonetics	Walkersville, MD	CC-4176
MCDB-131 medium	Merck-Sigma Aldrich	St. Louis, MO	M8537
Trypsin-EDTA (0.05%)	Thermo Fisher Scientific-Gibco	Grand Island, NY	25300054

Table 2.3 Molecular reagents and kits

Reagents	**Manufacturer**	**Location**	**Catalogue number**
RNA extraction and cDNA synthesis			
GenElute Mammalian Total RNA Miniprep Kit	Merck- Sigma Aldrich	St. Louis, MO	RTN350
iScript Reverse Transcription Supermix	Bio-Rad Laboratories	Hercules, CA	1708841
RNeasy Mini Kit	QIAGEN	Hilden, Germany	74106
TRIzol Reagent	Thermo Fisher Scientific- Ambion	Carlsbad, CA	15596-018
Polymerase chain reaction (PCR)			
Deoxynucleotide-triphosphate mix (dNTP)	QIAGEN	Hilden, Germany	201901
GelPilot 100 bp Plus Ladder	QIAGEN	Hilden, Germany	239045
GelPilot 1kb Plus Ladder	QIAGEN	Hilden, Germany	239095
GenElute Gel Extraction Kit	Merck-Sigma Aldrich	St. Louis, MO	NA1111
HotStarTaq Plus DNA Polymerase kit	QIAGEN	Hilden, Germany	203607
SYBR Safe DNA Gel Stain	Thermo Fisher Scientific -Invitrogen	Carlsbad, CA	S33102

Table 2.3 Molecular reagents (cont.)

Reagents	Manufacturer	Location	Catalogue number
Quantitative polymerase chain reaction (RT-qPCR)			
iQ SYBR Green Supermix	Bio-Rad Laboratories	Hercules, CA	170-8884
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad Laboratories	Hercules, CA	172-5274
Transfection reagents / siRNA			
Ets-1 Silencer Select siRNA (s4847)	Thermo Fisher Scientific - Ambion	Foster City, CA	4392420
Ets-1 Silencer Select siRNA (s4848)	Thermo Fisher Scientific - Ambion	Foster City, CA	4392420
Silencer Select negative control siRNA	Thermo Fisher Scientific - Ambion	Foster City, CA	4390843
Lipofectamine RNAiMAX	Thermo Fisher Scientific - Invitrogen	Vilnius, Lithuania	13778150
Opti-MEM reduced serum medium	Thermo Fisher Scientific - Invitrogen	Carlsbad, CA	31985070

Table 2.4 Recombinant human cytokines

Reagents	**Manufacturer**	**Location**	**Catalogue number**	**Working concentration**
Tumour necrosis factor alpha (TNF-α)	R&D Systems	Minneapolis, MN	RDS210TA100	10 ng/mL
Interleukin 1 beta (IL-1β)	R&D Systems	Minneapolis, MN	RDS201LB025	5 - 10 ng/mL

Table 2.5 Primary and secondary antibodies

Reagents	**Manufacturer**	**Location**	**Catalogue number**	**Working concentration**
Mouse anti-human CD54 (ICAM-1) clone LB-2	BD Pharmingen	San Jose, CA	559047	1 µg/ml
Mouse IgG2b$_k$ anti-Dansyl clone 27-35	BD Pharmingen	San Jose, CA	555740	1 µg/ml
Mouse anti-human CD31 clone WM59	BD Pharmingen	San Jose, CA	555444	10 µg/ml
Mouse IgG1$_k$ clone MOPC-21	BD Pharmingen	San Jose, CA	554121	10 µg/ml
Polyclonal Rabbit Anti-Human von-Willebrand Factor (VWF)	Agilent - Dako	Glostrup, Denmark	A 0082	15.5 µg/ml
Rabbit IgG	Vector Laboratories	Burlingame, CA	I-1000-5	15.5 µg/ml
Goat anti-Mouse IgG (H+L) Highly Cross-Absorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific - Molecular Probes	Eugene, OR	A11029	2.5 µg/ml
Goat anti-Rabbit IgG (H+L) Cross-Absorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific - Molecular Probes	Eugene, OR	A11008	2 or 5 µg/ml

2.1.2 Recipe for selected buffers & solutions

Phosphate buffered saline (PBS) **[10X]**

80 g	Sodium chloride
2.0 g	Potassium chloride
14.4 g	Disodium hydrogen phosphate
2.4 g	Potassium dihydrogen phosphate
800 ml	Water

Adjust pH to 7.4

Adjust volume to 1L with additional distilled H_2O.

1X PBS is prepared by diluting 100 ml 10X PBS with 900 ml H_2O.

Tris-acetate-EDTA (TAE) buffer **[50X]**

242 g	Tris base
57.1 ml	Glacial acetic acid
18.6 g	EDTA
500 ml	Distilled water

Adjust volume to 1L with additional distilled H_2O.

1X TAE buffer solution is prepared by diluting 20 ml 50X TAE buffer with 980 ml H_2O.

5% Skim milk

2.5 g	Instant skim milk powder
50 ml	Water

2% Agarose gel

2 g	Agarose
100 ml	TAE buffer
10 µL	SYBR safe DNA gel stain

2% Bovine serum albumin in PBS

1 g	Bovine serum albumin
50 ml	PBS

2.1.3 Primers

Both forward and reverse primers were either designed or sourced from existing literature. All primers were verified using the United States National Library of Medicine National Centre for Biotechnology Information (NCBI) BLAST® web interface (Madden, 2002). Primers were purchased from Sigma-Genosys (The Woodlands, TX). Primer pair sequences, expected product sizes and efficiency in reverse transcription (RT)-quantitative real-time polymerase chain reaction (qPCR) are listed in **Table 2.6**.

2.2 Methods

2.2.1 *In silico* analysis

Transcription factors predicted to bind to the *ICAM1* gene promoter were identified using 3 publicly available software programs: ConTra (Kreft et al., 2017), InnateDB (Breuer et al., 2013), and ChEA3 (Keenan et al., 2019). Each database was set to optimise identification of the potential TFs or TF binding sites in the *ICAM1* gene promoter, using the default settings recommended by the respective software. ConTra is a phylogenetic footprinting tool that identifies sequences within the promoter conserved across species, suggestive of TF binding. InnateDB predicts potential TF binding from curated data generated in *in vitro* and *in vivo* molecular interaction studies. ChEA3 is a platform used for identifying TFs that regulate changes in gene expression patterns observed in RNA-seq and microarray experiments.

A promoter analysis was conducted to identify TFs that bind to *ICAM1* promoter using ConTra version 3 on 15/7/2019. The sequence 10,078 bp immediately upstream of the *ICAM1* TSS was searched for conserved TFs shared across human and mouse species.

Table 2.6 Primer sequences, expected amplicon sizes and efficiency in RT-qPCR if applicable.

Primer pair	Sequences	Amplicon size (bp)	Efficiency (%)
ICAM1 F1/R1	**Forward:** 5'-CAACCTCAGCCTCGCTATGG-3' **Reverse:** 5'-TCACACTGACTGAGGCCTT-3'	851 & 164	-
ICAM1 F2/R2 (Lu et al., 2014)	**Forward:** 5'-CCTTCCTCACCGTGTACTGG-3' **Reverse:** 5'-AGCGTAGGGTAAGGTTCTTGC-3'	90	83.8
ICAM1 F1/R3	**Forward:** 5'-CAACCTCAGCCTCGCTATGG-3' **Reverse:** 5'-TCAGGGAGGCGTGGCTTGTGTGTT-3'	928	-
ICAM1 F1/R4	**Forward:** 5'-CAACCTCAGCCTCGCTATGG-3' **Reverse:** 5'-TGGGGTTCAACCTCTGGTCATT-3'	113	88.5
ETS1 F1/R1(Zhou et al., 2018)	**Forward:** 5'-GTGCTGACCTCAATAAGGA-3' **Reverse:** 5'-GCTGATAAAAGACTGACAGGAT-3'	134	96.9
GAPDH (Silverman et al., 2003)	**Forward:** 5'-AGCTGAACGGGAAGCTCACTGG-3' **Reverse:** 5'-GGAGTGGGTGTCGCTGTTGAAGTC-3'	209	85.1
RPLP0	**Forward:** 5'-GCAGCATCTACAACCCTGAA-3' **Reverse:** 5'-GCAGATGGATCAGCCAAGAA-3'	235	91.8

Abbreviations: ICAM1 = intercellular adhesion molecule 1; ETS1 = protein C-ets-1; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; RPLP0 = ribosomal protein lateral stalk subunit P0

Similarly, TFs were identified *in silico* via InnateDB version 5.4 on 13/08/2019, using its database of molecular interaction pathways of proteins that are known to regulate *ICAM1* gene expression. The TF binding analysis function was selected, and the gene ID of *ICAM1* (ENSG00000090339) was imported for cross-reference to Ensembl release 104 (Howe et al., 2021).

Lists of molecules, previously generated by RNA-sequencing at the home laboratory at Flinders University for primary human retinal endothelial cells treated with TNF-α or IL-1β, were input into ChEA3 on 6/11/2020 to identify TFs that potentially interacted with the *ICAM1* gene promoter by overlaying changes in gene expression across the whole transcriptome. Transcription factors were ordered by "mean rank", which represented an average of rankings assigned on the strength of scientific evidence across 6 data repositories or libraries, with the one additional requirement that evidence included chromatin immunoprecipitation-RNA sequencing (ChIP-RNA-seq) data. The top 50 TFs were prioritised for further evaluation.

2.2.2 Culture of human retinal endothelial cells

Primary human retinal endothelial cells isolated from eyes of deceased humans were used in this study. These eyes were sourced from the Eye Bank of South Australia (Adelaide, Australia), and used with the approval of the Southern Adelaide Clinical Human Research Ethics Committee (Protocol Number: 175.13). Cell isolation was performed by Dr. Yuefang Ma, following a previously published method (Bharadwaj et al., 2013a). In essence, tissues were digested with collagenase Type II, followed by cell capture with magnetic beads conjugated to anti-human CD31 antibody. Primary isolates of retinal endothelial cells from human retina are described in **Table 2.7** and **Table 2.8**. Donor age ranged from 30 to 77, with both male and female donors included. The human retinal endothelial cell line used in this study was previously generated from a primary human retinal endothelial cell isolate, as detailed by Bharadwaj and colleagues (Bharadwaj et al., 2013a). The cell line was generated by transformation with a mouse retroviral construct that encoded human papillomavirus genes (LXSN16E6E7). Human retinal endothelial cells were grown on tissue culture-treated plastic,

Table 2.7 Gender and age of donors, and death to isolation time of human retinal endothelial cell isolates that were used in RT-PCR studies.

Cell isolates treated with TNF-α			
Donors	**Gender**	**Age at death (years)**	**Death to isolation time (hours)**
Donor 1	Male	55	8
Donor 2	Female	59	20
Donor 3	Female	50	16
Donor 4	Female	62	11
Donor 5	Male	77	22
Cell isolates treated with IL-1β			
Donors	**Gender**	**Age at death (years)**	**Death to isolation time (hours)**
Donor 1	Female	35	11
Donor 2	Female	64	18
Donor 3	Female	62	11
Donor 4	Female	64	10
Donor 5	Female	55	19
Donor 6	Male	49	9

Table 2.8 Gender and age of donors, and death to isolation time of human retinal endothelial cell isolates that were used in ICAM-1 cellular immunoassays.

Cell isolates treated with TNF-α			
Donors	**Gender**	**Age at death (years)**	**Death to isolation time (hours)**
Donor 1	Female	66	13
Donor 2	Male	30	21
Donor 3	Male	46	29
Donor 4	Male	50	38
Donor 5	Male	55	11
Donor 6	Female	51	15
Cell isolates treated with IL-1β			
Donors	**Gender**	**Age at death (years)**	**Death to isolation time (hours)**
Donor 1	Female	35	11
Donor 2	Female	64	18
Donor 3	Female	62	11
Donor 4	Female	64	10
Donor 5	Male	49	9

in MCDB-131 medium supplemented with 10% FBS and endothelial growth factors, at 37 ℃ and 5% CO_2 in air, and passaged when near confluence with 0.05% trypsin-EDTA. The primary cells were used in experiments at passage 2-3. For storage, cells were frozen in medium with 10% dimethyl sulphoxide (DMSO) and stored in liquid nitrogen vapour phase. Viability of all cells was checked by light microscopy before they were used in experiments.

2.2.3 RNA extraction

Total RNA was extracted from cells using RNA extraction kits, performed according to the manufacturer's instructions: for the human retinal endothelial cell line, GenElute Mammalian Total RNA Miniprep Kit (Merck - Sigma Aldrich) or TRIzol Reagent (Thermo Fisher Scientific - Ambion); and for the primary human retinal endothelial cells, RNeasy mini kit (QIAGEN). Cells were lysed in Buffer RLT lysis solution from RNeasy Mini kit (QIAGEN) after addition of 2-ME or TRIzol Reagent, and lysates were frozen at -80 ℃ ahead of RNA extraction. Concentration of the extracted RNA was determined on the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Purity was assessed on the 260/280 ratio, with a range of 1.8 to 2.0 considered to indicate good quality. RNA was frozen at -80 ℃ until ready for use.

2.2.4 Reverse transcription

Reverse transcription was performed using the iScript Reverse Transcription Supermix for RT-qPCR, following the procedure recommended by the manufacturer, with an input amount of 100 ng of RNA template producing 20 μL of cDNA. For each sample, a technical duplicate cDNA was prepared and pooled. The cDNA was stored at -20 ℃ until ready for use.

2.2.5 Standard polymerase chain reaction

Standard PCR was conducted using the HotStarTaq DNA polymerase kit. Each reaction included the following reagents: 1.5μL of template human retinal endothelial cell cDNA (diluted 1:10), 2.5 μL of 10x PCR buffer, 0.125 μL of Taq DNA polymerase, 1 μL of 10 mM dNTP, 2 μL of 25 mM $MgCl_2$, 2 μL each of 10 μM forward and reverse primers, and nuclease-free water to a final volume of 25 μL.

The PCR was performed on a T100 Touch thermal cycler (Bio-Rad Laboratories, Singapore). Cycling conditions were as follows: a pre-cycling hold for 5 minutes at 95 °C; 40 cycles of denaturation for 30 seconds at 95 °C, gradient annealing for 30 seconds at 3 temperatures between 56 °C and 62 °C, and extension for 1 minute at 72 °C; and a post-cycling hold for 5 minutes at 72 °C.

2.2.6 Agarose gel electrophoresis

The PCR products were loaded onto a 2% agarose gel. DNA ladders were also loaded, either a GelPilot 1 kb ladder for products larger than 1 kbp or GelPilot 100 bp plus ladder for PCR products smaller than 1 kbp. Electrophoresis was conducted over 45 minutes at 120 V, using the Bio-Rad Mini-Sub Cell GT electrophoresis system (Hercules, CA) and the Thermo Fisher Scientific Owl EC300XL power supply (Waltham, MA). DNA in the gel was visualised using the Thermo Fisher Scientific-Invitrogen Safe Imager 2.0 blue light Transilluminator (Waltham, MA), image taken with Canon ImageBrowser EX (Hong Kong, PRC). To isolate the DNA product from agarose gel following electrophoresis, bands of interest were cut out using a clean razor blade, and excess gel was trimmed. DNA was recovered using the GenElute Gel Extraction Kit, leading to a purified DNA sample according to standard manufacturers' guidelines.

Sequencing of purified DNA was performed by Flinders Sequencing Facility, SA Pathology (Adelaide, Australia). The sequence data were imported into SnapGene®, version 5.0.4 for analysis.

2.2.7 Quantitative real-time polymerase chain reaction

Each qPCR reaction was carried out with either iQ SYBR Green Supermix or SsoAdvanced Universal SYBR Green Supermix. For the first Supermix, reaction reagents consisted of: 2 µL of cDNA (diluted 1:10), 4 µL of iQ SYBR Green Supermix, 1.5 µL each of 10 µM forward and reverse primers, and nuclease-free water to a final volume of 20 µL. For the second Supermix reaction reagents consisted of: 2 µL of cDNA (diluted 1:10), 4-10 µL SsoAdvanced SYBR Green Supermix, 0.75 µL each of 10 µM forward and reverse primers, and and nuclease-free water to a final volume of 20 µL. The PCR plates were prepared in an Aura PCR cabinet (EuroClone-BioAir, Pero, Italy). All plasticware and

equipment were exposed to 20 minutes of ultraviolet light prior to use. The qPCR was performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with the following cycling conditions: a pre-cycling incubation for 30 seconds or 5 mins at 95 °C; 40 cycles of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 62 °C, and extension for 30 seconds at 72 °C. All samples were amplified in duplicate. Reference genes included in each qPCR run were GAPDH and RPLP0. Melt curves were generated with 1-second holds at 0.5 °C increments between 70 °C and 95 °C to confirm amplification of a single PCR product. PCR products were subsequently run on a 2% agarose gel to confirm expected product size.

2.2.8 Immunohistochemistry

Primary human retinal endothelial cells were grown to confluency in a black, clear flat bottom 96-well plate and washed in PBS before being fixed with 2% PFA at room temperature for 10 minutes. The cells were then washed again with PBS, and air dried for 15 minutes. The cells were then blocked with 2% BSA in PBS. One specific or negative control primary mouse or rabbit antibody diluted in blocking solution was applied to each well, and the plate was incubated for 1 hour at room temperature. After two PBS washes, Alexa Fluor 488-conjugated goat anti-mouse or anti-rabbit antibody in blocking solution was added to wells, which were incubated for 30 minutes in the dark at room temperature. Wells were then washed twice with PBS, and fixed in 2% PFA for 5 minutes, and counterstained with 300 nM DAPI before a final wash in PBS. One drop of Fluoromount mounting medium was added to each well in preparation for photography. Wells were imaged under fluorescence on an Olympus IX53 inverted tissue culture microscope (Tokyo, Japan), captured with UC50 CCD camera (Munster, Germany).

2.2.9 ICAM-1 cellular immunoassay

Monolayers of human retinal endothelial cells were seeded at 80-100% confluency in wells of a black, clear flat bottom 96-well plates, and treated with TNF-α (10 ng/ml), IL-1β (5-10 ng/ml), or fresh medium alone for 24 hours at 37 °C and 5% CO_2 in air. The plates were then washed twice with PBS,

fixed in 1% PFA for 30 minutes, and washed twice again with PBS. The plates were wrapped with parafilm and stored at 4°C until ready for labelling.

Cell monolayers were blocked with 5% skim milk in PBS for 30 minutes. The monolayers were then incubated with mouse anti-human ICAM-1 antibody or mouse monoclonal IgG2bk in blocking solution for 45 minutes at room temperature and washed 3 times in PBS. Monolayers were then incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody in blocking solution for 30 minutes at room temperature before washing three times in PBS. Finally, the monolayers were treated with 300 nM DAPI in PBS for 5 minutes and washed three times in PBS. Incubations were performed on a Ratek OM5 orbital shaker (Victoria, Australia) at slow rotation.

Monolayer fluorescence was detected using the VICTOR X3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA) with an excitation wavelength of 485 nm and emission wavelength of 535 nm (Alexa Fluor 488 filter), and an excitation wavelength of 355 nm and emission wavelength of 460 nm (DAPI filter) for 1 second each. The DAPI filter readings were used to adjust for cell numbers across the wells. The mean background fluorescence was determined by averaging the readings from the negative control antibody-labelled wells. True fluorescence was determined by subtracting the mean background fluorescence from the fluorescence readings of anti-ICAM-1 antibody-labelled wells.

2.2.10 Transfection of human retinal endothelial cells with small interfering RNA

Transfection of human retinal endothelial cell line with siRNA were carried out in a 12-well plate for the RNA studies and 96-well black plates for the ICAM-1 cellular immunoassays. Cells were seeded the day before transfection for 70% to 80% confluency. Cells were transfected with either 12 ρmol of siRNA in the 12-well plate, or 1 ρmol in the 96-well plate. Single or a combination of two (1:1 ratio) ETS-1-targeted siRNAs were used. A non-targeted siRNA was included as negative control. Equal volume suspensions of siRNA and Lipofectamine RNAiMAX Reagent in OptiMEM were mixed thoroughly and incubated at room temperature for 5 minutes to ensure a homogenous siRNA lipofectamine complex mixture. Transfected cells were incubated at 37 °C and 5% CO_2 in air for 24

hours, subsequently treated with TNF-α (10 ng/ mL), IL-1β (10 ng/ mL), or fresh media alone, and incubated for a further 24 hours. After a total transfection time of 48 hours, medium was removed, and cells were either treated with Lysis solution from RNA miniprep kit (Merck - Sigma Aldrich), supplemented with 2-ME for RNA extraction or fixed with 1% PFA for 30 minutes for immunolabelling.

2.2.11 Data analysis

All analyses were performed using GraphPad Prism, version 9.0 (GraphPad Software, San Diego, CA). The comparisons between two groups were performed using a paired or unpaired Student t-test. The comparisons across multiple groups were made with the two-way ANOVA test, with post-hoc analyses performed by Sidak tests. A p-value less than 0.05 was taken to indicate a statistically significant difference.

CHAPTER 3

Expression of ICAM-1 by human retinal endothelial cells

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CHAPTER 3: EXPRESSION OF ICAM-1 BY HUMAN RETINAL ENDOTHELIAL CELLS

3.1 Introduction

Intercellular adhesion molecule is an adhesion molecule on the cell surface, belonging to the Ig superfamily. It is expressed predominantly by leucocytes and endothelial cells (Roebuck and Finnegan, 1999), and has important roles in multiple cellular immune processes (Lawson and Wolf, 2009). However, ICAM-1 is also expressed in a range of other cells, including epithelial cells, glial cells and fibroblasts, as well as platelets (Mruk et al., 2014). Like most adhesion molecules, ICAM-1 is expressed at relatively low basal level on endothelial cells (Hubbard and Rothlein, 2000).

Various stimuli can trigger an increase in cellular expression of ICAM-1. In particular, inflammatory cytokines elicit an increase in ICAM-1 expression in a cytokine- and cell-specific manner (Hubbard and Rothlein, 2000, Scholz et al., 1996, Dustin et al., 1988, Figenschau et al., 2018). Indeed, an increased level of ICAM-1 expression in the tissue is characteristic of inflammation (Lawson and Wolf, 2009). Among the inflammatory cytokines, TNF-α and IL-1β interact with their respective receptors to play major roles in mediating systemic inflammation (Nizamutdinova et al., 2007) and intraocular inflammation (Khera et al., 2010). During inflammation in many organs, one key activity of TNF-α and IL-1β is to induce ICAM-1 upregulation on local endothelial cells to enhance leucocyte infiltration (Bharadwaj et al., 2013a). Of specific relevance to the eye and posterior uveitis, TNF-α and IL-1β have been shown to significantly increase ICAM-1 expression on retinal endothelial cells *in vitro* (Bharadwaj et al., 2013a, Xie et al., 2014).

Several ICAM-1 isoforms exist, formed through alternatively spliced transcript variants and post-translation modifications, as shown primarily in mouse models (King et al., 1995). A similarity of 65% in the nucleotide sequence between mice and humans suggests some functional correlation between ICAM-1 in the two species (Greenwald and Yuki, 2016). The functional properties of different mouse ICAM-1 isoforms have been discussed in detail by Ramos and colleagues, drawing

on studies using transgenic mice with exon deletions (Ramos et al., 2014). For example, wild type mice injected with lipopolysaccharide, known to increase ICAM-1 expression, have a 50% survival rate, but exon 5-deletion mice have a 23% survival rate, while exon 4-deletion mice have a 100% survival rate (Robledo et al., 2003).

Full-length membrane-bound ICAM-1 has been the central focus in studies directed at determining ICAM-1 function. Expression of membrane-bound ICAM-1 is linked to the binding of β_2 family of integrins, such as LFA-1, Mac-1, found solely in leucocytes. The soluble form of ICAM-1 (sICAM-1) lacks a transmembrane domain. sICAM-1 can be generated from alternatively splicing of membrane ICAM-1 mRNA, but also from the proteolytic cleavage of membrane-bound ICAM-1 (Witkowska and Borawska, 2004). In contrast to the work undertaken to delineate ICAM-1 isoforms in mice, there has not been much progress in characterising human ICAM-1 isoforms to date beyond description of the full-length isoform (Ramos et al., 2014). There are at least two ICAM-1 isoforms in humans, based on the discovery in 1986 by Dustin and colleagues, who performed immunoprecipitation on protein extracted from human dermal fibroblast and the U937 myelomonocytic cell line (Dustin et al., 1986). However, it was not until 2007, when Wakamatsu and colleagues discovered a new ICAM-1 transcript that was 1296 base pairs long, in a large-scale sequencing project of human synovial membrane tissue collected from patients with rheumatoid arthritis, termed ICAM-1-202 (Wakamatsu et al., 2009). The ICAM-1-202 transcript has 5 exons, translating into a protein of 310 amino acids. Despite the demonstration of this isoform in humans, there are no further publications extending the research to understand the expression and function of ICAM-1-202 in other cell types or tissues.

The aim of the work presented in this chapter was to characterise ICAM-1 transcript variants in human retinal endothelial cells. Given that full-length ICAM-1 expression can be induced, it was also interesting to examine whether a second ICAM-1 isoform would be similarly inducible. Thus, RT-qPCR was employed to quantify expression of full-length ICAM-1 and a second ICAM-1 transcript

variant in human retinal endothelial cells stimulated with TNF-α and IL-1β. Finally, the variation in ICAM-1 protein levels on human retinal endothelial cells stimulated with these cytokines was explored using cellular immunoassay.

3.2 Results

3.2.1 ICAM-1 transcript variants in human retinal endothelial cells

To characterise ICAM-1 transcript variants in human retinal endothelial cells, standard RT-PCR was employed to amplify the coding region of ICAM-1 mRNA, where an alternative splicing site is believed to exist at the junction between exon 1 and 4 (**Figure 3.1A**), giving rise to formation of ICAM-1-202. A human retinal endothelial cell line, generated in-house (Bharadwaj et al., 2013a), was used for the initial studies. Primers were designed using NCBI BLAST and interrogated using its Basic Local Alignment Search tool to ensure the specified target was detected by the primers and sensitive to show any significant number of mismatches to the primer set. These primers are listed in **Table 2.6**. Forward and reverse primers were designed to generate amplicons of differing size depending on the splicing arrangement of ICAM-1 mRNA in human retinal endothelial cells (**Figure 3.1B and Figure 3.1C**). These PCR products were visualised by agarose gel electrophoresis and two discrete bands were seen, corresponding to the product sizes, 851 bp, representing the full-length ICAM-1 transcript, and a shorter product of 185 bp, at first assumed to represent the ICAM-1-202 transcript (**Figure 3.2**).

Sequencing of the shorter 185 bp PCR product revealed an alternative splice site between exon 2 and exon 4, suggestive of a novel human ICAM-1 transcript variant. This particular variant differed from the ICAM-1-202 transcript (Howe et al., 2021), as it contained a 12 bp fragment of exon 2, and an alternative splice junction. Based on the sequence, the size of the product is 164 bp. In comparison to the full-length ICAM-1 transcript, this transcript lacks 687 bp of sequence, spanning 252 bp of exon 2, all of exon 3, and 129 bp of exon 4, as illustrated in **Figure 3.1C**.

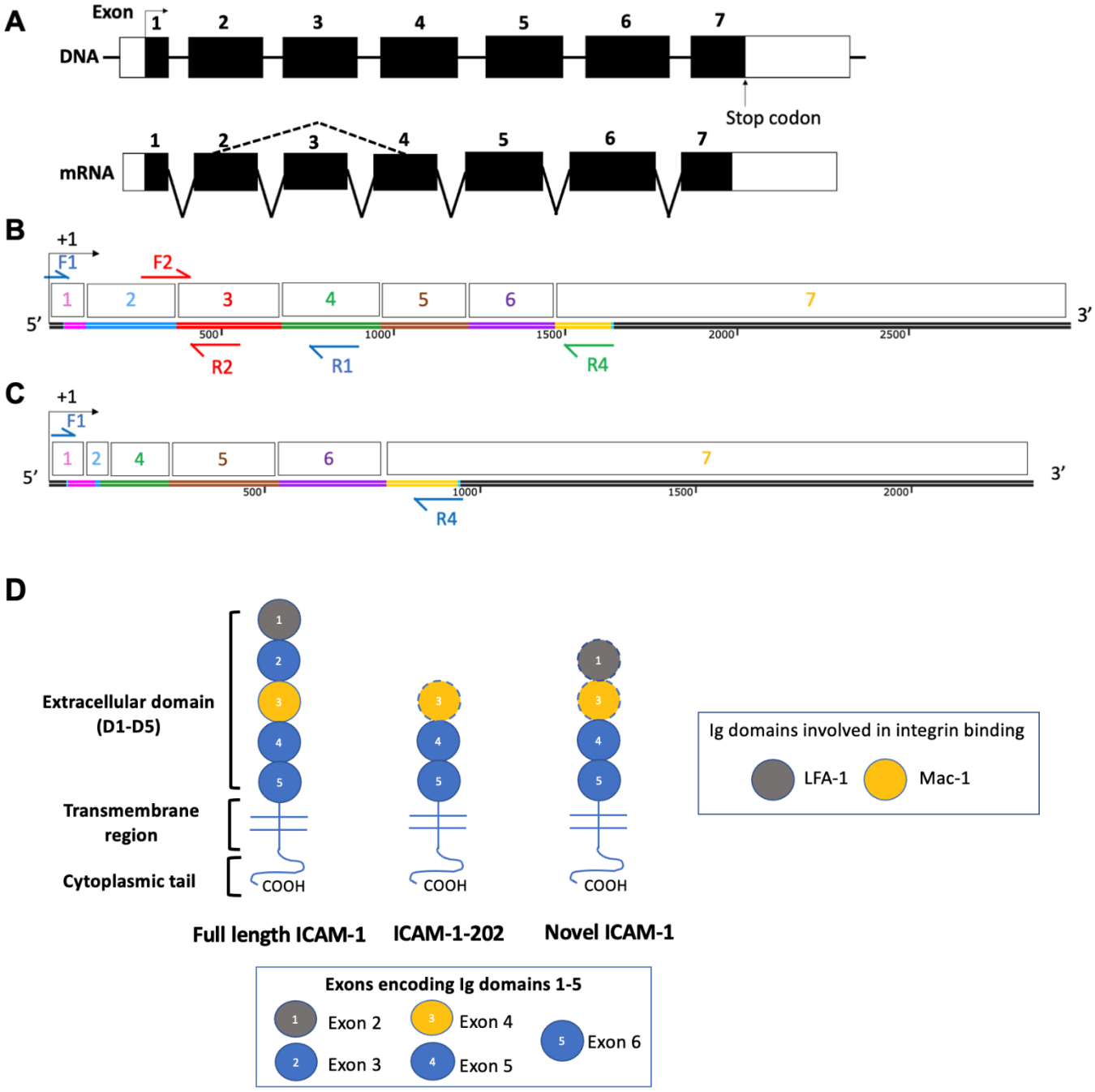

Figure 3.1 (**A**) Schematic representation of the *ICAM1* gene and messenger RNA. Rectangle = exon; black box = coding region; solid straight line = intron; solid bent line = splicing for full-length transcript; dashed line = splicing for ICAM-1-202 transcript. (**B**) mRNA of full-length ICAM-1 transcript, 2967 bp in length (**C**) mRNA of novel ICAM-1 transcript variant, 2280 bp in length. (**D**) ICAM-1 proteins from full-length ICAM-1 transcript and ICAM-1-202 transcript, and ICAM-1 protein predicted from novel ICAM-1 transcript variant.

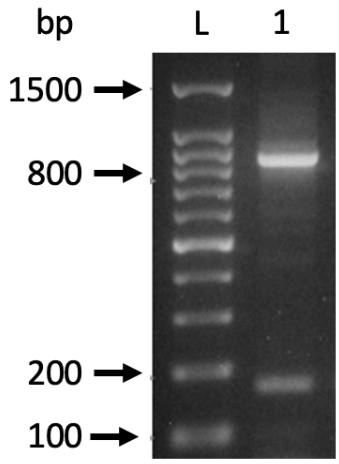

Figure 3.2 Expression of ICAM-1 transcripts in human retinal endothelial cell line. Gel image shows RT-PCR products on 2% agarose gel. L = DNA ladder; 1 = PCR products for full-length ICAM-1 (expected size: 851 bp) and ICAM-1-202 transcript (expected size: 185 bp) using primer set ICAM1 F1/R1. PCR no template control was negative.

Another primer set was designed, with the same forward primer used to detect the novel ICAM-1 transcript, and a different reverse primer spanning the 5' stop codon of exon 7. Using standard RT-PCR, a 928 bp gel product was detected (**Figure 3.3).** DNA sequencing of this product validated the existence of a novel ICAM-1 transcript, with the sequence corresponding to the sequence of the 164 bp product (**Figure 3.2**). The full coding sequence of this novel ICAM-1 transcript was thus confirmed, and is presented in **Appendix A**. The predicted translated protein sequence can be found in **Appendix B**.

To confirm that the novel ICAM-1 variant was expressed in human retinal endothelial cells, RT-PCR was performed using RNA extracted from primary human retinal endothelial cell isolates (**Section 2.2.2**). These cells were prepared from cadaveric donor eyes. The isolation procedure involved enzymatic digestion of retinae, followed by magnetic capture of endothelial cells using anti-human CD31 antibody-tagged magnetic beads from the digested tissue. First, the endothelial cell phenotype of cells isolated by this method was confirmed on the basis of characteristic cobblestone morphology and expression of the endothelial cell markers, von Willebrand Factor (**Figure 3.4**) and CD31 (not shown).

Expression of the full-length ICAM-1 transcript was identified in primary retinal endothelial cells isolated from 5 different human donors, as well as the human retinal endothelial cell line (**Section 2.2.2**) by standard RT-PCR using primers designed to detect the full-length transcript: forward primer in exon 2 and reverse primer in exon 3, expected product size of 90 bp in length, labelled as ICAM1 F2/R2 in **Table 2.6** (**Figure 3.5**). Expression of the novel ICAM-1 transcript variant was identified in cells isolated from the same donors and the cell line, but treated with TNF-α, using primers designed to detect the variant transcript: forward primer in the 5'UTR of exon 1 and reverse primer in exon 4, expected product size of 113 bp, labelled as ICAM1 F1/R4 in **Table 2.6** (**Figure 3.6**). Treatment with TNF-α resulted in increased expression (see next section), and thus gave best

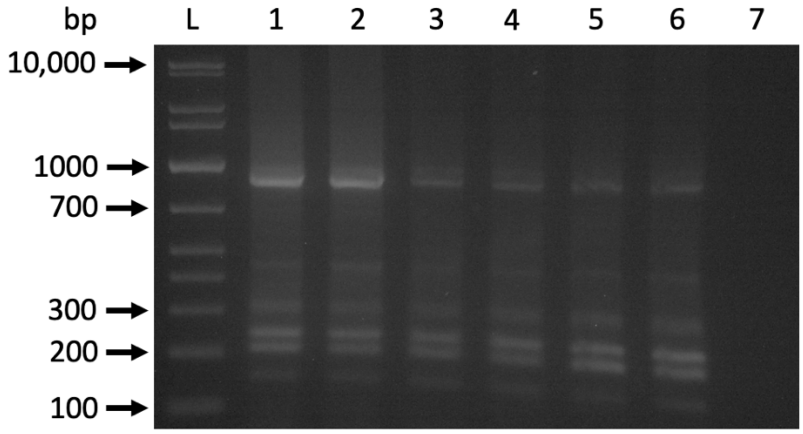

Figure 3.3 Expression of novel ICAM-1 transcript variant in human retinal endothelial cell line**.** Gel image shows RT-PCR products on 2% agarose gel. L = DNA ladder; 1-6 = PCR products for alternative ICAM-1 transcript (expected size: 928 bp) using primer set ICAM1 F1/R3; 7 = PCR no-template control.

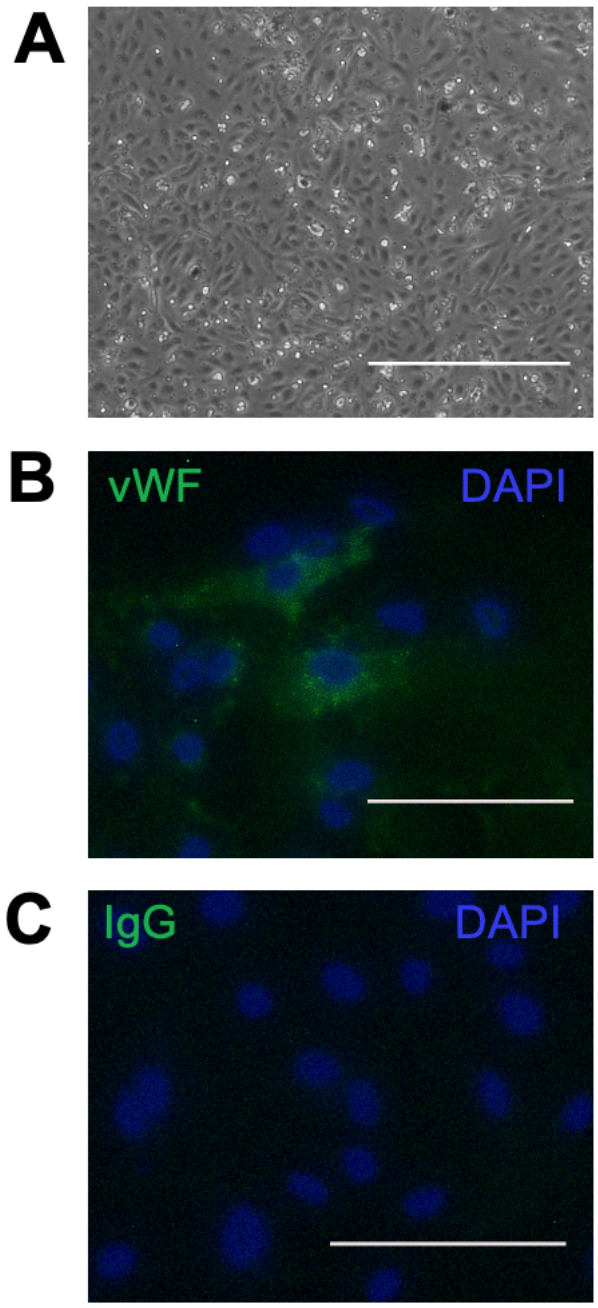

Figure 3.4 Phenotype of human retinal endothelial cells. Representative photomicrograph images of primary human retinal endothelial cells under (**A**) white light (100x original magnification, scale bar = 50 µm), and (**B and C**) epifluorescence illumination after immunolabelling with (**B**) anti-human von Willebrand Factor antibody (vWF) or (**C**) negative control antibody (IgG) (green), and DAPI nuclear counterstain (blue) (200x original magnification, scale bar = 200 µm).

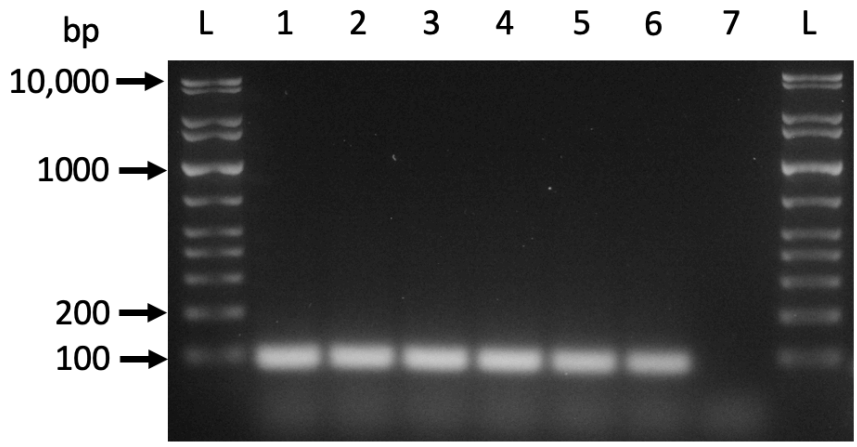

Figure 3.5 Expression of full-length ICAM-1 transcript in primary human retinal endothelial cells and human retinal endothelial cell line. Gel image shows RT-PCR products on 2% agarose gel. L = DNA ladder; 1-5 = primary retinal endothelial cell isolates from 5 human donors; 6 = human retinal endothelial cell line; 7 = PCR no-template control. Expected product size: 90 bp.

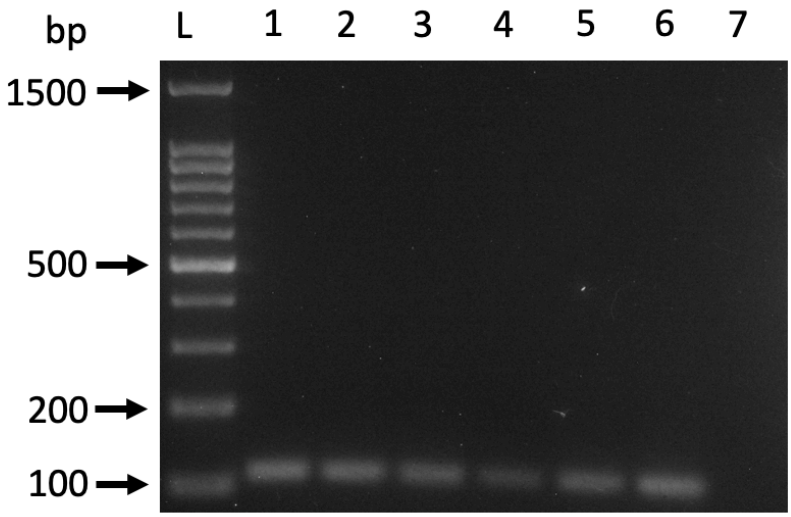

Figure 3.6 Expression of novel ICAM-1 transcript variant in TNF-α-treated primary human retinal endothelial cells and human retinal endothelial cell line. Gel image shows RT-PCR products on a 2% agarose gel. L = DNA ladder; 1-5 = primary retinal endothelial cells from 5 human donors; 6 = human retinal endothelial cell line; 7 = PCR no-template control. Expected product size: 113 bp.

visualisation for the gel image. These findings validated that human retinal endothelial cells expressed the novel ICAM-1 transcript variant, as well as full-length ICAM-1 transcript.

3.2.2 ICAM-1 transcript expression in cytokine-stimulated human retinal endothelial cells

Human retinal endothelial cell line was first employed to investigate changes in ICAM-1 transcript expression in response to stimulation with inflammatory cytokines. Cells were treated with TNF-α (10 ng/mL) or IL-1β (5 ng/mL), or fresh medium only as control, for a period of 4 hours, and expression of the full-length ICAM-1 transcript and the novel ICAM-1 transcript variant was measured by RT-qPCR, using primer sets, ICAM1 F1/R1 and ICAM1 F1/R4. A significant increase in full-length ICAM-1 transcript expression was observed in human retinal endothelial cell line treated with either TNF-α or IL-1β, in comparison to the control condition (**Figure 3.7**, $p \leq 0.0054$).

The same study was repeated using the human primary retinal endothelial cell samples. A significant increase in expression of both full-length and novel ICAM-1 transcripts was observed in primary human retinal endothelial cells treated with TNF-α, compared to the medium only control condition (**Figure 3.8**). The increase in full-length ICAM-1 transcript expression was approximately 10.2-fold overall (**Figure 3.8A**, $p = 0.003$), and the increase in novel ICAM-1 transcript expression was approximately 5.0-fold overall (**Figure 3.8C**, $p = 0.025$). It was not possible to perform a statistical analysis for individual isolates, due to limited availability of cells. However, full-length ICAM-1 transcript was expressed at a higher level after treatment with TNF-α for all five cell isolates (**Figure 3.8B)**. The novel ICAM-1 transcript was also expressed at a higher level after treatment in four isolates (**Figure 3.8D**), with an inconclusive result for a fifth isolate. The magnitude of the increase in ICAM-1 transcript expression following TNF-α treatment varied substantially across donors, from 3.2-fold to 20.3-fold compared to control for the full-length transcript, and from 1.9-fold to 45.4-fold for the novel transcript variant.

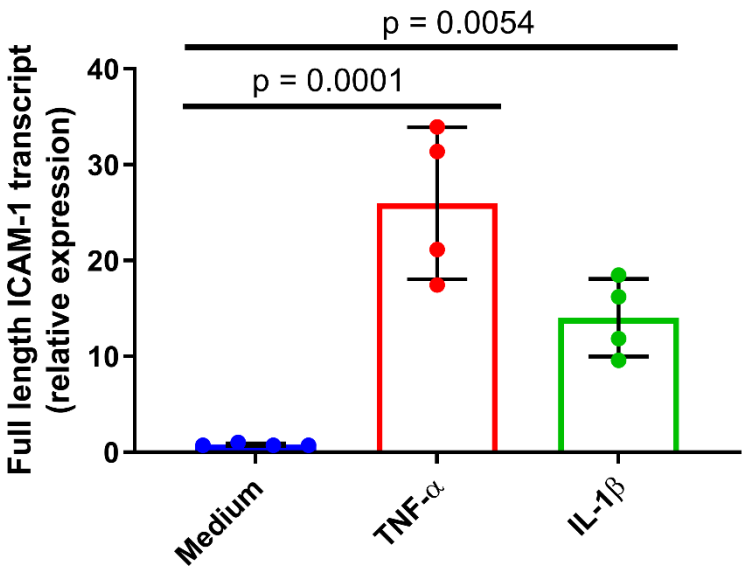

Figure 3.7 Expression of full-length ICAM-1 transcript in human retinal endothelial cell line is increased following stimulation with TNF-α and IL-1β. Graph shows relative expression of transcript in the human retinal endothelial line following 4-hour treatment with TNF-α (red) or IL-1β (green), in comparison to fresh medium alone (blue) (n = 4 replicates/condition). Reference genes were RPLP0 and GAPDH. Bars represent mean relative expression. Error bars indicate standard deviation. Data were analyzed using a one-way ANOVA, with Holm-Sidak post-hoc testing.

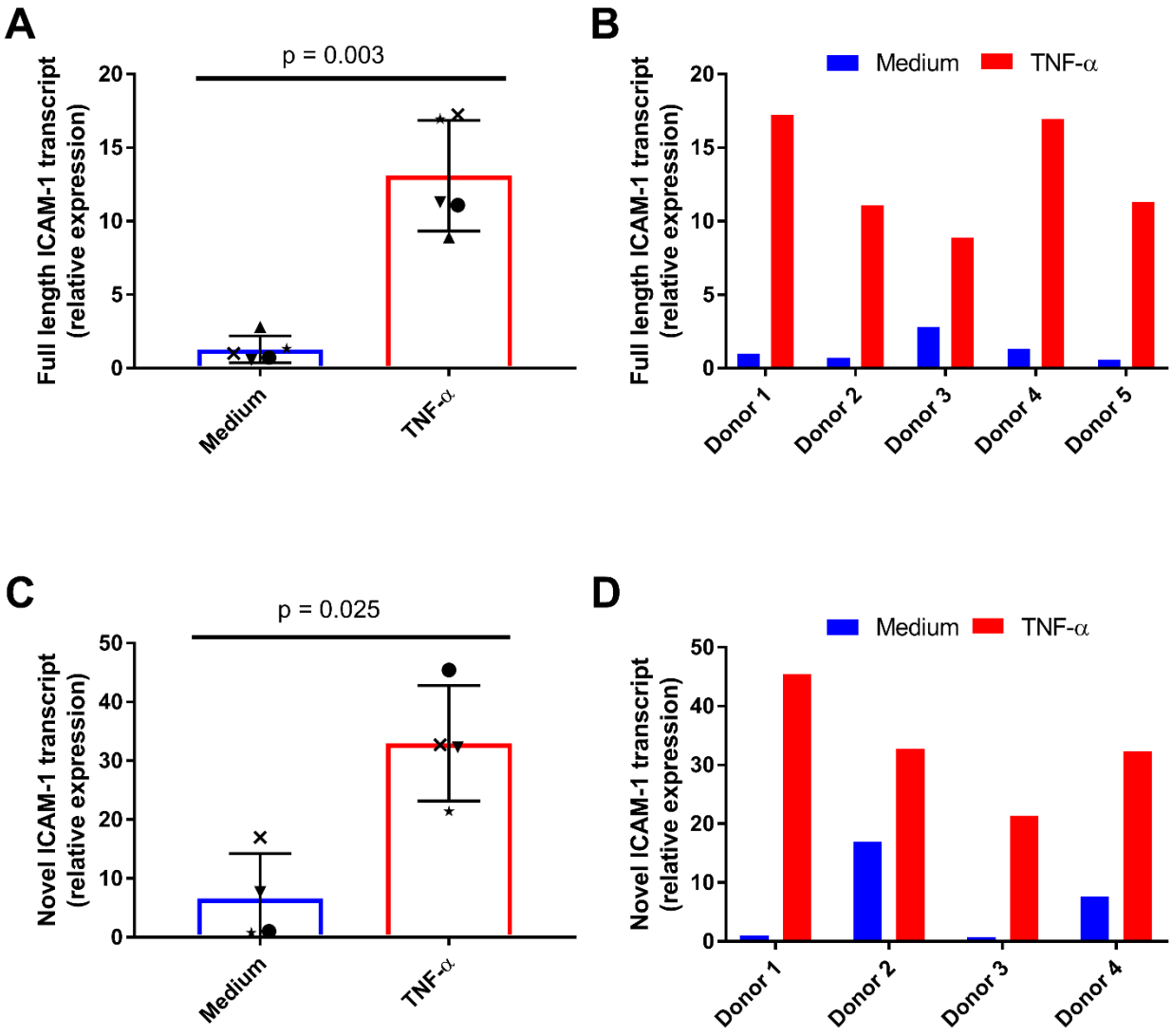

Figure 3.8 Expression of full-length ICAM-1 and novel ICAM-1 transcripts increased significantly in primary human retinal endothelial cells stimulated with TNF-α. Graphs show (**A & B**) full-length ICAM-1 transcript expression or (**C & D**) novel ICAM-1 transcript expression following 4-hour treatment with TNF-α (red) or fresh medium alone (blue) for (**A & C**) all isolates (n = 5 donors) or for (**B & D**) individual isolates (n = 1 donor). Reference genes were RPLP0 and GAPDH. In **A** and **C**, bars represent mean relative expression and error bars indicate standard deviation. Data were analysed by paired *t*-test.

A significant increase in both full-length and novel ICAM-1 transcript expression was also confirmed following IL-1β stimulation of primary retinal endothelial cell isolates (**Figure 3.9**). A 2.7-fold overall increase in full-length ICAM-1 transcript level was observed compared to the medium only control condition (**Figure 3.9A**, $p = 0.022$), while a 8.7-fold overall increase was recorded for the novel ICAM-1 transcript (**Figure 3.9C**, $p = 0.048$). The full-length and novel ICAM-1 transcript variants increased in all five cell isolates stimulated with IL-1β compared to control (**Figure 3.9B & 3.9D**). The increase in full-length ICAM-1 transcript expression was between 1.4-fold and 5.9-fold across the four isolates (**Figure 3.9B**), while the increase in novel ICAM-1 transcript varied between 1.9-fold and 50.4-fold (**Figure 3.9D**).

3.2.3 ICAM-1 protein expression on cytokine-stimulated human retinal endothelial cells

Since both full-length and novel ICAM-1 transcripts showed a significant increase in the cytokine-stimulated primary human retinal endothelial cells, this study explored whether ICAM-1 protein showed a similar increase in expression. The change in membrane-bound ICAM-1 expression on primary human retinal endothelial cells stimulated for 24 hours with TNF-α (10 ng/mL) or IL-1β (5 ng/mL) was measured using a cellular immunoassay. Monolayers of human retinal endothelial cells were treated with cytokine for 24 hours, fixed with PFA, and labelled with anti-human ICAM-1 antibody and fluorescently tagged secondary antibody. Fluorescence intensity emitted by the monolayers indicated level of ICAM-1 protein on the cell surface. Both TNF-α and IL-1β induced a significant increase in membrane bound ICAM-1 protein expression in six and five human retinal endothelial isolates respectively, compared to the medium only control (**Figure 3.10A and Figure 3.10C**, $p \leq 0.01$). However, the increase in ICAM-1 protein expression varied across the different isolates (**Figure 3.10B & 3.10D**). The increase in ICAM-1 protein across donor isolates treated with TNF-α ranged from 3.5-fold to 41.4-fold (**Figure 3.10B**, significant for all six isolates); while human retinal endothelial donor isolates treated with IL-1β ranged from 1.3-fold to 6.3-fold difference compared to medium treated control (**Figure 3.10D**, significant for three of five isolates).

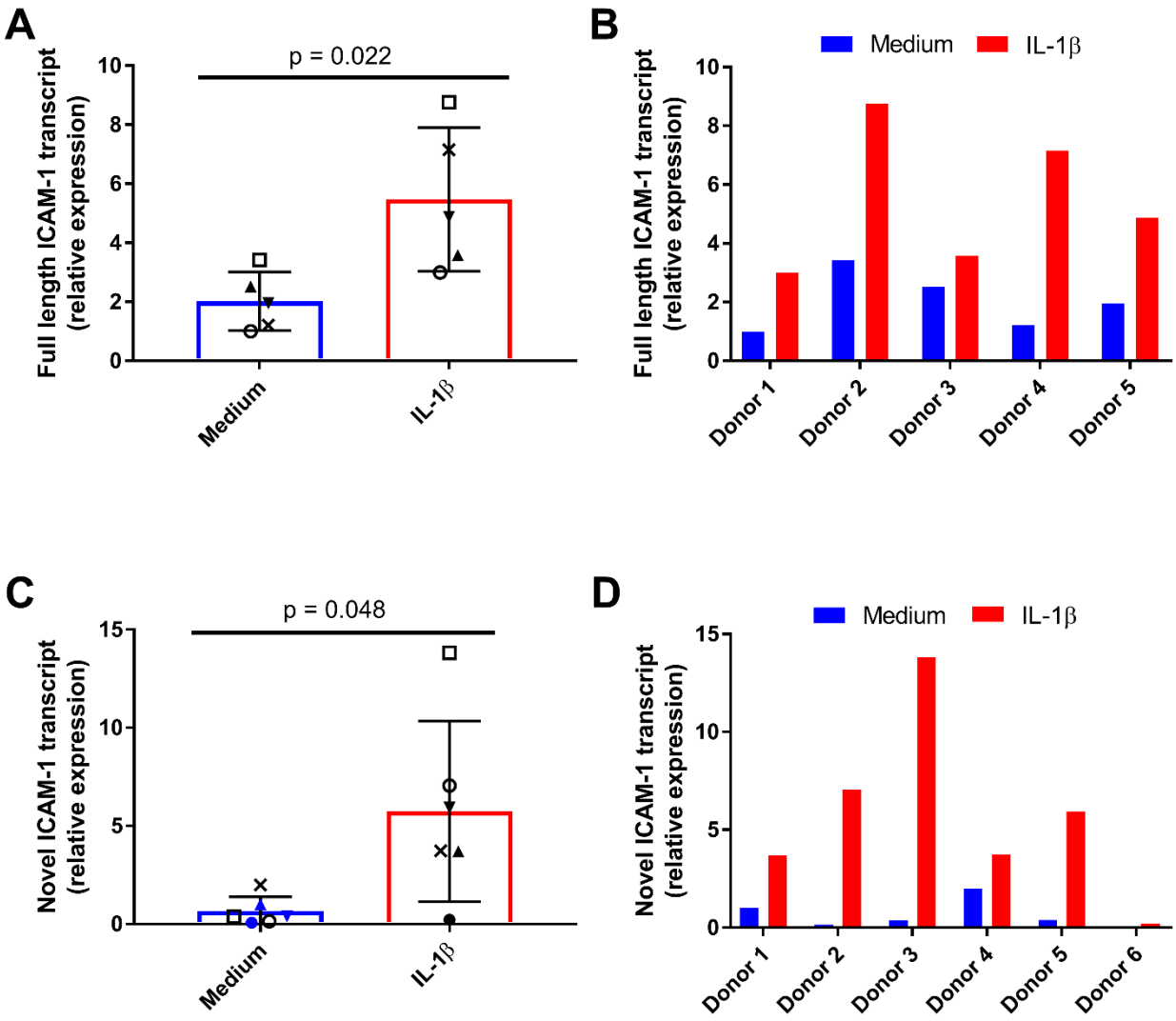

Figure 3.9 Expression of full-length ICAM-1 and novel ICAM-1 transcripts increased significantly in primary human retinal endothelial cells stimulated with IL-1β. Graphs show (**A & B**) full-length ICAM-1 transcript expression or (**C & D**) novel ICAM-1 transcript expression following 4-hour treatment with IL-1β. (red) or fresh medium alone (blue) for (**A & C**) all isolates (n = 5-6 donors) or for (**B & D**) individual isolates (n = 1 donor). Reference genes were RPLP0 and GAPDH. In **A** and **C**, bars represent mean relative expression and error bars indicate standard deviation. Data were analysed by paired *t*-test.

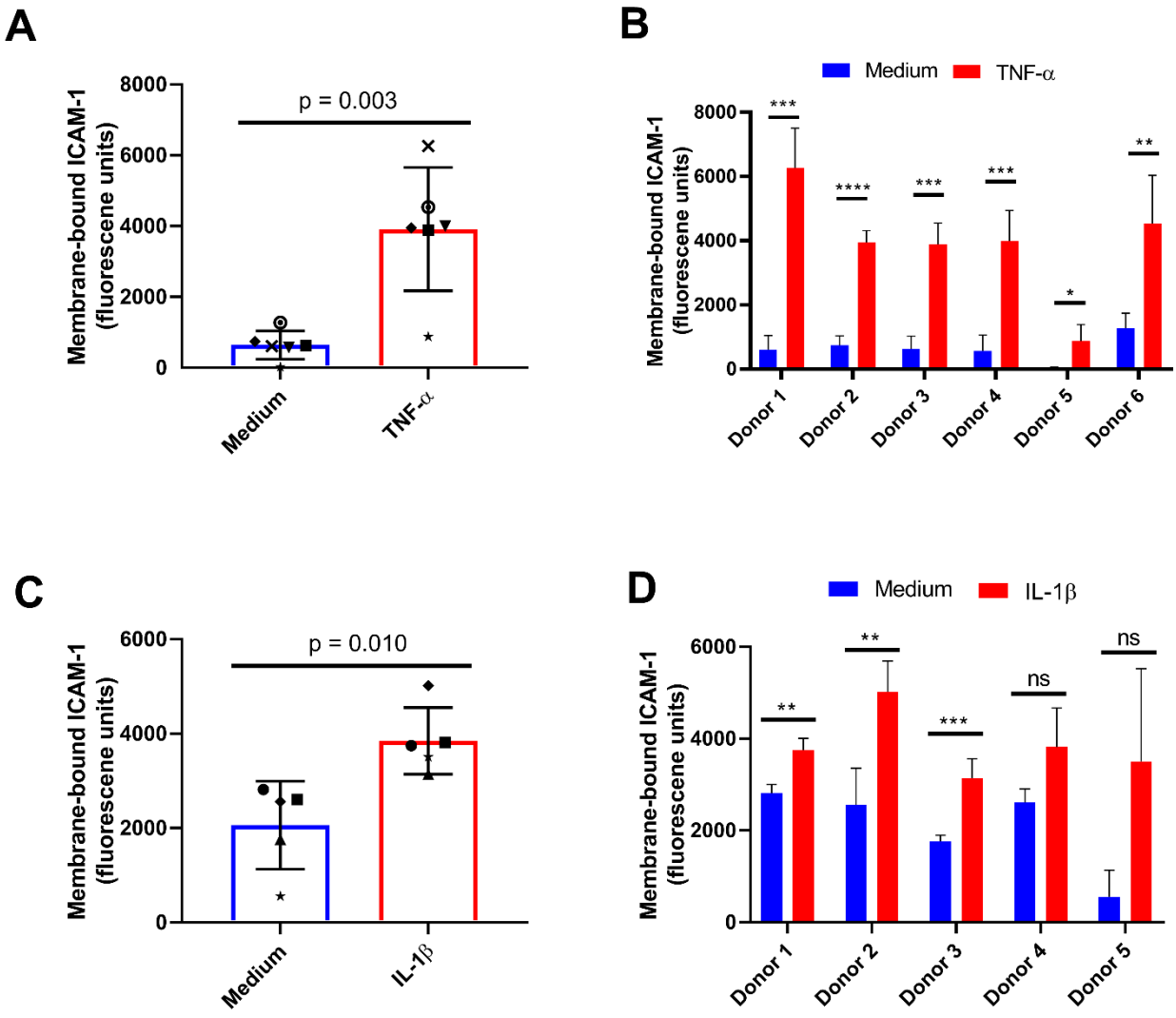

Figure 3.10 Expression of membrane-bound ICAM-1 protein significantly increased in primary retinal endothelial cells following inflammatory cytokine treatment. Monolayers of human retinal endothelial cells were treated for 24 hours with TNF-α or IL-1β (red) or fresh medium alone (blue). Membrane bound ICAM-1 was indirectly immunolabeled, and fluorescence of the endothelial monolayer was determined by microplate reader. Graphs show fluorescence levels for (**A & C**) all isolates (n = 5-6 donors) or for (**B & D**) individual isolates (n = 1 donor, 4 monolayers/condition) following treatment with (**A & B**) TNF-α or (**C & D**) IL-1β. Bars represent mean relative fluorescence following correction for background fluorescence and adjustment for total cell number. Error bars indicate standard deviation. Data were analyzed by (**A & C**) paired *t*-test or (**B & D**) unpaired *t*-test: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant.

3.3 Discussion

The work described in this chapter aimed at measuring ICAM-1 transcript and protein expression in human retinal endothelial cells, including following stimulation with TNF-α and IL-1β. The primary role of ICAM-1 in directing leucocyte migration to sites of inflammation, including the retina in non-infectious posterior uveitis, has been well-highlighted in the literature. Two ICAM-1 transcript variants were identified in human retinal endothelial cells, both in primary cell isolates and a cell line. As expected, one variant was the full-length ICAM-1 transcript. A most interesting finding was a novel transcript variant of ICAM-1, confirmed to be encoded by 6 of the 7 exons in the *ICAM1* gene.

The inflammatory cytokines, TNF-α and IL-1β, stimulated an increase in ICAM-1 expression by primary human retinal endothelial cells. Expression of full-length ICAM-1 transcript in the cell, and also ICAM-1 protein on the cell membrane, was significantly elevated above the control levels. Treatment with both cytokines also led to a significant increase in novel ICAM-1 transcript variant expression. Full-length ICAM-1 transcript is expressed in human retinal endothelial cells at basal and cytokine-induced conditions across various studies (Duguid et al., 1991, Smith et al., 2012b, Smith et al., 2007, Ashander et al., 2016, Karthikkeyan et al., 2018). However, variation in full-length ICAM-1 transcript and protein expression was yet to be determined in primary isolates of human retinal endothelial cells.

There were differences in the level of induction of ICAM-1 transcripts and protein following TNF-α and IL-1β stimulation across the primary human retinal endothelial cell isolates. Due to limited numbers of cells and isolates, specific statistical analyses were not carried out. However, this observation is consistent with variation in the immune response between different people, which can be attributed to multiple factors, such as age (Carr et al., 2016), genetics (Roederer et al., 2015), environment (Brodin et al., 2015) and biological variations (Kanodia et al., 2019, Kaczorowski et al., 2017, Brodin and Davis, 2017). The method used for preparing retinal endothelial cell isolates was consistent across donors, but multiple steps are involved, and thus minor technical variations are

unavoidable and could also contribute to molecular differences between isolates. Nonetheless, the significant upregulation in ICAM-1 transcript and protein expression in primary retinal endothelial cells overall, comparing control versus TNF-α or IL-1β treatment, was consistent with the elevated full-length ICAM-1 transcript expression in the human retinal endothelial cell line under the same conditions.

The finding of novel ICAM-1 transcript variant in primary human retinal endothelial cell isolates and the human retinal endothelial cell line (**Section 2.2.2**) revealed a splice site between exon 2 and exon 4. However, the structure of this novel isoform is yet to be described in published papers, and based on its mRNA sequence, the protein encoded by this novel ICAM-1 transcript is predicted to have structural differences from ICAM-1-202. This predicted human ICAM-1 isoform has a similar structure to one of the 6 mouse ICAM-1 isoforms (King et al., 1995). This mouse membrane-bound ICAM-1 protein lacks Ig domain 2, being encoded by an alternatively spliced transcript, with no exon 3. Further investigation of the novel human ICAM-1 transcript might involve an RLM-RACE reaction to produce cDNA for the entire transcript, for PCR and sequencing. Following on from this, one could investigate the function of the novel ICAM-1 isoform by inserting DNA sequence into a plasmid vector, and over-expressing the protein in cultured cells.

The ICAM-1 isoform predicted from the novel transcript may have a role in regulating leucocyte migration, suggested by the elevated transcript expression in human retinal endothelial cells upon cytokine stimulation. It has the same start codon, methionine, in exon 1 as in full-length ICAM-1 transcript. Exon 1 of ICAM-1 is known to encode the signal sequence (Puthothu et al., 2006), and some spliced isoforms are able to bind the major ligand, LFA-1 (van Den Engel et al., 2000). Gay and colleagues observed a delay in wound healing, with decreased wound elasticity, in mice lacking the entire *ICAM1* gene and thus all ICAM-1 isoforms, but less than what was observed in mice deficient in selected isoforms (Gay et al., 2011). This study highlighted the possible involvement of ICAM-1 isoforms in physiological mechanisms and pathological processes.

In summary, this work has confirmed the expression of full-length ICAM-1 transcript plus a novel ICAM-1 transcript variant in primary human retinal endothelial cells. More studies need to be conducted to further understand the functional role of the novel ICAM-1 isoform in health and in disease states, such as non-infectious posterior uveitis. Cells stimulated with inflammatory cytokines, TNF-α and IL-1β, expressed increased ICAM-1 transcript and protein. A significant increase in ICAM-1 expression was also observed following both TNF-α and IL-1β stimulation in a human retinal endothelial cell line. Thus, for subsequent work, the cell line was used as a model to investigate the effect of manipulating gene transcription on ICAM-1 expression by human retinal endothelium.

CHAPTER 4

Prediction of transcription factors that regulate *ICAM1* gene expression

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CHAPTER 4: PREDICTION OF TRANSCRIPTION FACTORS THAT REGULATE *ICAM1* GENE EXPRESSION

4.1 Introduction

Transcription factors are proteins that recognise and bind specific DNA sequence motifs called TF binding sites, which are usually 5 to 20 bp in length (Cuellar-Partida et al., 2012). Transcription factor binding at these promoter sites can promote or inhibit RNA polymerase activity to upregulate or downregulate specific target genes (Maston et al., 2006, Castellanos et al., 2020). Thus, TFs play a vital role in regulating gene expression, and the identification of functional TF binding sites is crucial to understanding the mechanistic pathway of transcriptional regulation. The development of high-throughput screening and sequencing laboratory techniques, including ChIP-chip and ChIP-seq, has resulted in the identification of many TF binding sites (Ren et al., 2000, Kharchenko et al., 2008, Park et al., 2013).

Chromatin immunoprecipitation is a key methodology for assessing protein-DNA binding and the effects of TF binding activity (Davis et al., 2018, Lambert et al., 2018b). It is worth noting that TF sequence recognition motifs are abundant in the genome, and not all motifs are functional TF binding sites that alter transcription activity (Whitfield et al., 2012, Cusanovich et al., 2014). Newer approaches to increase the prediction of TFs involved in specific gene regulation have been investigated via the combination of chromatin immunoprecipitation (ChIP) data and motif hits from gene expression assays, such as RNA-seq (Balwierz et al., 2014, Jiang and Mortazavi, 2018). Jolma and colleagues identified additional specific binding for a huge number of TFs, more than previously known, through combining data of sequence-specific binding of human TF from systematic evolution of ligands by exponential enrichment (SELEX), with data from a ChIP-seq dataset (Jolma et al., 2013). Other high-throughput assays include nascent transcription assays measuring *bona fide* transcription, before RNA synthesis (Core et al., 2008), and the Cap-associated approaches, CAGE and GRO-CAP, which target the 5' cap of transcripts (Core et al., 2014, Tome et al., 2018). However, limitations to the various laboratory methodologies include high cost and the lack of specific reagents

for numerous TFs, leading to biological experiments being unable to detect all genomic TFBSs (Wang et al., 2015a).

In silico prediction algorithms have proven to be extremely valuable tools for guiding investigations of gene transcriptional regulation (Fang et al., 2016, Keilwagen et al., 2019). Various computational methods have been employed to identify additional members of an existing TF binding motif or to find new TF binding motifs (Rohr et al., 2013, Fornes et al., 2020, Koo and Ploenzke, 2020). Another approach, called TF enrichment analysis uses gene expression datasets to predict TFs (Li et al., 2019, Keenan et al., 2019). This phase of the work sought to identify TFs that regulated *ICAM1* gene expression through a series of *in silico* analysis techniques, including phylogenetic footprinting, a predictive tool for TF binding sites, and TF enrichment analysis based on gene expression datasets. Literature around the candidate TFs identified *in silico* could then be reviewed, to prioritise the TF candidate for subsequent *in vitro* targeting in human retinal endothelial cells.

4.2 Results

4.2.1 Putative transcription factor binding sites in the *ICAM1* gene promoter

Bioinformatics tools were used to narrow down a TF candidate that would be tested for inducible regulation of *ICAM1* gene transcription in human retinal endothelial cells. First, software programs that could identify putative TF binding sites in the promoter of the human *ICAM1* gene were employed. ConTra, which uses phylogenetic footprinting, generated a list of 10 TFs that bound sites conserved in 10,000 bp of sequence immediately upstream of the *ICAM1* gene TSS across humans and mice. These TFs are presented in **Table 4.1**. InnateDB, which identifies potential TF binding from curated *in vivo* and *in vitro* experimental data, gave a total of 12 TFs predicted to regulate expression of the human *ICAM1* gene. These TFs are presented in **Table 4.2**.

4.2.2 Putative transcription factors regulating *ICAM1* gene expression

Since the lists of TFs predicted using ConTra and InnateDB were relatively short, an alternative approach using TF enrichment analysis was implemented. This made use of disease-relevant datasets

Table 4.1 Transcription factors predicted to bind to the *ICAM1* gene promoter, based on analysis using ConTra. Motifs for these 10 TFs were highly conserved in 10 kB of DNA sequence upstream of the *ICAM1* TSS in mice and humans.

Transcription factors
REL-associated protein (RELA)
Glycolytic genes transcriptional activator (GCR)2
Purine rich box-1 (PU.1)/ SFFV proviral integration site -1 (Spi-1)
Signal transducer and activator of transcription (STAT)
Nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB)
Floral homeotic protein PISTILLATA (PI)
Pectin degradation regulator 1 (PDR1)
SPIB Transcription Factor (Spi-B)
SPIC Transcription Factor (Spi-C)
E74 like ETS transcription factor 1 (Elf-1)

Table 4.2 Transcription factors predicted to bind to the *ICAM1* gene promoter, based on analysis using InnateDB. A total of 12 TFs were predicted to interact with human *ICAM1* gene promoter. The TF class indicates a potentially shared regulatory pathway.

Transcription factors	**Transcription factor class**
REL-associated protein (RELA)	Unknown
CCAAT Displacement protein (CUTL1)	Unknown
NF-kappaB p65	REL
NF-kappaB (NF-kB)	REL
Collier/Olf-1/EBF1 (COE1)	Unknown
Nuclear factor of activated T-cells (NF-AT)1	REL/NFAT
Proto-oncogene c-Maf / V-maf musculoaponeurotic fibrosarcoma (Maf)	Unknown
Olf-1 / early B-cell factor 1 (EBF1)	Unknown
ETS like-1 protein (ELK1)	ETS
Bromodomain PHD Finger (BPTF)	CH
REL protooncogene (REL)	REL
Telomere length regulation protein (Tel-2a)	ETS

that were available in the home laboratory. ChEA3 was input with the transcriptomes of primary human retinal endothelial cells treated with TNF-α or IL-1β for 60 minutes or 24 hours. The analysis yielded extensive lists of TFs: 89 for 60 mins, 88 for 24-hour TNF-α-treatment datasets and 88 for 60 mins, 86 for 24-hour IL-1β-treatment datasets. Of the top 50 TFs predicted to regulate changes in *ICAM-1* gene expression in human retinal endothelial cells following TNF-α stimulation, 40 TFs were identified at both 60 minutes and 24 hours, as presented in **Table 4.3**. Of the top 50 TFs predicted to regulate changes in gene expression following IL-1β stimulation, 41 TFs were identified at both time points, as presented in **Table 4.4**. A final list of 35 TFs predicted to regulate *ICAM1* gene expression in human retinal endothelium in response to TNF-α and IL-1β was produced from Table 4.3 and Table 4.4, and given as **Table 4.5**.

4.3 Discussion

The work presented in this chapter involved *in silico* prediction of TFs that may regulate *ICAM1* gene expression. The final goal was to select a candidate TF that could be evaluated for an effect on ICAM-1 protein levels in human retinal endothelial cells. Retinal endothelial ICAM-1 has a primary role in directing migration of leucocytes into the posterior eye in non-infectious uveitis. The chosen TF ideally should not propagate unwanted downstream effects, namely affecting machinery that is required for normal cellular activities, including cell survival. As STAT and NFkB families of TFs are vital in roles for immunity and survival, it would be unwise to select TFs from these classes.

Of the three techniques used to identify a suitable TF for targeting *ICAM1* gene expression in human retinal endothelial cells, ChEA3 proved to be the most informative. In comparison, information from the other two databases was more limited and left few TFs to select from. ChEA3, developed by Mount Sinai Center for Bioinformatics (Keenan et al., 2019), utilises the method of TF enrichment analysis, which sorts and ranks TFs related to the user's gene or gene list of interest, using an approach that integrates existing libraries of previously noted TF predictions and TF binding sites. ChEA3 integrates six main gene set libraries in the web-based application programming interface. These

Table 4.3 Transcription factors predicted to bind to the *ICAM1* gene promoter, based on analysis of the transcriptome of human retinal endothelial cells stimulated with TNF-α using ChEA3. Ranked list combines TFs common to 60 minutes and 24 hours of stimulation with the cytokine.

Transcription factors
Interferon regulatory factor 1 (IRF1)
Interferon regulatory factor 8 (IRF8)
Signal transducer and activator of transcription 1 (STAT1)
Signal transducer and activator of transcription 5A (STAT5A)
Nuclear receptor subfamily 1 group H member 3 (NR1H3)
Ikaros zinc finger 1 (IKZF1)
SRY-Box transcription factor 17 (SOX17)
Signal transducer and activator of transcription 3 (STAT3)
CCAAT enhancer binding protein beta (CEBPB)
Activating transcription factor 3 (ATF3)
V-Jun avian sarcoma virus 17 oncogene homolog (JUN)
B-cell lymphoma 6 (BCL6)
ETS Proto-Oncogene 2 (ETS2)
Kruppel-like factor 2 (KLF2)
ETS Proto-Oncogene 1 (ETS1)
Peroxisome proliferator activated receptor delta (PPARD)
T-cell factor 7 (TCF7)
SFFV proviral integration site -1 (Spi-1)
Forkhead box P3 (FOXP3)
Aryl hydrocarbon receptor (AHR)

Table 4.3 (cont.)

Transcription factors
Early growth response 1 (EGR1)
T-cell acute lymphocytic leukemia 1 (TAL1)
Nuclear receptor subfamily 3 group C member 1 (NR3C1)
Signal transducer and activator of transcription 4 (STAT4)
CCAAT enhancer binding protein alpha (CEBPA)
Friend leukemia integration 1 (FLI1)
Vitamin D receptor (VDR)
Signal transducer and activator of transcription 6 (STAT6)
CCAAT enhancer binding protein delta (CEBPD)
GATA-binding factor 6 (GATA6)
REL-associated protein (RELA)
BTB domain and CNC homolog 1 (BACH1)
E74 like ETS transcription factor 1 (ELF1)
Runt-related transcription factor 1 (RUNX1)
Retinoic acid receptor alpha (RARA)
Lymphoblastic leukemia derived sequence 1 (LYL1)
Kruppel-like factor 4 (KLF4)
Kruppel-like factor 5 (KLF5)
GATA-binding factor 3 (GATA3)
SMAD3 family member 3 (SMAD3)

Table 4.4 Transcription factors predicted to bind to the *ICAM1* gene promoter, based on analysis of the transcriptome of human retinal endothelial cells stimulated with IL-1β using ChEA3. Ranked list combines TFs common to 60 minutes and 24 hours of stimulation with the cytokine.

Transcription factors
Nuclear receptor subfamily 1 group H member 3 (NR1H3)
Signal transducer and activator of transcription 5A (STAT5A)
Interferon regulatory factor 8 (IRF8)
CCAAT enhancer binding protein beta (CEBPB)
Signal transducer and activator of transcription 1 (STAT1)
Activating transcription factor 3 (ATF3)
Interferon regulatory factor 1 (IRF1)
SRY-Box transcription factor 17 (SOX17)
Peroxisome proliferator activated receptor gamma (PPARG)
V-Jun avian sarcoma virus 17 oncogene homolog (JUN)
ETS Proto-Oncogene 2 (ETS2)
Aryl hydrocarbon receptor (AHR)
T-cell factor 21 (TCF21)
Signal transducer and activator of transcription 3 (STAT3)
Ikaros zinc finger 1 (IKZF1)
Early growth response 1 (EGR1)
B-cell lymphoma 6 (BCL6)
T-cell acute lymphocvtic leukemia 1 (TAL1)
CCAAT enhancer binding protein delta (CEBPD)
GATA-binding protein 2 (GATA2)
BTB domain and CNC homolog 1 (BACH1)

Table 4.4 (cont.)

Transcription factors
Forkhead box P3 (FOXP3)
GATA-binding factor 6 (GATA6)
CCAAT enhancer binding protein alpha (CEBPA)
Hypoxia-inducible factor 1 alpha (HIF1A)
Nuclear receptor subfamily 3 group C member 1 (NR3C1)
SMAD Family Member 3 (SMAD3)
Kruppel-like factor 4 (KLF4)
ETS Proto-Oncogene 1 (ETS1)
GATA-binding factor 3 (GATA3)
Runt-related transcription factor 2 (RUNX2)
Peroxisome proliferator activated receptor delta (PPARD)
REL-associated protein (RELA)
SFFV proviral integration site -1 (SPI1)
T-cell factor 7 (TCF7)
Kruppel-like factor 5 (KLF5)
Nuclear factor erythroid 2 like 2 (NFE2L2)
Signal transducer and activator of transcription 4 (STAT4)
Friend leukemia integration 1 (FLI1)
Runt-related transcription factor 1 (RUNX1)
Kruppel-like factor 2 (KLF2)

Table 4.5 Common list of transcription factors predicted to bind to the *ICAM1* gene promoter in human retinal endothelial cells stimulated with TNF-α or IL-1β. Unranked list combines TFs common to Table 4.3 and Table 4.4.

Transcription factors
Interferon regulatory factor 1 (IRF1)
Interferon regulatory factor 8 (IRF8)
Signal transducer and activator of transcription 1 (STAT1)
Signal transducer and activator of transcription 5A (STAT5A)
Nuclear receptor subfamily 1 group H member 3 (NR1H3)
Ikaros zinc finger 1 (IKZF1)
SRY-Box transcription factor 17 (SOX17)
Signal transducer and activator of transcription 3 (STAT3)
CCAAT enhancer binding protein beta (CEBPB)
Activating transcription factor 3 (ATF3)
V-Jun avian sarcoma virus 17 oncogene homolog (JUN)
B-cell lymphoma 6 (BCL6)
ETS Proto-Oncogene 2 (ETS2)
Kruppel-like factor 2 (KLF2)
CCAAT enhancer binding protein delta (CEBPD)
ETS Proto-Oncogene 1 (ETS1)
T-cell factor 7 (TCF7)
SFFV proviral integration site -1 (SPI1)
Forkhead box P3 (FOXP3)
Aryl hydrocarbon receptor (AHR)
Early growth response 1 (EGR1)
T-cell acute lymphocvtic leukemia 1 (TAL1)
Friend leukemia integration 1 (FLI1)

Table 4.5 (cont.)

Transcription factors
Signal transducer and activator of transcription 4 (STAT4)
CCAAT enhancer binding protein alpha (CEBPA)
Nuclear receptor subfamily 3 group C member 1 (NR3C1)
SMAD Family Member 3 (SMAD3)
GATA-binding factor 6 (GATA6)
REL-associated protein (RELA)
BTB domain and CNC homolog 1 (BACH1)
Runt-related transcription factor 1 (RUNX1)
Kruppel-like factor 4 (KLF4)
Kruppel-like factor 5 (KLF5)
GATA-binding factor 3 (GATA3)
Peroxisome proliferator activated receptor delta (PPARD)

include data from co-expression analysis, ChIP-seq, and user-submitted queries (Keenan et al., 2019).

One co-expression data library ARCHS4, containing data from human samples, looks at TF-gene co-expression across all GEO RNA-seq. Another co-expression data library, GTEx, gathers its data from TF-gene co-expression across all genotype-tissue expression. The three libraries including ChIP-seq data are taken from ENCODE (Davis et al., 2018), curated individual literature findings, and ReMap. Finally, the last library integrated in ChEA3 is Enrichr, whereby TF gene co-occurrence is predicted based on crowd-submitted gene sets aiming to identify genes that are associated with TFs in submitted queries. The mining process, in conjunction with the aid of machine learning algorithms, yields a "best performance benchmark" among the six libraries (Keenan et al., 2019), providing high accuracy predictions of TFs which share a similar regulatory function to the users' gene set of interest.

This method of TF enrichment analysis was therefore the most appropriate method to identify a candidate TF for this project. The TFs that ChEA3 analysis predicted to regulate *ICAM1* expression originated mainly from the NF-kB, STAT, C/EBP, interferon regulatory factor (IRF), Jun, and ETS families. Apart from the aforementioned importance of STAT and NFkB families of TFs in cell survival, other TFs that were identified included the C/EPB class, which are transcriptional regulators that play a pivotal role in metabolism, regeneration, and differentiation (Zhao et al., 2019b). Another class of TF, the IRF family, is involved in various phases of the immune response, such as immune cell differentiation and coordinating the response to pathogens (Jefferies, 2019). Among the members of the IRF family, IRF3, IRF5 and IRF7 are of high importance to type I IFN production. Another family member, IRF8, has key importance in the regulation of the immune response, given its involvement in myeloid cell phenotype and development (Jefferies, 2019). Whilst ChEA3 indicated IRF-1 might be associated in the regulation of the *ICAM1* gene, opposing results were presented by Nizamutdinova and colleagues (Nizamutdinova et al., 2012). They found that the promoter region of *ICAM1* contains neither IRF-1 nor the GATA binding motif, but both TFs have a binding motif located in the *VCAM1* gene promoter region.

Based on the available literature, the candidate TF was selected from the ETS family: Ets-1. In normal physiology, ETS family TFs are involved in multiple biological processes, such as haematopoiesis, angiogenesis, apoptosis and tissue remodelling (Dittmer, 2003, Findlay et al., 2013). De Launoit and colleagues previously discovered two ETS binding sites in the proximal *ICAM1* gene promoter upstream of an iron-responsive element (de Launoit et al., 1998). They also demonstrated that Ets proteins, including Ets-1, Ets-2, PEA3 and ERM, were able to activate the human *ICAM1* gene promoter (de Launoit et al., 1998). Ets-1 is the most studied of the ETS family members of TFs, and the published literature supports the notion that Ets-1 function primarily as a transcriptional activator (see Chapter 6 for additional discussion). Hence, Ets-1 was chosen as the TF target for studies aimed at reducing ICAM-1 transcript and protein levels in human retinal endothelial cells by manipulating *ICAM1* gene transcription, which are presented in the next chapter.

CHAPTER 5

Effect of targeting Ets-1 on ICAM-1 expression by human retinal endothelial cells

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CHAPTER 5: EFFECT OF TARGETING ETS-1 ON ICAM-1 EXPRESSION BY HUMAN RETINAL ENDOTHELIAL CELLS

5.1 Introduction

The protein, ETS protooncogene 1, transcription factor, is commonly termed protein C-ets-1 in the literature and abbreviated Ets-1. It belongs to the Ets family of TFs, which are encoded by 28 genes in humans (Garrett-Sinha, 2013). This particular gene family is highly conserved across evolution (King et al., 2008). Among the family members, the portion of *Ets1* that binds to DNA forms a winged helix-turn-helix motif structure (Aravind et al., 2005). The Ets TF binding domain is a well-conserved DBD shared by Ets family members, known as Ets-binding site (EBS) in the promoter region of target genes (Laitem et al., 2009). Ets-1 is involved in multiple cellular activities including cell migration (Liu et al., 2016), differentiation (John et al., 2008), proliferation (Higuchi et al., 2007), invasion (Xu et al., 2019) and apoptosis (O'Hara et al., 2019). Apart from the aforementioned functions, Ets-1 also plays a role in tumour progression and angiogenesis (Dittmer, 2015, Zhou et al., 2018).

Garrett-Sinha has described the human Ets1 isoforms in a very comprehensive review (Garrett-Sinha, 2013). The major isoform of *Ets1* is a protein encoded by 8 exons, the first exon being exon A, and the last seven exons named exons III to IX. This isoform of Ets-1 protein, which is referred to as p51 or p54, is 441 amino acids in length. A second isoform, p42, which is formed by alternative splicing of exon VII, is described as being more active in DNA binding than the full-length protein (Wasylyk et al., 1992). A third isoform, p68, contains additional exons Ia, I and II, but not exon A (Leprince et al., 1988). However, the role which these additional sequences play in any functional protein is not clear. The final Ets-1 isoform, p27, lacks several regions (a ERK2 phosphorylation site, an acidic transactivation domain and also the pointed domain), but still contains the DBD (Laitem et al., 2009). Hence, it is able to regulate gene expression, but acts as a negative inhibitor of p54 (Laitem et al., 2009). Both p68 and p27 isoforms are produced in low amounts in tissues according to the literature (Garrett-Sinha, 2013).

The Ets-1 protein is produced in a range of tissues, from lymphoid tissues to blood vessels, and also solid tumours (Garrett-Sinha, 2013). In humans, high expression of Ets-1 is detected mainly in lymphoid tissues, including in B-cells and T-cells (Mayeux et al., 2015, Sunshine et al., 2019). Stimuli involved in induction of Ets-1 include TNF-α, and growth factors, such as hepatocyte growth factor and platelet-derived growth factor (Goetze et al., 2001a, Paumelle et al., 2002, Goetze et al., 2001b). In such instances, the factors driving Ets-1 production are linked to the activation of the Ras/Raf/MEK1/ERK1/2 pathway (Plotnik et al., 2014).

Loss of Ets-1 is associated with several consequences in cellular immunity, including an increased number of plasma cells in lymphoid organs (Luo et al., 2014). Also, a partial impairment in bone marrow B-cell development was observed following loss of Ets-1 (Eyquem et al., 2004). The role of Ets-1 in the helper T-cell mediated response is quite complex. Ets-1 is able to cooperate with another TF, T-bet, to enhance expression of Th1-specific cytokine interferon-γ (Grenningloh et al., 2005), but it can also recruit HDAC enzymes to repress expression of Th2-specific genes (Lee et al., 2012). These observations and others made in the area of tumorigenesis demonstrate that Ets-1 can act as a transcriptional activator or a transcriptional repressor (Lelievre et al., 2001, Li et al., 2000, Sinh et al., 2017, Zhao et al., 2019a).

Despite showing the presence of Ets binding sites (EBS) on ICAM-1 promoter (de Launoit et al., 1998), the role of Ets-1 in *ICAM1* gene regulation is not fully understood. It was not until 2009, Yockell and colleagues investigated the effect of inactivation of both previously identified EBS (Yockell-Lelievre et al., 2009). They found a reduction on IFN-γ-induced ICAM-1 transcription following inactivation of both EBS at position -138 and -158. They also provided evidence of a physical interaction between Ets-1 and STAT-1 TFs in coimmunoprecipitation studies (Yockell-Lelievre et al., 2009). Another example demonstrating Ets-1 regulation of ICAM-1 was demonstrated by Zhou and colleagues in non-small cell lung cancer cell lines (Zhou et al., 2018). In Ets-1 overexpressing cells, ICAM-1 expression was increased, resulting in effects on cell-cell and cell-

extracellular matrix interactions (Zhou et al., 2018). Recently, a study was carried out by Srivastava and colleagues to understand the impact of Ets-1 signalling on cell adhesion molecule expression in patients with vitiligo (Srivastava et al., 2020). In the study, Ets-1 knock-down was achieved by using a combination of four Ets-1 siRNA in melanocytes, resulting in a significant reduction in Ets-1 and ICAM-1 mRNA expression. These studies suggest Ets-1 functions as a transcriptional activator of the *ICAM1* gene promoter.

The work presented in this chapter explored the effect of silencing Ets-1 transcription on *ICAM1* gene expression in human retinal endothelial cells. A human retinal endothelial cell line (**Section 2.2.2**) was used for this work, given its resemblance to primary cell responses to inflammatory cytokines, as described in Chapter 3. First, expression of Ets-1 is expressed in TNF-α or IL-1β-treated human retinal endothelial cells was determined. Then Ets-1 transcript expression after cell transfection with Ets-1-targeted siRNA was examined. Finally, the effect of Ets-1 silencing on ICAM-1 transcript and protein expression was measured in the human retinal endothelial cells.

5.2 Results

5.2.1 Ets-1 transcript expression in human retinal endothelial cells

The *in silico* analysis presented in Chapter 4 predicted *ICAM1* binding sites and TFs that regulate *ICAM1* gene expression. Combining this analysis with findings from a literature search, Ets-1 was selected as the target for studies aimed at reducing ICAM-1 expression in human retinal endothelial cells by manipulating *ICAM1* gene transcription. First, standard RT-PCR was used to confirm that human retinal endothelial cells expressed Ets-1, using RNA extracted from the human retinal endothelial cell line (**Section 2.2.2**) after 24-hour treatment with TNF-α, IL-1β or fresh medium alone (**Figure 5.1**).

Next, changes in Ets-1 transcript expression in primary human retinal endothelial cells under inflammatory conditions were investigated. In primary cell isolates treated with TNF-α or IL-1β for 4 hours, Ets-1 transcript expression was significantly increased, compared to fresh medium alone

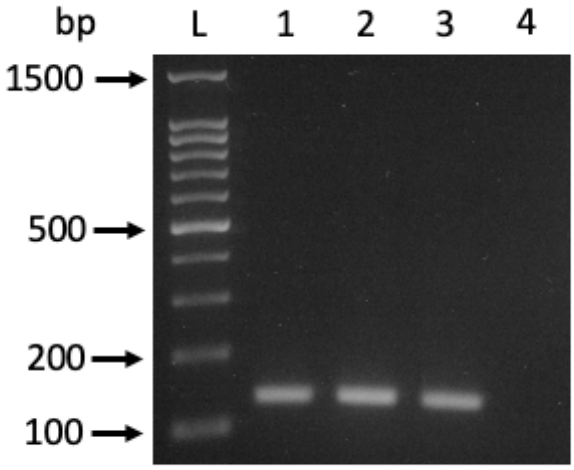

Figure 5.1 Ets-1 expression in a human retinal endothelial cell line following treatment with TNF-α or IL-1β. Gel image shows RT-PCR products on a 2% agarose gel. L = DNA ladder; 1-3 = human retinal endothelial cell line treated with (1) fresh medium only, (2) TNF-α or (3) IL-1β; 4 = PCR no-template control. Expected size: 134 bp.

controls (**Figure 5.2A & 5.2C**, $p \leq 0.032$). It was not possible to perform a statistical analysis for individual isolates, due to limited availability of cells. However, on the basis of individual results, all primary cell isolates stimulated with TNF-α showed an increase in Ets-1 transcript expression that varied between isolates (**Figure 5.2B**). Interestingly, the same cannot be said about primary cell isolates treated with IL-1β, as it is noted that one isolate showed a reduction in Ets-1 transcript expression after stimulation (**Figure 5.2D**).

5.2.2 ICAM-1 transcript expression in Ets-1-silenced human retinal endothelial cells

To measure the effect of silencing Ets-1 on human retinal endothelial cell ICAM-1 expression, the cell line (**Section 2.2.2**) was transfected with two targeted siRNA, individually or in combination, or with non-targeted negative control siRNA for 48 hours. To mimic the Ets-1 effect on ICAM-1 regulation during inflammation, the cells were stimulated with TNF-α or IL-1β, added 24 hours after the siRNA were applied. The siRNAs used in this experimental study, Ets-1 siRNA (1) and Ets-1 siRNA (2), were obtained from a commercial source (Thermo Fisher Scientific - Ambion) that had validated the specificity of the targeted siRNA for knock-down of Ets-1 transcripts. A significant and substantial reduction in Ets-1 transcript expression was noted in human retinal endothelial cells transfected with each individual Ets-1 siRNA and the combination versus non-targeted negative control siRNA across all conditions: Ets-1 siRNA (1), ≥ 90.8%; Ets-1 siRNA (2), ≥ 95.7%; Ets-1 siRNA (1) and (2), ≥ 92.2% (**Figure 5.3**, $p \leq 0.005$).

First, expression of full-length ICAM-1 transcript was determined by RT-qPCR upon silencing Ets-1 transcript in the human retinal endothelial cell line. As expected, transcript expression was relatively low in cells treated with medium alone, and considerably higher in cells stimulated with TNF-α or IL-1β. Transfection with Ets-1 siRNA (1) resulted in a significant increase in transcript expression in the cell line under unstimulated and TNF-α-stimulated conditions ($p \leq 0.021$), but not when treated with IL-1β (**Figure 5.4A**). The Ets-1 siRNA (2) transfection showed a significant increase in full-length ICAM-1 transcript in the human retinal endothelial cell line across all treatment conditions

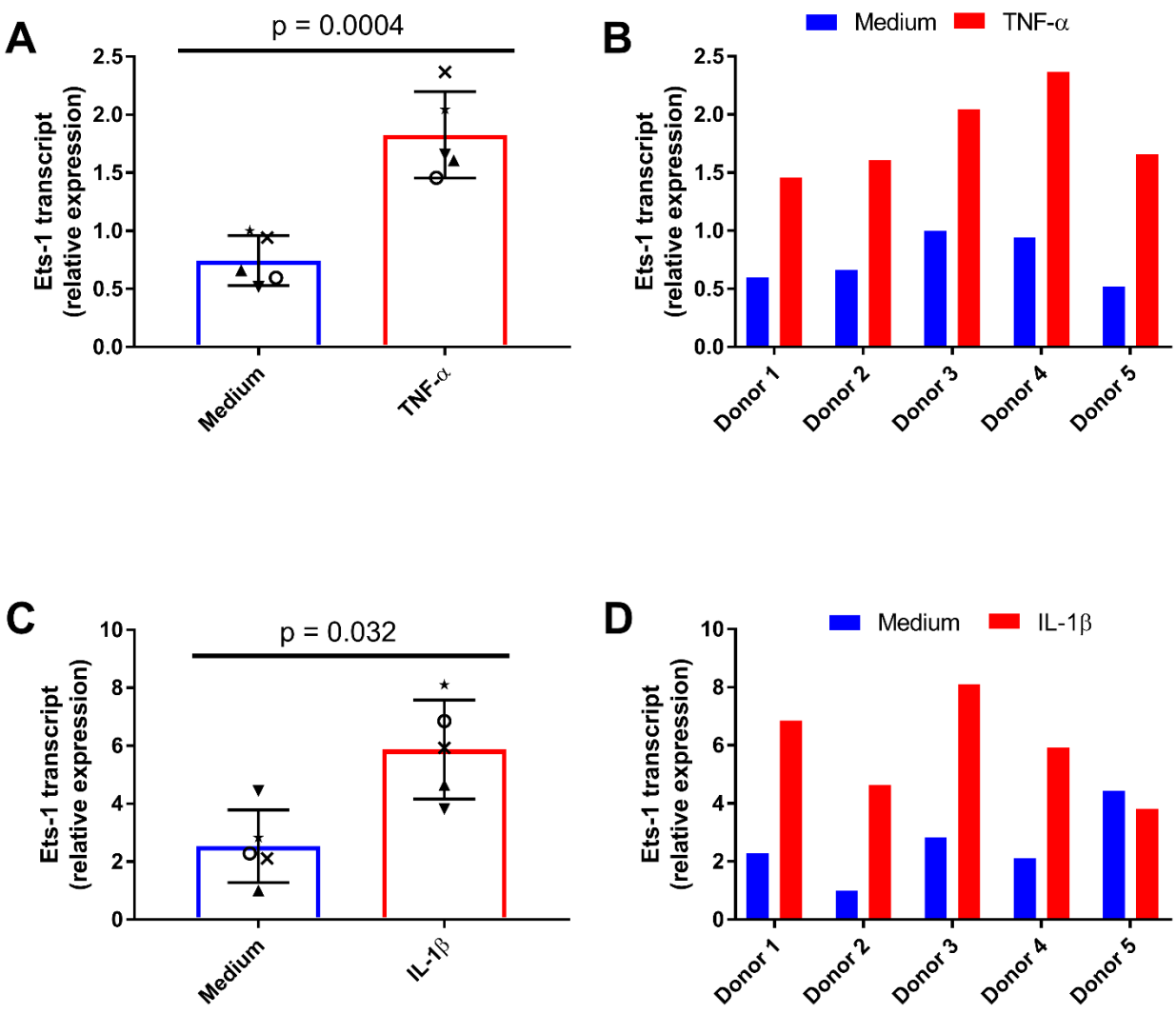

Figure 5.2 Expression of Ets-1 transcript increased significantly in primary human retinal endothelial cells stimulated with TNF-α or IL-1β. Graphs show Ets-1 transcript expression by RT-qPCR following 24-hour treatment with TNF-α or IL-1β (red), or fresh medium only (blue) in (**A & C**) all isolates (n = 5 donors) or (**B & D**) individual isolates (n = 1 donor). Reference genes were RPLP0 and GAPDH. In **A** and **C**, bars represent mean relative expression and error bars indicate standard deviation. Data were analysed by paired *t*-test.

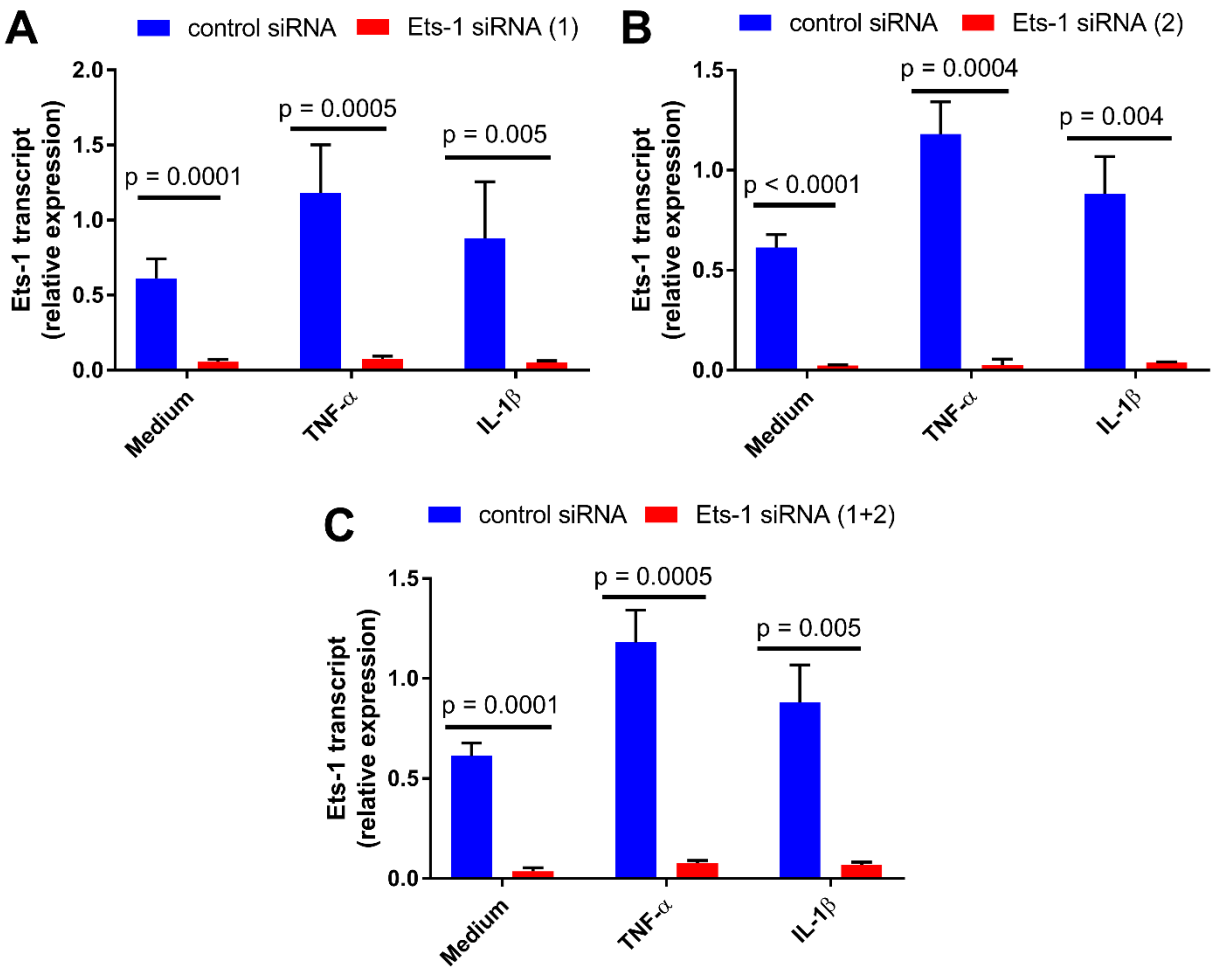

Figure 5.3 Expression of Ets-1 transcript decreased significantly in human retinal endothelial cell line stimulated with TNF-α or IL-1β and treated with Ets-1-targeted siRNA. The human retinal endothelial cell line was transfected with Ets-1 siRNA (1), Ets-1 siRNA (2), Ets-1 siRNA (1) and (2), or non-targeted negative control siRNA for 24 hours, followed by 24-hour stimulation with TNF-α, IL-1β or fresh medium alone. Graphs show expression of Ets-1 transcript by RT-qPCR for treatment with (**A**) Ets-1 siRNA (1), (**B**) Ets-1 siRNA (2), and (**C**) Ets-1 siRNA (1) and (2), versus negative control siRNA (n = 4 cell monolayers/condition). Reference genes were RPLP0 and GAPDH. In **A** and **C**, bars represent mean relative expression and error bars indicate standard deviation. Data were analysed by unpaired *t*-test.

(**Figure 5.4B**, $p \leq 0.018$). Transfection with combined Ets-1 siRNA (1) and (2) led to a significant increase in ICAM-1 transcript expression in cells treated with IL-1β ($p = 0.020$), a trend to increase in cells treated with TNF-α ($p = 0.060$), but no change in the medium alone control (**Figure 5.4C**).

Next, the effect of Ets-1 transcript silencing on expression of the novel ICAM-1 transcript in the human retinal endothelial cell line was investigated. There was no increase in transcript expression following transfection with Ets-1 siRNA (1) in the cell line (**Figure 5.5A**, $p > 0.05$). However, expression of the transcript increased significantly in cells transfected with Ets-1 siRNA (2) and stimulated with TNF-α, IL-1β or medium alone (**Figure 5.5B**, $p \leq 0.047$). Human retinal endothelial cells transfected with the combination of Ets-1 siRNA (1) and (2) and stimulated with IL-1β also showed a significant increase in expression of the novel ICAM-1 transcript (**Figure 5.5C**, $p = 0.022$).

5.2.3 ICAM-1 protein expression on Ets-1-silenced human retinal endothelial cells

Finally, the effect of Ets-1 knock-down with TNF-α or IL-1β treatment on expression of membrane-bound ICAM-1 protein was investigated in the human retinal endothelial cell line (**Section 2.2.2**). This was performed using an immunoassay that measured the level of ICAM-1 protein on the endothelial cell surface. In the experiment that involved TNF-α stimulation, no significant difference in ICAM-1 protein expression was detected in the fresh medium-treated cells for Ets-1 knock-down with Ets-1 siRNA (1) and/or (2), in comparison to the negative control siRNA ($p > 0.05$). Retinal endothelial cells transfected with individual or the combination of Ets-1 siRNAs and then stimulated with TNF-α showed a significant increase in membrane-bound ICAM-1 in comparison to the negative control siRNA (**Figure 5.6**, $p \leq 0.0001$).

Expression of membrane-bound ICAM-1 protein expression in Ets-1-transfected and IL-1β-stimulated retinal endothelial cells was studied in a separate experiment. Again, there were no significant changes in the fresh medium-treated cells that were transfected with the negative control siRNA or the Ets-1 siRNAs, singly or in combination ($p > 0.05$). However, there was a significant

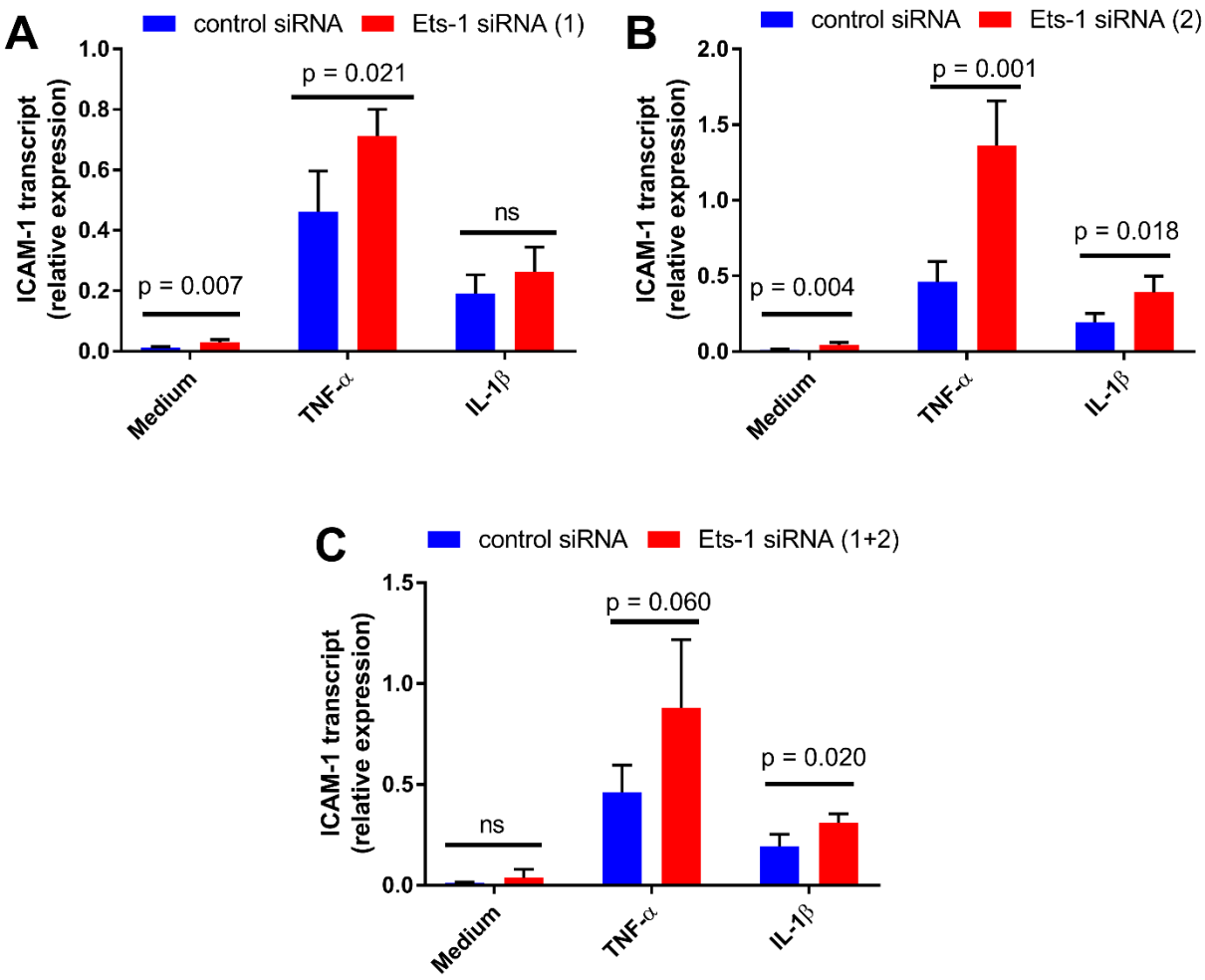

Figure 5.4 Expression of the full-length ICAM-1 transcript increased significantly in human retinal endothelial cell line stimulated with TNF-α or IL-1β and treated with Ets-1-targeted siRNA. The human retinal endothelial cell line was transfected with Ets-1 siRNA (1), Ets-1 siRNA (2), Ets-1 siRNA (1) and (2), or non-targeted negative control siRNA for 24 hours, followed by 24-hour stimulation with TNF-α, IL-1β or fresh medium alone. Graphs show expression of full-length ICAM-1 transcript by RT-qPCR for treatment with (**A**) Ets-1 siRNA (1), (**B**) Ets-1 siRNA (2), and (**C**) Ets-1 siRNA (1) and (2), versus negative control siRNA (n = 4 cell monolayers/condition). Reference genes were RPLP0 and GAPDH. In **A** and **C**, bars represent mean relative expression and error bars indicate standard deviation. Data were analysed by unpaired *t*-test. ns = not significant.

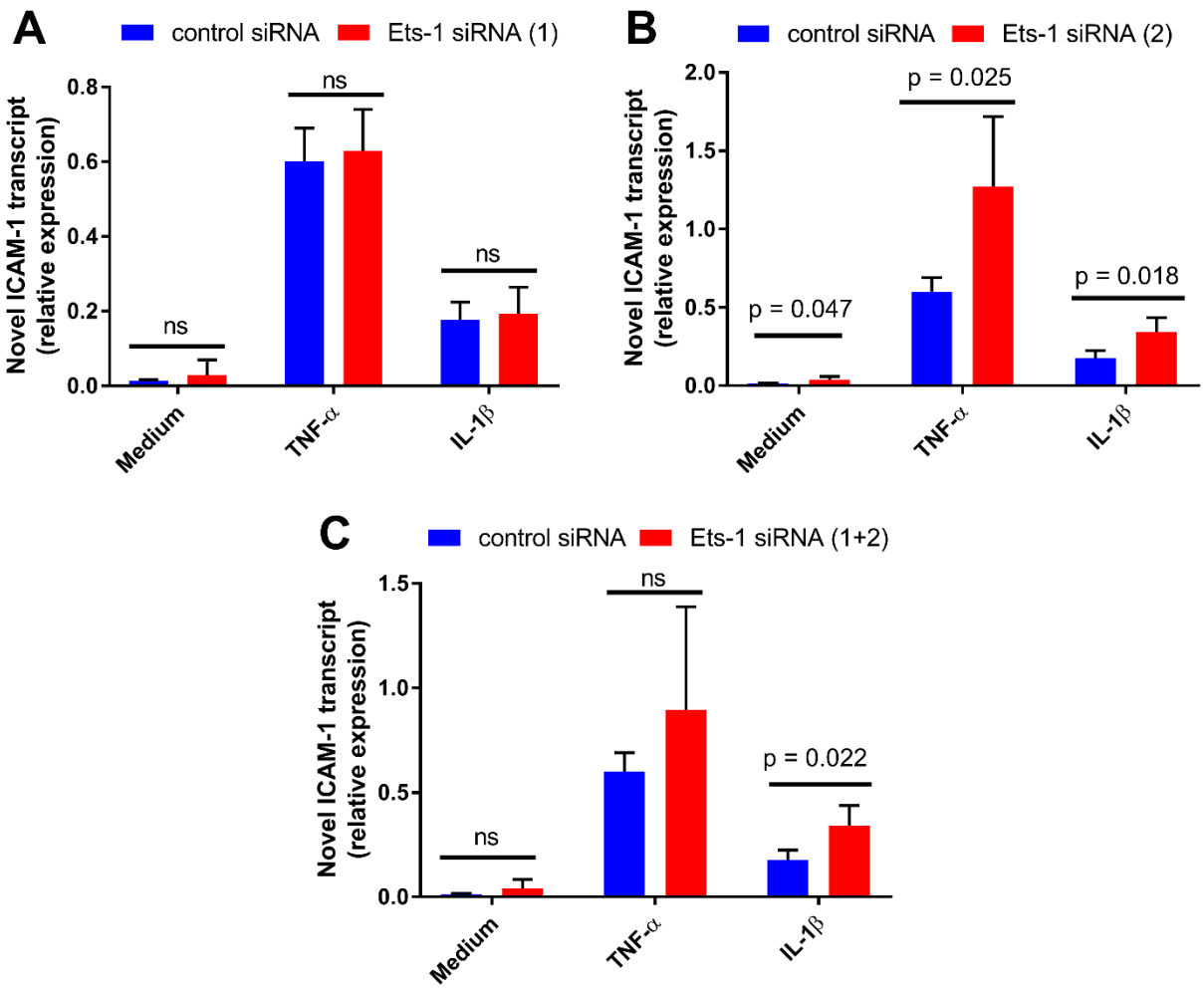

Figure 5.5 Expression of the novel ICAM-1 transcript increased significantly in human retinal endothelial cell line stimulated with TNF-α or IL-1β and treated with Ets-1-targeted siRNA. The human retinal endothelial cell line was transfected with Ets-1 siRNA (1), Ets-1 siRNA (2), Ets-1 siRNA (1) and (2), or non-targeted negative control siRNA for 24 hours, followed by 24-hour stimulation with TNF-α, IL-1β or fresh medium alone. Graphs show expression of the novel ICAM-1 transcript by RT-qPCR for treatment with (**A**) Ets-1 siRNA (1), (**B**) Ets-1 siRNA (2), and (**C**) Ets-1 siRNA (1) and (2), versus negative control siRNA (n = 4 cell monolayers/condition). Reference genes were RPLP0 and GAPDH. In **A** and **C**, bars represent mean relative expression and error bars indicate standard deviation. Data were analysed by unpaired *t*-test. ns = not significant.

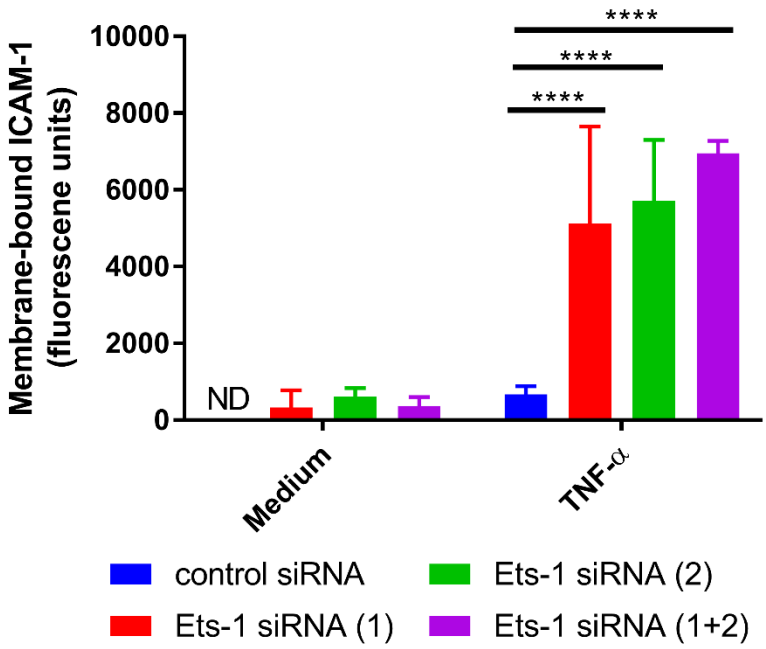

Figure 5.6 Expression of the membrane-bound ICAM-1 protein increased significantly in human retinal endothelial cell line stimulated with TNF-α and treated with Ets-1-targeted siRNA. The human retinal endothelial cell line was transfected with Ets-1 siRNA (1), Ets-1 siRNA (2), Ets-1 siRNA (1) and (2), or non-targeted negative control siRNA for 24 hours, followed by 24-hour stimulation with TNF-α or fresh medium alone. Graph shows monolayer fluorescence expression for treatment with control siRNA (blue), Ets-1 siRNA (1) (red), Ets-1 siRNA (2) (green), and Ets-1 siRNA (1) and (2) (purple) (n = 4 cell monolayers/condition). Bars represent mean fluorescence units and error bars indicate standard deviation. ND = not detectable. Data were analyzed using a one-way ANOVA, with Holm-Sidak post-hoc testing. Only significant differences are shown. ****$p < 0.0001$.

increase in ICAM-1 protein expression in Ets-1 siRNA (2)-transfected human retinal endothelial cells compared to negative control siRNA in the IL-1β-treated group (**Figure 5.7**, p = 0.0043). Also, ICAM-1 protein expression increased significantly in cells transfected with the combination of Ets-1 siRNA (1) and (2), compared to those transfected with negative control siRNA (**Figure 5.7**, p = 0.0015). In summary, for one or both targeted siRNA, Ets-1 knock-down augmented the expression of membrane-bound ICAM-1 in human retinal endothelial cells that were stimulated with TNF-α or IL-1β. However, knock-down did not change the level of the protein in cells that were not exposed to the inflammatory cytokines.

5.3 Discussion

The aim of the work presented in this chapter was to validate Ets-1 as a suitable TF candidate for targeting in human retinal endothelial cells. Given the results of the *in silico* analysis of RNA-seq data from human retinal endothelial cells treated with inflammatory cytokines, coupled with evidence in the literature that *Ets1* is involved in *ICAM1* gene regulation, this TF seemed to be a promising therapeutic target for non-infectious posterior uveitis. The hypothesis was that Ets-1 acted as a transcriptional activator of the *ICAM1* gene, and by silencing it, ICAM-1 expression in human retinal endothelial cells would be reduced. Thus, a series of experiments was conducted to understand the effect of silencing Ets-1 on ICAM-1 transcript and protein expression in human retinal endothelial cells. First, expression of Ets-1 transcript in human retinal endothelial cell line under basal and inflammatory conditions, induced with either TNF-α or IL-1β, was confirmed. A significant increase in Ets-1 transcript in primary human retinal endothelial cells was observed following stimulation with TNF-α or IL-1β. Utilising single or a combination of siRNAs to silence Ets-1, a significant reduction, on average 94.2%, in Ets-1 transcript levels was achieved.

Following Ets-1 silencing with one siRNA (Ets-1 siRNA (2), representing Thermo Fisher Scientific Ets-1 silencer select siRNA s4848), there was a consistent significant increase in ICAM-1 transcript in human retinal endothelial cells under TNF-α or IL-1β-stimulated, and unstimulated conditions,

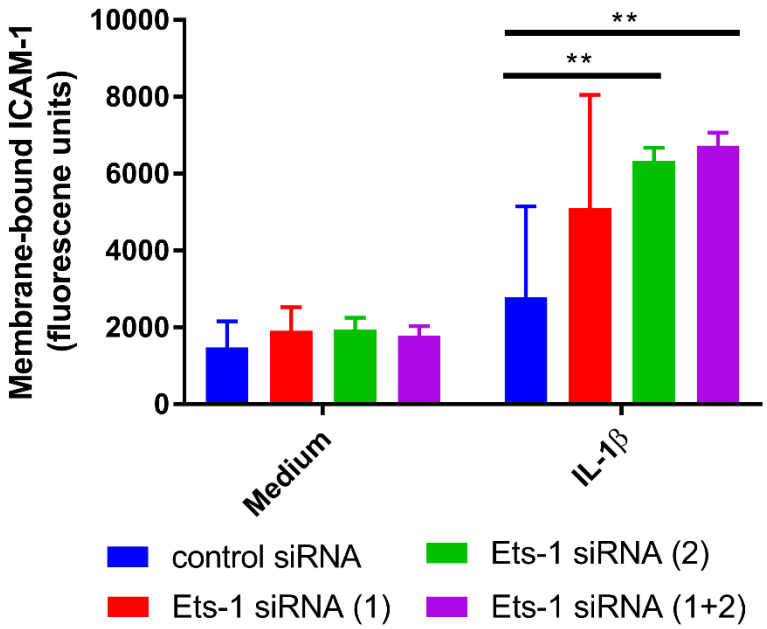

Figure 5.7 Expression of the membrane-bound ICAM-1 protein increased significantly in human retinal endothelial cell line stimulated with IL-1β and treated with Ets-1-targeted siRNA. The human retinal endothelial cell line was transfected with Ets-1 siRNA (1), Ets-1 siRNA (2), Ets-1 siRNA (1) and (2), or non-targeted negative control siRNA for 24 hours, followed by 24-hour stimulation with IL-1β or fresh medium alone. Graph shows monolayer fluorescence expression for treatment with control siRNA (blue), Ets-1 siRNA (1) (red), Ets-1 siRNA (2) (green), and Ets-1 siRNA (1) and (2) (purple) (n = 4 cell monolayers/condition). Bars represent mean fluorescence units and error bars indicate standard deviation. Data were analyzed using a one-way ANOVA, with Holm-Sidak post-hoc testing. Only significant differences are shown. **$p < 0.01$.

compared with control siRNA treatment. Another Ets-1 siRNA (Ets-1 siRNA (1), representing Thermo Fisher Scientific Ets-1 silencer select siRNA s4847) and the combination of the two Ets-1 siRNA induced human retinal endothelial cells' ICAM-1 transcript under some conditions. Neither siRNA, alone or in combination, decreased *ICAM1* gene expression in human retinal endothelial cells. Looking at the newly discovered novel ICAM-1 transcript variant, only the Ets-1 siRNA (2) managed to show a significant increase in cell expression levels under control and cytokine stimulatory conditions in comparison to the control siRNA.

Whilst it was helpful to understand the effect of Ets-1 silencing on ICAM-1 transcript expression, to get the full picture of the effect of Ets-1 targeting, expression of ICAM-1 protein had to be investigated. Specifically, ICAM-1 protein on the cell surface was measured, since this form of ICAM-1 is involved in transendothelial migration of leucocytes. These studies showed that knocking down Ets-1 with a single or a combination of siRNA led to a significant increase in membrane-bound ICAM-1 in human retinal endothelial cells induced by TNF-α. In Ets-1-silenced human retinal endothelial cells treated with IL-1β, a significant increase in membrane-bound ICAM-1 was noted after transfection with Ets-1 siRNA (2), or Ets-1 siRNA (1) and (2) combined.

There was no significant difference in constitutively expressed ICAM-1 protein upon knocking down Ets-1 in human retinal endothelial cells. This suggests targeting Ets-1 during inflammation would not interfere with normal physiological functions in human retinal endothelial cells. The result is different from that obtained for the ICAM-1 transcripts, as described above, which may reflect technical considerations and/or biological factors. Technical issues could relate to various aspects of the different methodologies, as well as higher sensitivity of the RT-qPCR versus cellular immunoassay to measure changes in ICAM-1. Biological issues, such as feedback mechanisms that regulate ICAM-1 transcript versus protein half-lives, may also be at play.

Most importantly, these experiments disproved the hypothesis. Inhibiting Ets-1 with siRNA resulted in increased, not reduced, ICAM-1 expression in human retinal endothelial cells. To date, there are

only a handful of experiments exploring the association of Ets-1 regulation of ICAM-1 expression, and these studies showed Ets-1 to be a transcriptional activator (Zhou et al., 2018, Srivastava et al., 2020). While there are no previous reports of Ets-1 repressing *ICAM1* gene expression, Ets-1 can repress the activity of other genes. One of the scenarios highlighting Ets-1 acting as a repressor was the downregulation of IL-1β-induced *MUC5AC* gene expression in a human airway epithelial cell line (Song et al., 2012). Tsao and colleagues showed that Ets-1 can directly repress the transcription of the *PRDM1* gene, which encodes B lymphocyte-induced maturation protein 1 (Blimp1) in human Th1 cells (Tsao et al., 2013). In their study, following the knock-down of Ets-1, the level of Blimp1 transcript was significantly higher than baseline expression. A recent bioinformatic analysis by Saelee and colleagues, combining Ets-1 ChIP-seq binding site data and an RNA-seq dataset of gene expression in *Ets1*-knockout B-cells, predicted genes that were regulated by Ets-1 (Saelee et al., 2017). Of the 263 genes with promoters containing Ets-1 binding sites, 126 genes were in fact repressed by Ets-1. Notably, of the few Ets-1 target genes identified in the analysis, Ets-1 suppressed *Egr1* and *Prdm1* expression.

There are multiple isoforms of Ets-1, as summarised in the review by Garrett-Sinha (Garrett-Sinha, 2013). The inconsistency in ICAM-1 transcript expression observed upon knocking down Ets-1 with the two different siRNA may relate in part to certain Ets-1 isoforms playing more dominant roles in *ICAM1* gene regulation in human retinal endothelial cells. Both Ets-1 siRNA used in the study are targeted to knock-down the transcript encoding the major Ets-1 isoform, p54, as well as p42. However, the Ets-1 siRNA (1) also targets p27, but not p68; and Ets-1 siRNA (2) also targets p68, but not p27. The difference in p27 targeting may be quite important because p27 blocks p54 transcriptional activation and promotes cytoplasmic translocation of p54 (Laitem et al., 2009). While it is assumed that human retinal endothelial cells express all Ets-1 isoforms, it should be noted that the single primer set that was used in this work to measure Ets-1 expression in cell isolates could not distinguish variant transcripts (Zhou et al., 2018).

While ICAM-1 protein expression increases in human retinal endothelial cells following silencing of Ets-1 and TNF-α or IL-1β stimulation, a limitation to this work is that the Ets-1 protein expression was not evaluated. However, Ets-1 protein is expressed in endothelial cells, as has been described in multiple publications (Harris et al., 2010, Dejana et al., 2007, Kathuria et al., 2004). Thus, it is reasonable to infer that the increase in ICAM-1 protein expression following Ets-1 siRNA transfection is the result of reduced production of Ets-1 protein. Future studies might include running Western blots or performing enzyme-linked immunosorbent assays to confirm that Ets-1 protein is indeed made by human retinal endothelial cells and that its level of expression changes in response to targeted siRNA transfection, as well as inflammatory cytokine treatments. In addition, a ChIP study would be needed to confirm an interaction between Ets-1 protein and the *ICAM1* gene promoter, since the effect of Ets-1 knock-down on cellular ICAM-1 expression could be indirect.

In summary, this work demonstrates that, quite unexpectedly, Ets-1 blockade does not reduce ICAM-1 expression in human retinal endothelial cells. When Ets-1 was targeted with an siRNA that silences the p54, p42 and p68 Ets-1 isoforms, expression of full-length and novel ICAM-1 transcript variants, along with membrane-bound ICAM-1 protein, increased in human retinal endothelial cells treated with TNF-α or IL-1β, or maintained without stimulation. However, constitutive expression of ICAM-1 protein was not altered by this Ets-1 knock-down. In future studies, it would be interesting to determine the expression of individual Ets-1 isoforms in human retinal endothelial cells, and to study the effect of siRNA that specifically target each isoform in these cells.

CHAPTER 6

General Discussion and Conclusion

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CHAPTER 6

6.1 Discussion

Non-infectious posterior uveitis is an inflammatory eye disease that frequently results in vision loss because it involves the retina (de Smet et al., 2011). This research involved the manipulation of *ICAM1* gene transcription, which could be the basis of a new treatment for this disease. In non-infectious posterior uveitis, leucocytes migrate across the retinal endothelium into the posterior eye (Bharadwaj et al., 2017, Bharadwaj et al., 2013b, Crane and Liversidge, 2008). This process is regulated primarily by ICAM-1, a well-studied adhesion molecule (Rahman and Fazal, 2009) Expression of ICAM-1 is primarily controlled at the level of gene transcription (Pate et al., 2010). Targeting the induction of ICAM-1 could reduce inflammation, while avoiding side effects expected from direct blockade. The work included studies of ICAM-1 transcript and protein expression in human retinal endothelial cells following stimulation with inflammatory cytokines, TNF-α or IL-1β, as described in Chapter 3. The TF that was targeted to manipulate *ICAM1* gene transcription was determined by TF enrichment analysis of RNA-seq data generated using human retinal endothelial cells treated with these cytokines, as reported in Chapter 4. The selected TF, Ets-1, was silenced in cytokine-stimulated human retinal endothelial cells using siRNA, to determine its effect on *ICAM1* gene regulation, as described in Chapter 5.

Expression of ICAM-1 by the retinal endothelium is induced in response to inflammatory cytokines, TNF-α or IL-1β, that are expressed in the eye during non-infectious posterior uveitis (Bharadwaj et al., 2013a, Xie et al., 2014). This work confirmed the previous observation at both transcript and protein levels in multiple primary human retinal endothelial cell isolates. There are multiple TFs identified in the literature, such as NF-kB, STAT and Ets TF family members, that act to regulate ICAM-1 levels through binding to *ICAM1* gene promoter sites (Kesanakurti et al., 2013, Sung and Kim, 2013, Yu et al., 2015, Sperone et al., 2011, Colas-Algora et al., 2020). Tumour necrosis factor alpha is said to bring forth the activation of the PKC signaling pathway (Lee et al., 2015), which in

turn induces ICAM-1 expression. Interleukin-1 beta is known to regulate ICAM-1 expression and its activation is linked to the involvement of the p38-MAPK pathway (Yang et al., 2010).

An *in silico* predictive analysis identified Ets-1 as a potential TF target for manipulating *ICAM1* gene expression in human retinal endothelial cells. Of the three *in silico* methods, TF enrichment analysis using ChEA3 software was the most informative. By importing four sets of differential gene expression lists generated for human retinal endothelial cells treated with TNF-α or IL-1β at 60 minutes and 24 hours, respectively, four lists of TFs were produced. Ets-1 was selected out of the list of the top 50 TFs that potentially regulated ICAM-1 expression, observed across both TNF-α and IL-1β-stimulated human retinal endothelial cells, and based on literature surrounding the regulatory pathways for the *ICAM1* gene. Although the ChEA3 analysis identified many TFs that potentially regulated *ICAM1* gene transcription, one TF had to be prioritised for the subsequent TF silencing study. Other TFs that were included on the list belonged to the IRF family, including IRF1 and IRF8. Due to the limited understanding of IRF family of TFs and its involvement in regulating ICAM-1 according to the literature, these TFs would potentially serve as a good candidate for future studies.

Prior to this work, no studies had been conducted to determine the effect of silencing Ets-1 on ICAM-1 expression in human retinal endothelial cells. However, limited evidence from work in other cell types, including lung cancer cells and melanocytes, indicated Ets-1 could act as an activator of *ICAM1* gene transcription (Zhou et al., 2018, Srivastava et al., 2020). This led to the hypothesis that Ets-1 acted as a transcriptional activator of the *ICAM1* gene, and by silencing it, ICAM-1 expression in human retinal endothelial cells would be reduced. However, Ets-1 siRNA knock-down with two different siRNA did not reduce ICAM-1 transcript or protein in TNF-α-stimulated and IL-1β-stimulated human retinal endothelial cells. Instead, levels of ICAM-1 increased consistently after Ets-1 knock-down with one targeted siRNA, and in some situations with the other targeted siRNA or a combination of the two siRNAs. While this finding was unexpected, Ets-1 has been shown to act as a transcriptional repressor of other genes, such as human *BAX* (Nakazawa et al., 2007), *MUC5AC*

(Song et al., 2012), and mice *Egr1* and *Prdm1* (Saelee et al., 2017). Ets-1 has been identified as a master regulator of endothelial cell gene transcription (Chen et al., 2017), and it may be important to maintain baseline ICAM-1 expression. Ets-1 may be acting as a transcriptional repressor, but it is also possible that the effect of Ets-1 blockade on *ICAM1* gene expression is indirect. Overall, this study is the first to demonstrate Ets-1 acts to reduce *ICAM1* gene expression.

This research was conducted to evaluate knock-down of Ets-1 with the aim of reducing induced *ICAM1* gene transcription, as the basis for a new local treatment for non-infectious posterior uveitis. Recently, Hsing and colleagues provided a comprehensive review on the latest cancer treatment strategies targeting Ets family of TFs, using small molecules, siRNA and peptidomimetics (Hsing et al., 2020). All the strategies that were described were focused on inhibiting Ets expression. Furthermore, none of the strategies involving Ets-1 were centred on upregulating Ets-1 expression. The findings from this study were the complete opposite of the expected results: by knocking down Ets-1, ICAM-1 expression increased. Increased ICAM-1 expression would accelerate leucocyte transmigration to the retina, in turn worsening inflammation in the tissue. Thus, Ets-1 presents a poor drug target for uveitis treatment, although one might consider a therapeutic that increased Ets-1 expression.

One option would be delivering an Ets-1 activator to the posterior eye by intravitreal injection to reduce retinal endothelial cell ICAM-1 expression. A previous experimental study by Chen and colleagues demonstrated that Ets-1 expression can be upregulated in human umbilical vein endothelial cells using synthetic modified mRNA (Chen et al., 2017). However, a significant reservation is the link between Ets-1 and development of cancer. The Ets-1 protein encoded by the *ETS1* oncogene regulates the expression of angiogenic and extracellular matrix remodelling factors, namely VEGF, MMP9 and MMP2 (Dittmer, 2015). While studies have centred on understanding factors regulated by Ets-1 in the tumour environment, few studies have characterised the fundamental mechanisms of *ETS1* gene expression (Kim et al., 2018). However, it is clear that Ets-1 is able to auto-regulate its own transcriptional activity via the Ras/Raf/MEK/ERK1/2 pathway (Plotnik et al.,

2014, Selvaraj et al., 2015), Thus, Ets-1 expression promotes metastasis across many cancers including breast (Kim et al., 2018), lung (Phuchareon et al., 2015), prostate (Smith et al., 2012a) and colorectal carcinomas (Gu et al., 2019).

Although this research shows that Ets-1 targeting in human retinal endothelium is unlikely to have application as a uveitis treatment, it is worth noting that knock-down did not alter constitutive expression of membrane-bound ICAM-1. There was an effect on ICAM-1 transcript levels in some experiments, but ICAM-1 protein levels did not change. Not altering baseline protein expression is a potential benefit if blocking ICAM-1-mediated leucocyte migration by targeting gene transcription.

A significant finding in this work is the discovery of a novel ICAM-1 transcript. In comparison to the full-length transcript with a coding region of 1599 bp, the novel transcript has a coding sequence of 912 bp, lacking most of exon 2, all of exon 3 and a small portion of exon 4. Both the full-length and the novel ICAM-1 transcripts contain the same start and stop codon. This suggests both transcripts share the same promoter. Hence, there is a likelihood that the novel ICAM-1 transcript variant is formed via alternative splicing from the full-length transcript. There was a significant increase in expression of the novel ICAM-1 transcript in primary cell isolates of human retinal endothelial cells treated with IL-1β and TNF-α. Interestingly, alternative splicing may be promoted during inflammation as illustrated by the study conducted by Janssen and colleagues, which showed that alveolar macrophages use alternate pre-mRNA spliced variants of multiple genes during lung inflammation (Janssen et al., 2020). The Ig domain 1, encoded by exon 2, binds LFA-1. Given that the novel ICAM-1 transcript variant includes part of exon 2, and since expression increases following TNF-α and IL-1β stimulation, it is possible this transcript variant encodes a protein that plays a functional role in leucocyte migration.

From the literature, it is clear that ICAM-1 plays a major role in directing migration of leucocytes that express Mac-1 and LFA-1 integrins across the retinal endothelium (Lawson and Wolf, 2009, Lefort and Ley, 2012, Verma and Kelleher, 2014, Buffone et al., 2019). Although functional studies

of leucocyte interactions with human retinal endothelial cells were not carried out as part of this thesis, future studies considering other TF targets should include endothelial adhesion and transmigration assays with different leucocyte subsets involved in uveitis, including T-cells, B-cells and monocytes. There are different assays that could be used, such as a leucocyte-endothelial cell adhesion assay, the Bowden chamber transwell migration assay, and flow chamber assays of leucocyte-endothelial cell interactions. These *in vitro* methods provide analysis of leucocyte adhesion to the endothelium and the leucocyte invasion capability in an accurate and high reproducibility manner (Pijuan et al., 2019). However, *in vitro* manipulations may affect the phenotype of the cells (Justus et al., 2014, Pijuan et al., 2019), and *in vivo* studies conducted in the EAU model also would be valuable.

6.2 Conclusion

In summary, this research cemented the idea of targeting TFs to reduce ICAM-1 expression as a potential approach for the treatment of non-infectious posterior uveitis. In spite of the literature supporting the notion of Ets-1 being a transcriptional activator of *ICAM1* gene expression, this study demonstrated that ICAM-1 transcript and protein level increased in human retinal endothelial cells when Ets-1 was silenced with siRNA. These observations indicate that there would be little value in exploring Ets-1 as a drug target for uveitis treatment. However, the work showed that basal ICAM-1 protein expression was unchanged when Ets-1 was knocked down, which implies no effect on homeostatic leucocyte migration of manipulating the TF. The role of Ets-1 cannot be underestimated, in that it may act as a regulatory TF, preventing overexpression of ICAM-1 in the retinal endothelium. This research also identified a novel ICAM-1 transcript variant in human retinal endothelial cells, which may encode a functional ICAM-1 isoform that contributes to the regulation of leucocyte transendothelial migration in uveitis. Future studies that build on this exciting finding may increase understanding of the role of ICAM-1 in inflammation.

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APPENDICES

Appendix A: Novel ICAM-1 transcript coding sequence

The sequence below indicates the full coding region of novel ICAM-1 transcript which is 912 bp in size according to DNA sequencing of human retinal endothelial cells treated with TNF-α for 24 hours.

ATGGCTCCCAGCAGCCCCCGGCCCGCGCTGCCCGCACTCCTGGTCCTGCTCGGGGCTCT
GTTCCCAGGACCTGGCAATGACCAGAGGTTGAACCCCACAGTCACCTATGGCAACGAC
TCCTTCTCGGCCAAGGCCTCAGTCAGTGTGACCGCAGAGGACGAGGGCACCCAGCGGC
TGACGTGTGCAGTAATACTGGGGAACCAGAGCCAGGAGACACTGCAGACAGTGACCA
TCTACAGCTTTCCGGCGCCCAACGTGATTCTGACGAAGCCAGAGGTCTCAGAAGGGAC
CGAGGTGACAGTGAAGTGTGAGGCCCACCCTAGAGCCAAGGTGACGCTGAATGGGGTT
CCAGCCCAGCCACTGGGCCCGAGGGCCCAGCTCCTGCTGAAGGCCACCCCAGAGGACA
ACGGGCGCAGCTTCTCCTGCTCTGCAACCCTGGAGGTGGCCGGCCAGCTTATACACAA
GAACCAGACCCGGGAGCTTCGTGTCCTGTATGGCCCCCGACTGGACGAGAGGGATTGT
CCGGGAAACTGGACGTGGCCAGAAAATTCCCAGCAGACTCCAATGTGCCAGGCTTGGG
GGAACCCATTGCCCGAGCTCAAGTGTCTAAAGGATGGCACTTTCCCACTGCCCATCGG
GGAATCAGTGACTGTCACTCGAGATCTTGAGGGCACCTACCTCTGTCGGGCCAGGAGC
ACTCAAGGGGAGGTCACCCGCAAGGTGACCGTGAATGTGCTCTCCCCCCGGTATGAGA
TTGTCATCATCACTGTGGTAGCAGCCGCAGTCATAATGGGCACTGCAGGCCTCAGCAC
GTACCTCTATAACCGCCAGCGGAAGATCAAGAAATACAGACTACAACAGGCCCAAAA
AGGGACCCCCATGAAACCGAACACACAAGCCACGCCTCCCTGA

Appendix B: Novel ICAM-1 protein sequence

This protein sequence is generated using ExPASy translate tool (Gasteiger et al., 2003).

Met A P S S P R P A L P A L L V L L G A L F P G P G N D Q R L N P T V T Y G N D S F S A K A S V S V T A E D E G T Q R L T C A V I L G N Q S Q E T L Q T V T I Y S F P A P N V I L T K P E V S E G T E V T V K C E A H P R A K V T L N G V P A Q P L G P R A Q L L L K A T P E D N G R S F S C S A T L E V A G Q L I H K N Q T R E L R V L Y G P R L D E R D C P G N W T W P E N S Q Q T P Met C Q A W G N P L P E L K C L K D G T F P L P I G E S V T V T R D L E G T Y L C R A R S T Q G E V T R K V T V N V L S P R Y E I V I I T V V A A A V I Met G T A G L S T Y L Y N R Q R K I K K Y R L Q Q A Q K G T P Met K P N T Q A T P P **Stop**