

The Characterisation, Pharmacology and Applications of Stem Cell Derived Endothelial Cells

A thesis submitted to Imperial College London for the degree
of Doctor of Philosophy in Clinical Medicine Research

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Declaration

I, Daniel Mark Reed, hereby declare that I wrote this thesis and the work and experiments described herein, except where appropriately referenced, were performed by myself. Information derived from other sources and work done in collaboration with others has been appropriately cited and acknowledged.

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Dedication

I dedicate this thesis to my Nan, Joyce Osbourne. I will always remember her support and influence during my early education that in so many ways shaped my approach in all my academic pursuits.

I also dedicate this thesis to my Mum and Dad, Mark and Sue Reed, whose continuous love and boundless support has made this thesis possible. And of course to my brother Jason Reed, for his unyielding ability to keep all those around him well and truly, grounded.

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Abstract

Stem cell derived endothelial cells have a growing number of applications in clinical medicine and biomedical research and will be critical in any organ regeneration programme. Endothelial cells can now be derived from a number of distinct stem cell populations including embryonic stem cells, blood progenitors and induced pluripotent stem cells. In order for stem cell derived endothelial cells to be used optimally it is important that they are fully assessed for the cardinal characteristics of endothelial cells on mature vessels.

With this in mind, in my thesis I have assessed the ability of stem cell derived endothelial cells to display hallmarks of authentic endothelial cells from vessels including, alignment under shear stress, responses to pathogen stimuli and vasoactive hormone release. My group has previously shown that endothelial cells from human embryonic stem cells did not respond to agonists of toll-like receptor-4 (TLR4). TLR4 is important to allow endothelial cells to sense infection, but is also associated with cardiovascular disease. In my PhD, I showed that, whilst these cells have no TLR4 they have a fully functional NOD1 receptor pathway which allows the cells to sense Gram-negative bacteria. This may be relevant to their use therapeutically and allowed me to speculate that these cells are immune competent, but through lack of TLR4 might be protected from vascular inflammation. In order for stem cell derived endothelial cells to be useful in *in vitro* assays however, it is important that they express all the key hallmarks of endothelial cells from vessels. Endothelial cells grown from blood progenitors or from induced pluripotent stem cells did have TLR4 responses and released vasoactive hormones at levels comparable to endothelial cells from vessels.

In the later part of my PhD, I applied endothelial cells derived from blood progenitor cells to key assays to study endothelial cell biology and pharmacology. This included an assay to detect cytokine storm reactions to drugs, which currently limits the development of biological drugs. This assay, employing cells derived from stem cells of individual patients also has applications in personalised medicine. In my final chapter, I was able to grow BOEC from a patient with a homozygous mutation that results in loss of function of the enzyme cPLA₂, which is thought to be critical to prostaglandin and prostacyclin release, and used these cells to study this pathway. I showed that cPLA_{2α} is the dominant isoform responsible

for the release of prostacyclin from endothelial cells and provided a proof-of-concept that BOEC can be used to phenotype patients.

In summary, my thesis includes characterisation of stem cell derived endothelial cells, and includes applications of adult progenitor derived cells in assays to study pharmacology and cell biology with a view to developing personalised medicines and cell therapies.

Publications

First author papers:

Reed DM, Foldes G, Gatheral T, Paschalaki KE, Lendvai Z, Bagyura Z, Nemeth T, Skopal J, Merkely B, Telcian AG, Gogsadze L, Edwards MR, Gough PJ, Bertin J Johnston SL, Harding SE, Mitchell JA (April 2014) *Pathogen sensing pathways in human embryonic stem cell derived-endothelial cells: Role of NOD1 receptors*. Plos One. April 1 2014 9 (4): e91119

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Co-author papers:

George PM, Oliver E, Dorfmüller P, Dubois OD, **Reed DM**, Kirkby NS, Mohamed N, Perros F, Antigny F, Fadel E, Schreiber B, Holmes AM, Southwood M, Hagan G, Wort SJ, Bartlett N, Morrell NW, Coghlan JG, Humbert M, Zhao L, Mitchell JA (Dec 2013). *Evidence for the involvement of Type I interferon in pulmonary arterial hypertension*. Circ Res. 2014 Feb 14;114(4):677-88

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Abstracts

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List of Abbreviations

***for ease of reading abbreviations are written out in full on first use in each chapter**

6-ketoPGF _{1α}	6-keto prostaglandin F _{1α}
AA	Arachidonic acid
ANOVA	Analysis of variance
ATCC	American type tissue culture collection
BM	Bone marrow
BOEC	Blood outgrowth endothelial cells
C12-iE-DAP	Lauroyl-γ-D-glutamyl-meso- diaminopimelyl-D-alanine
CARD	Caspase recruitment domain
COPD	Chronic obstructive pulmonary disease
COX-1/2	Cyclooxygenase-1/2
CRS	Cytokine release syndrome
DAMP	Danger associated molecular pattern
DAP	Diamino-pimelate
DAPI	4',6'-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EBM	Endothelial basal medium
EET	Epoxyeicoastienoic acids
EGM-2	Endothelial growth medium-2
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
ERK	Extracellular signal-related kinases
ET-1	Endothelin-1
FBS	Foetal bovine serum
FCS	Foetal calf serum
HAEC	Human aortic endothelial cells
hESC	Human embryonic stem cells

hESC-EC	Human embryonic stem cell derived endothelial cells
HETE	Hydroxyeicosatetraenoic acids
HLF	Human lung fibroblast
HMVEC	Human lung microvascular endothelial cells
HPASM	Human pulmonary artery smooth muscle cells
HUVEC	Human umbilical vein endothelial cells
iE-DAP	γ -D-glutamyl-meso-diaminopimelyl-D-alanine
IRF	interferon regulatory factor
IFN	Interferon
IKK	I κ B kinase
IL-1	Interleukin -1
iNOS	Inducible nitric oxide synthase
IP3	Inositol trisphosphate
iPSC	Induced pluripotent stem cell
iPSC-EC	Induced pluripotent stem cell derived endothelial cells
IRAK	Interleukin-1 receptor associated kinase
JNK	C-jun N-terminal kinase
LC-MS/MS	Liquid chromatography mass spectrometry/mass spectrometry
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAL	MyD88 adaptor like
MAPK	Mitogen activated protein kinase
MDP	Muramyl Di-peptide
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response gene (88)
NF- κ B	Nuclear factor kappa B
NLR	NOD like receptor
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOD1	Nucleotide oligomerisation domain 1
OD	Optical density

OEC	Outgrowth endothelial cells
PAH	Pulmonary arterial hypertension
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PG	Prostaglandin
PGI ₂	Prostacyclin
PIP3	Phosphatidylinositol (3, 4, 5)-trisphosphate
PLA ₂	Phospholipase A ₂
PRR	Pattern recognition receptor
RIP	Receptor interacting protein
RPMI	Roswell Park Memorial Institute Medium
siRNA	Small interfering RNA
TAB	TAK1-binding protein
TAK1	Transforming growth factor β activated kinase 1
TBK	TANK-binding kinase
TGF β	Tumour growth factor-beta
TIR	Toll/Interleukin-receptor
TLR	Toll like receptor
TNF α	Tumour necrosis factor alpha
TRAM	TRIF related adaptor molecule
TRIF	TIR-containing adaptor protein inducing interferon- β
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

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CHAPTER 1: INTRODUCTION

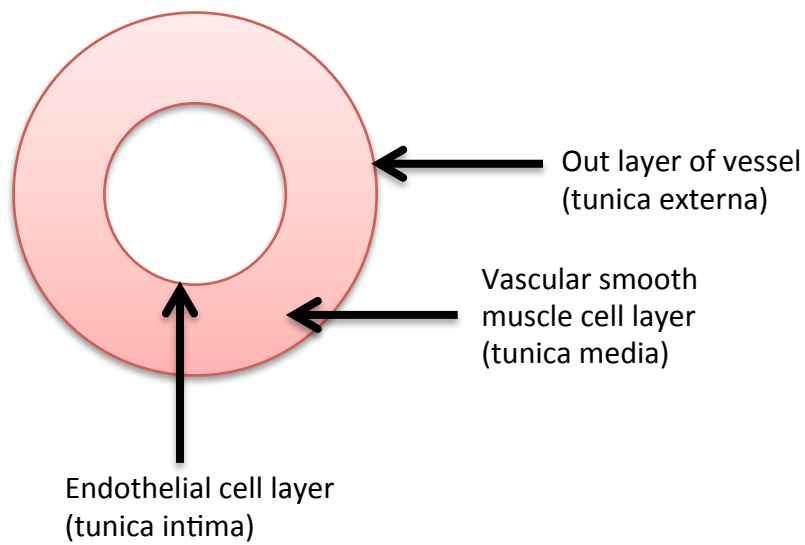
1.1 OVERVIEW

Endothelial cells line the surface of every vessel in the body. Through the release of vasoactive mediators and other important functions, including sensing of pathogens, endothelial cells are important for the health of almost every organ in the body. In line with this, endothelial cells derived from stem cells have been considered as therapies and tools for cardiovascular research, and will be important to integrate organs engineered from stem cells with host vasculature. In this introduction, I describe these key endothelial cell functions in detail and develop the idea of stem cell derived endothelial cells as therapies and as *in vitro* tools in pharmacological bioassays to study cardiovascular pathways and develop and test new therapies.

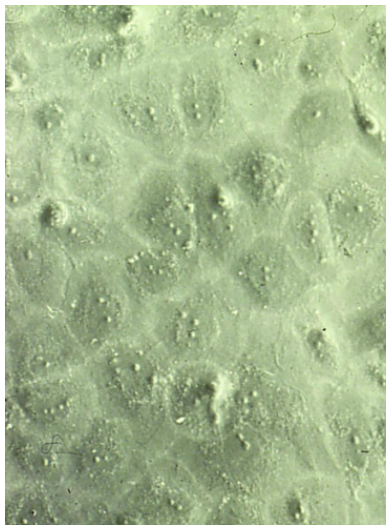
1.2 ENDOTHELIAL CELL FUNCTION

Endothelial cells are critical regulators of the cardiovascular system. Endothelial cells, and indeed the term endothelium, were first introduced in the 1800s. Lord Florey described endothelial cells as ‘a sheet of nucleated cellophane’ wrap for the blood vessel (Florey, 1966). This view changed dramatically in 1973 with the isolation of endothelial cells from human umbilical vein, which formed the basis of two seminal research papers by Jaffe and co-workers (Jaffe et al., 1973a; Jaffe et al., 1973b). The anatomical location of the endothelial cell layer in vessels as well as the appearance of these cells in culture is shown in Figure 1.1. The ensuing study of endothelial cells from human umbilical vein (Nachman, 2012) as well as other sources has meant that we now know endothelial cells represent a ubiquitous endocrine organ, essential for cardiovascular homeostasis.

A



B



Cobblestone
morphology of human
endothelial cells

Figure 1.1 (A) Cross section of structure of human arteriole. **(B)** Cobblestone morphology of human umbilical vein endothelial cells (HUVEC) in culture. Image from Nachman and Jaffe 2004 under licence from the Copyright Clearance Center (Nachman and Jaffe, 2004)

1.1.1 Barrier function

One of the most important functions of endothelial cells is to provide a restrictive semi-permeable physical barrier between the blood and the vessel wall. Endothelial cells fulfil this function by forming a universal monolayer that covers the luminal surface of the entire vascular tree. Connections between endothelial cells are strengthened with tight junctions between cells. Proteins involved in the formation of these junctions include vascular endothelial-cadherin (VE-cadherin or CD144), which can also be used as a marker of endothelial cell membranes. Whilst providing a barrier, the endothelium must also permit the transport of molecules, and cells from the luminal to the abluminal side of the vessel wall. Molecules and cells can pass between (paracellular permeability) cells and selected molecules can also pass through (intracellular permeability) cells.

Paracellular permeability of the endothelial monolayer can be increased or decreased depending on external stimuli (Komarova and Malik, 2010; Konstantoulaki et al., 2003). The leakiness of the endothelial cell monolayer to molecules and cells in this regard is regulated in two ways: (i) by phosphorylation of cadherin proteins which result in internalisation and (ii) by actin rearrangement which break apart the junctions between the cells. These two pathways can be activated by a range of stimuli including VEGF, thrombin and histamine which typically increase intracellular Ca^{2+} and activate protein kinase C. Transcellular permeability allows the transport of selected macromolecules through cells. This energy dependent process requires trafficking of vesicles called caveolae. Caveolae are cholesterol rich pits present both on the endothelial cell surface and as free vesicles in the cytoplasm. The assembly of these vesicles, and subsequent transport, is regulated by the structural protein caveolin-1. Both paracellular and intracellular pathways are important to endothelial

cell barrier function and are thought to cross-talk to regulate over barrier function in health and disease (Komarova and Malik, 2010).

1.1.2 Endothelial cells and shear stress

In vessels, endothelial cells exist in a dynamic environment that includes not only interactions with cells, but also physical forces. These include cyclic stretch, due to pulsatile nature of blood flow, and shear stress, which is the frictional force of the blood flow on the endothelial cell surface. The degree and direction of shear stress that endothelial cells are exposed to is thought to have profound effects on both morphology, function, sensitivity to inflammation and the development of atherosclerosis (Dai et al., 2004; Wasserman and Topper, 2004). This phenomenon is well illustrated in the endothelium of the aortic arch. Cells lining the greater curvature of the arch are exposed to directional, also referred to as laminar, shear stress are elongated and aligned in the direction of the blood flow. Endothelial cells in regions of directional shear stress are protected from inflammation and atherosclerosis. By contrast, endothelial cells lining the lesser curvature of the aortic arch are exposed to non-directional, also referred to as oscillatory or turbulent, shear stress are of a cobblestone appearance and are primed for inflammation (Hajra et al., 2000) and atherosclerosis (Cheng et al., 2006; Dai et al., 2004; Dai et al., 2007). This phenomenon can be modelled *in vitro* using a number of approaches (Gimbrone et al., 2000). Our group has adopted a simple approach where complex shear stress patterns can be applied easily to endothelial cell cells in regular plastic culture plates placed on an orbital shaker (Potter et al., 2011). The different phenotypes of endothelial cells that occur at different regions of the aortic arch are illustrated in Figure 1.2

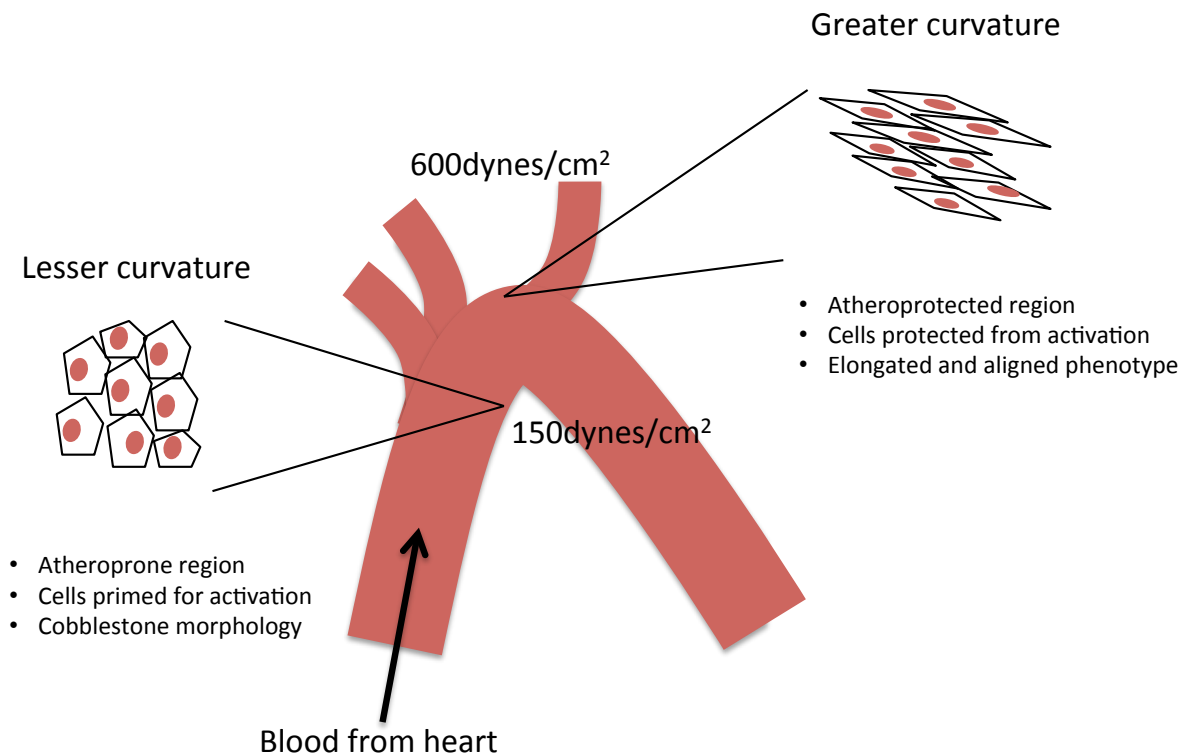


Figure 1.2 Schematic representation of endothelial cell morphology and phenotype in the greater curvature and lesser curvature of the aortic arch.

1.1.3 Endothelial cells and vasoactive hormones

In addition to a physical barrier endothelial cells also form a metabolic barrier. In this regard endothelial cells have the capacity to metabolise and convert circulating hormones, such as angiotensin and bradykinin via angiotensin converting enzyme. Endothelial cells also release three key vasoactive hormones, which act to regulate vascular tone and platelet reactivity. These are (i) prostacyclin (Moncada et al., 1976) (ii) nitric oxide (Palmer et al., 1987) and (iii) endothelin (ET)-1 (Yanagisawa et al., 1988). During the course of my PhD thesis I have used each of these hormones or their synthetic enzymes as markers of endothelial phenotype. I

have therefore included more detail below on the background of NO, prostacyclin and ET-1 in the vasculature.

1.1.4 Prostacyclin

Receptor pathways utilised by prostacyclin

Prostacyclin belongs to a family of mediators known as the prostaglandins, which have a range of effects on human physiology. It was pioneering work by Sir John R Vane's group that identified prostacyclin as a molecule secreted by endothelial cells (Moncada et al., 1976). Prostacyclin has a myriad of protective effects on the cardiovascular system including; vasodilation, inhibition of platelet aggregation and inhibition of vascular smooth muscle proliferation. Prostacyclin mediates these actions via ligating two receptors; IP receptors which are expressed on the cell surface and peroxisome proliferator activated receptor- β (PPAR β) which is a cytosolic receptor (Ali et al., 2006; Mitchell et al., 2008). IP receptors belong the seven transmembrane domain receptor family of G-protein coupled receptors (GPCRs) and are expressed on vascular smooth muscle and platelets (Stitham et al., 2007) (Figure 1.3). Activation of IP receptors, which are coupled to G_s, results in activation of adenylate cyclase and thus a rise in intracellular levels of the second messenger, cyclic adenosine monophosphate (cAMP) which leads to activation of protein kinase A. Activated protein kinase A phosphorylates Ca²⁺ channels leading to rapid reduction in intracellular Ca²⁺ concentration in vascular smooth muscle cells. PPAR β is a nuclear receptor that mediates the effects of prostacyclin, along with other biologically active lipids, in a least three ways. Firstly activation of PPAR β releases bound and repressed BCL6. Once released BCL6 is free to act as a transcriptional repressor of genes encoding inflammatory

proteins including MCP-1 (Lee et al., 2003). Secondly PPAR β acts to regulate the transcription of target genes, including CD36 and AGNP4 via binding to specific response elements. Finally, PPAR β can act as an repressor of PKC α through a direct interaction (Ali et al., 2009).

Synthesis of prostacyclin

Prostacyclin synthesis is mediated by the concerted actions of three enzymes. Firstly phospholipase A₂ (PLA₂) liberates the substrate, arachidonic acid, from membrane phospholipids. Due to the potent biological actions of mediators derived from arachidonic acid levels are actively kept low within the cell and once PLA₂ activity has declined arachidonic acid is rapidly re-acetylated back to the plasma membrane. Secondly, cyclooxygenase (COX) metabolises arachidonic acid in a two-step process, first to PGG₂ and then PGH₂. Finally PGH₂ is metabolised by prostacyclin synthase (PGIS) to prostacyclin. As part of my thesis, I have had the opportunity to grow endothelial cells from blood progenitors from a unique patient with a homozygous polymorphism in the gene that regulates cPLA₂ (cPLA_{2 α}). In light of this I have introduced PLA₂ in some additional detail below.

Phospholipase A₂ (PLA₂)

PLA₂ was first identified in the 1800s as a component of snake venom (Stephens et al., 1898) and later identified as an enzyme present in mammalian fluids (Seilhamer et al., 1989). We now know that there are numerous individual isoforms of PLA₂ that are categorised into (i) cytosolic PLA₂ (cPLA₂), (ii) calcium-independent PLA₂ (iPLA₂) and (iii) secreted PLA₂ (sPLA₂). sPLA₂ and cPLA₂ are calcium dependent enzymes. The concentration of calcium required for

activation of sPLA₂ often depends on the substrate phospholipid (Lambeau and Gelb, 2008) whereas cPLA₂ can be activated by low levels of Ca²⁺ in cells and is sensitive to short-duration transient changes in intracellular Ca²⁺ (Evans et al., 2001; Leslie, 1997). The classification of PLA₂ enzymes is complex and has recently been revised giving 11 groups in total based on protein structure and covering plants and animals (Six and Dennis, 2000).

Group II, V, X (sPLA₂), Group IV (cPLA₂) and Group VI (iPLA₂) are the most relevant in humans. As mentioned above cPLA₂ is activated by increases in cytosolic calcium. As such it is thought that this isoform is responsible for arachidonic acid release and subsequent eicosanoid biosynthesis in response to a range of agonists that cause increases in intracellular Ca²⁺ (Leslie, 1997). In line with this recent work from our group using blood from a patient with a rare condition, cryptogenic multifocal ulcerating stenosing enteritis, and a homozygous mutation in the PLA2G4A gene which results in a complete loss of cPLA_{2α} activity, demonstrated that this isoform is responsible for prostaglandin release from platelets stimulated with collagen (Brooke et al., 2012). In the course of my PhD thesis I have grown endothelial cells from progenitors in this patients' blood and used them to investigate the role of cPLA_{2α} in prostacyclin release by endothelial cells (see Chapter 6). This was particularly important to do since others have suggested that the calcium independent form of PLA₂ (iPLA₂) mediates prostacyclin release from endothelial cells stimulated with thrombin (Sharma et al., 2011). A fuller description of the clinical manifestations and the genetic profiling data that defines this patient and their relatives is shown in the methods section of Chapter 6.

Cyclooxygenase (COX)

The conversion of arachidonic acid to PGH_2 occurs as a two-step process; the first step occurs via formation of an unstable cyclic endoperoxide, PGG_2 . This is then reduced to PGH_2 . PGH_2 is then converted by various isomerase and synthase, enzymes to a range of prostanoids. This process is catalysed by the enzyme COX. It is the expression of particular isomerase and synthase enzymes which then determines which specific prostanoids a cell will produce. In the case of endothelial cells, prostacyclin synthase (PGIS) is highly expressed resulting in prostacyclin being the predominant COX product released by endothelial cells.

Two distinct isoforms of COX exist and these arise from separate genes. COX-1, which is constitutively expressed, and COX-2, which is inducible under inflammatory conditions (Kujubu et al., 1991; Xie et al., 1991). Since COX-2 is induced in inflammation it is the therapeutic target for common anti-inflammatory medications of the non-steroidal anti-inflammatory drugs (NSAIDs) class. These include ibuprofen and diclofenac. However, these drugs also inhibit COX-1 resulting in increased risk of gastrointestinal side effects (Wallace et al., 1998). COX-2 selective drugs such as celecoxib and rofecoxib were introduced to spare COX-1 and therefore have reduced gastrointestinal side effects (Mitchell et al., 1993). However, inhibition of COX-2 by selective drugs such as celecoxib and rofecoxib or traditional anti-inflammatory drugs such as diclofenac and ibuprofen has been associated with increased risk of cardiovascular events. The mechanism explaining these increased cardiovascular events is not clear although it has been suggested by some that COX-2 is the principle isoform that drives prostacyclin release in the vasculature and that its inhibition removes a cardioprotective brake (Funk and FitzGerald, 2007; Yu et al., 2012). However, direct evidence to support this is difficult to find. Instead where directly investigated COX-1

appears to be the principal isoform that drives prostacyclin release *in vitro* and *in vivo* (Kirkby et al., 2012). COX-2 is also expressed in the kidney (Harris, 2006) where its inhibition leads to increases in blood pressure (Krum et al., 2006). Effects of COX-2 inhibitors on the kidney may therefore also explain the increased cardiovascular events associated with anti-inflammatory drugs.

1.1.5 Nitric Oxide (NO)

NO and prostacyclin are co-released by endothelial cells, and in partnership, regulate vascular tone, platelet function and VSMC dilation and proliferation. NO release by endothelial cells is regulated by NO synthase (NOS) (Pollock et al., 1991) of which three isoforms exist; NOSI, expressed in neurons, and also referred to as nNOS; NOSII, expressed under inflammatory conditions and also known as iNOS; and NOSIII, expressed in endothelial cells, also referred to as eNOS. eNOS and nNOS are both calcium/calmodulin dependent isoforms whilst iNOS activity is calcium independent due to calcium activated calmodulin being bound to it shortly after translation. eNOS is a membrane bound isoform thought to be associated with caveolin in lipid rafts whilst nNOS and iNOS are cytosolic isoforms.

The key sensor pathway in cells of NO is guanylate cyclase (Hanafy et al., 2001). Guanylate cyclase catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) which acting as a classical second messenger, activates protein kinase G. Activated protein kinase G, as with protein kinase A, phosphorylates calcium

channels leading to rapid reduction in intracellular calcium levels which leads to an overall reduction in intracellular Ca^{2+} and vasodilation.

1.1.6 Endothelin-1 (ET-1)

As a constrictor, ET-1 release by the endothelium is in balance with prostacyclin and NO to maintain cardiovascular homeostasis. Even prior to the full identification and isolation of ET-1 it was known that endothelial cells release a vasoconstrictor molecule (De Mey et al., 1982). Then in 1988 the sequence of a potent vasoconstrictor peptide termed ET-1 was published (Yanagisawa et al., 1988). ET-1 is a 21 amino acid peptide released primarily by endothelial cells, although other cell types can make it, particularly under inflammatory conditions (Lüscher and Barton, 2000). ET-1 is the dominant cardiovascular isoform. ET-1 is generated from immature precursors via a two-step proteolytic pathway. The ET-1 gene liberates prepro-ET-1 mRNA which encodes a 212 amino acid peptide. Once transcribed the immature protein is secreted in to the cytoplasm as pro-ET-1 (Inoue et al., 1989) where it undergoes proteolytic cleavage by a furin-like endopeptidase to liberate BIG-ET-1. The final, and rate limiting step involves cleavage of BIG-ET-1 by endothelin converting enzymes (ECE) to liberate ET-1.

The vasoactive hormones pathways discussed above are illustrated in Figure 1.3

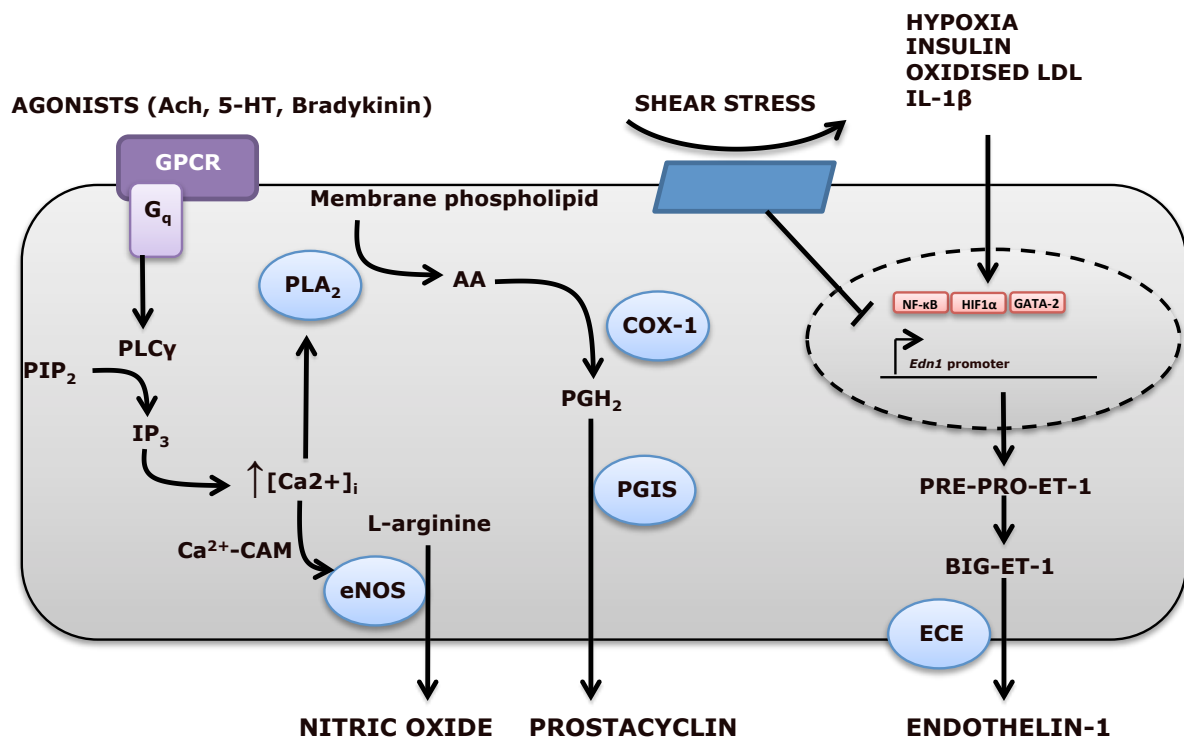


Figure 1.3. Summary of endothelial hormone pathways. Endothelial cells release nitric oxide, prostacyclin and endothelin-1, which are regulated by signalling pathways (including GATA-3, HIF1 α and NF- κ B mediated gene transcription) and the concerted actions of key enzymes. Abbreviations: G-protein coupled receptors (GPCRs), acetylcholine (Ach), phospholipase-Cy (PLC γ), Phosphatidylinositol 4,5-bisphosphate (PIP $_2$), inositol 1,4,5-trisphosphate (IP $_3$), diacylglycerol (DAG), cytosolic Ca $^{2+}$ -dependent PLA $_2$ (cPLA $_2$), cyclooxygenase (COX), arachidonic acid (AA), prostacyclin synthase (PGIS), Ca $^{2+}$ -calmodulin (Ca $^{2+}$ -CAM), endothelial nitric oxide synthase (eNOS), endothelin converting enzyme (ECE), endothelin-1 (ET-1), hypoxia inducible factor (HIF).

1.1.7 Endothelial cells and innate immunity

Another important function of endothelial cells is their critical role in innate immunity. Endothelial cells are the interface between pathogens and circulating leukocytes. Thus, endothelial cells need to be able to sense pathogens and in response to this release chemokines and express adhesion receptors to recruit leukocytes to the site of infection. The sensing of pathogens is mediated by a group of receptors known as 'pattern recognition

receptors' (PRRs) that respond to unique molecular motifs in pathogens. The best studied of these are the Toll like receptors (TLRs) and the nucleotide oligomerisation domain (NOD) receptors, many of which are expressed and functional on endothelial cells (Opitz et al., 2009). In my thesis I have studied the pharmacology of TLRs and NOD receptors in endothelial cells derived from stem cells and their role in infection with live bacteria. In this section of the introduction I will therefore include some general details of PRRs and their signalling pathways.

1.3 INNATE IMMUNITY AND PATTERN RECOGNITION RECEPTORS (PRR)

Until recently it was thought that the initial response of tissues, so called innate immune responses, was non-specific and of little importance in human immunity. Prior to pioneering work by Charles Janeway, explaining the requirement for co-stimulation, it was thought the adaptive immune system was wholly sufficient for host defense (Janeway, 1989). Janeway proposed that mobilisation of adaptive immunity in fact relied on the activation of an innate, evolutionary preserved family of receptors that could sense pathogens and orchestrate adaptive immune pathways. Crucially, it was shown that T-cells could not respond to a specific antigen without co-stimulation with lipopolysaccharide (LPS), which represents a small molecular motif found in Gram-negative bacterial cell membranes (Liu and Janeway, 1991). LPS and other molecular motifs unique to pathogens are referred to as pathogen associated molecular patterns (PAMPs).

Work subsequently began on the receptors responsible for the recognition of PAMPs; so-called pattern recognition receptors (PRR). The first work on PRRs was done on immune responses in the fruit fly *Drosophila melanogaster*. In 1996, Jules Hoffmann demonstrated for the first time that the immune response of the *Drosophila* fly to fungal infection was obliterated in the presence of loss of function mutations in a gene called Toll (Lemaitre et al., 1996). The toll gene had been characterised previously as essential in dorsal-ventral patterning (Anderson et al., 1985; Nüsslein-Volhard et al., 1980) and was known to have encoded a receptor with an intracellular domain similar to human interleukin (IL)-1 receptors (Gay and Keith, 1991). At the same time, a human homologue of the Toll receptor found in *Drosophila* was cloned. This receptor contained a leucine rich receptor (LRR) and an intracellular domain similar to Toll and IL-1 receptors, now termed Toll/IL-R (TIR) domains

(Medzhitov et al., 1997). This TIR domain was subsequently shown to activate the transcription factor NF- κ B and so regulate inflammatory genes, and that this activation provided the co-stimulatory signal for T-cell activation (Medzhitov et al., 1997). The significance therefore of innate immune pathways in human immunity was thus established. The family of human Toll receptor homologues were named Toll-like receptors. In parallel to work in human systems two groups working independently showed that TLR4 in mice was the critical receptor that mediates the effects of LPS (Kawai et al., 1999; Poltorak et al., 1998). In light of this pioneering work, Bruce Beutler and Jules Hoffmann were awarded the Nobel Prize in 2011 for their observation that TLR4 is the LPS receptor.

1.3.1 Toll-like receptors

After the discovery of the LPS receptor, TLR4, a number of additional receptors with shared sequence homology, and their agonists, were subsequently identified and characterised. To date, 10 functional TLRs have been identified with PAMPs identified for 9 of them. Selective TLR expression and activation enables cells to respond to an array of PAMPs on or within bacteria, viruses and fungi. Agonists of PRRs and their specific receptors are listed in Chapter 2 in Table 2.3

TLRs are type I membrane bound receptors, expressed either on the cell membrane (TLR1, TLR2, TLR4, TLR5, TLR6) or the endoplasmic reticulum membrane (TLR3, TLR7, TLR8, TLR9) (Akira and Takeda, 2004). A summary of TLR signalling is shown in Figure 1.4. TLRs expressed on the cell surface are typically responsive to bacteria and PAMPs on, or contained within, bacteria, whilst endosomal TLRs respond to nucleic acids in invading viruses. The function of TLR10 in humans is unknown. TLRs are activated by ligands as either homo- or hetero

dimers. TLR6 forms heterodimers with TLR2 and TLR1 whilst TLR3, 4 and 5 form homodimers. TLR8 can also form dimers with TLR7 and TLR9.

TLRs signal via recruitment of the adapter proteins MyD88 (with or without the adaptor protein Mal) and/or TIR-domain-containing adapter-inducing interferon- β (TRIF) (with or without the adapter protein TRAM) that results in downstream activation transcription factors associated with innate immunity and inflammation. For TLRs, MyD88 and TRIF are the two main adapter protein pathways and are described in more detail below.

MyD88 signalling

MyD88 was first identified as a differentiating factor in myeloid cells. In 1999 it was demonstrated in MyD88 (-/-) mice that responses to ligands of TLR2, 4, 5, 7 and 9 were completely abrogated (Kawai et al., 1999). Activation of MyD88 leads to activation of a complex of IL-1 receptor associated proteins (IRAK) and activation of tumour necrosis factor (TNF) receptor associated factor-6 (TRAF-6) (Cao et al., 1996). The interaction between TLRs and MyD88 occurs via a TIR to TIR domain interaction and between MyD88 and IRAK via a DD-domain interaction. MyD88 therefore provides a critical link between TLRs and their associated downstream pathways. Another adapter protein, MAL (or TIRAP) acts a bridging protein to anchor MyD88 to TIR domains on TLR2 and TLR4 since in mice deficient in MAL responses to TLR2 activation is absent, and to TLR4 activation delayed (Fitzgerald et al., 2001). TRAF-6, through association with TGF- β -activated kinases (TAK) and a series of TAK-binding proteins (TAB), provides a signal for phosphorylation of inhibitor of NF- κ B kinases (IKKs) (Kanayama et al., 2004). IKKs in turn phosphorylate the NF- κ B inhibitor, I κ B, resulting in its degradation. NF- κ B can then translocate to the nucleus and activate target genes. IKKs

can also activate NF- κ B independently of I κ B via phosphorylation of co-activator proteins such as CBP/p300, which interact with NF- κ B in the nucleus or via phosphorylation, and inhibition of repressor proteins such as SMRT (Ghosh and Hayden, 2008).

TLR mediated activation of NF- κ B is now known to extend beyond innate and adaptive immunity, suggesting new roles for PRRs in human cell signaling and physiology. TAK1, for example, may also activate a mitogen activated protein kinase (MAPK) cascade leading to activation of JNK, p38 MAPK, ERK, AP-1 that also regulate immunity and inflammatory genes (Wang et al., 2001).

TRIF signalling

The TIR domain of TLRs can also interact with the adaptor protein TRIF. TRIF was first identified as a requirement for TLR3 signalling and activation of the IFN β promoter in response to poly(I:C) which mimics viral dsRNA (Yamamoto et al., 2002) and to viral infection (Hoebe et al., 2003). In fact, TLR3 signals exclusively through TRIF since cytokine release is abolished in macrophages harbouring the germline mutation Lps2 which results in a frameshift error in TRIF (Hoebe et al., 2003). In macrophages harbouring the same mutation, responses to LPS are also truncated (Hoebe et al., 2003). This indicates that for TLR4 signalling in response to LPS, both MyD88 and TRIF pathways have important roles to play.

Importantly, TRIF results predominantly in activation of the IFN- β promoter (Yamamoto et al., 2002) via IRF transcription factors. This induces the expression of cytokines associated

with viral immunity including IFNs and IFN related protein-10 (IP10) (Akira and Takeda, 2004; Paul-Clark et al., 2012).

These pathways are summarised in Figure 1.4

1.3.2 TLR4, endothelial cells and cardiovascular disease

TLR4 has also been directly associated with cardiovascular diseases such as atherosclerosis where increased TLR4 activation and expression are reported (Edfeldt et al., 2002; Michelsen et al., 2004a). Indeed, TLR4-/- (Michelsen et al., 2004b), TLR2-/- (Mullick et al., 2008) and MyD88-/- (Michelsen et al., 2004b) mice are protected against atherosclerosis. The development and use of TLR knockout mice models help to explain early studies conducted in the late 1980s and early 1990s implicating a role for subclinical bacterial infection in atherosclerosis (Saikku et al., 1988; Thom et al., 1992). Specifically, a number of papers have shown that Gram-negative Chlamydia, which activates TLR4, is present in atherosclerotic lesions (Libby et al., 1997). These papers paved the way for early clinical trials designed to assess the potential preventive benefits of antibiotic therapy in atherosclerosis (Andr ws et al., 2005). Whilst, on the whole, the clinical trials with antibiotics were not successful in preventing cardiovascular disease, the link between pathogens and atherosclerosis has continued to be investigated.

TLR4 can also be activated by endogenous ligands that are associated with atherosclerosis, including oxidised LDL (Xu et al., 2001) and heat shock protein-90 (Sasu et al., 2001) high levels of which can drive vascular inflammation and atherosclerosis (Salonen et al., 1992; Wick, 2006).

Single nucleotide polymorphisms in genes encoding TLRs are also associated with diseases (Paul-Clark et al., 2012). The role of mutation in TLR function is typified by the well-studied polymorphism in the TLR4 gene which results in Asp-Gly substitution at position 299. Asp299Gly in fact confers hyporesponsiveness to LPS and results in reduced risk of atherosclerosis (Kiechl et al., 2002) although in 2012 a meta-analysis found a lack of association between this polymorphism and atherosclerosis was reported (Zhang et al., 2012). Nonetheless, increases in TLR4 activation and expression on endothelial cells and macrophages in atherosclerosis is clear (Edfeldt et al., 2002).

Atherosclerosis can also occur after transplant and is a major clinical limitation of organ transplant surgery (Rahmani et al., 2006). Interestingly, a 2005 study evaluating the role of TLR4 in atherosclerosis in patients following kidney transplant found that patients with Asp299Gly had reduced risk of post-transplant atherosclerosis but increased risk of severe infection (Ducloux et al., 2005). The role of TLR4 in disease is very much a double-edged sword, and in the context of vessel and organ regeneration this deserves careful consideration.

1.3.3 Nucleotide-binding leucine rich repeat (NLR) receptors

Another, more recently discovered family of PRRs are the NLR receptors. This family of receptors was identified from studies in plants (van der Biezen and Jones, 1998), which have no adaptive immune system and thus rely solely on innate immunity to overcome infection. In plants, NLRs are associated with resistance to disease. Screening of the human genome has led to the identification of NLR containing receptors in humans. These include the caspase recruitment domain-4 (CARD4) or NOD1 receptor and the CARD15 or NOD2

receptor (Harton et al., 2002). Whilst TLRs use TIR domains to interact with their adapter proteins, NLRs typically use CARD domains to interact with other adapter/signalling proteins, the best studied of these being RIP2.

NOD1-RIP2 signalling

The authors that first demonstrated that NOD1 and NOD2 activate NF- κ B (Inohara et al., 1999) also demonstrated that they require the then recently identified CARD domain containing serine/threonine kinase, receptor interacting protein-2 (RIP2) (Inohara et al., 1998).

NOD1 receptors are known to be activated by a muropeptide (L-Ala-G-Glu-meso-diaminopimelate (DAP)) found uniquely in Gram negative bacterial peptidoglycan. Once activated NOD1 binds the RIP2 via a CARD-CARD domain interaction. RIP2 then carries out a polyubiquitination which allows recruitment of the TAB-TAK complex described above and subsequent activation of NF- κ B and other downstream transcription factors (Hasegawa et al., 2008). NOD2 receptors are activated by muramyl dipeptides, which are expressed in the peptidoglycan structures of some Gram-positive and Gram-negative bacteria and are reviewed by Opitz. et al (Opitz et al., 2009) . The NOD1 pathway is summarised in Figure 1.4

After the characterisation of NOD1 and NOD2 the existence of many more NLRs emerged (Becker and O'Neill, 2007). All NLRs comprise a central nucleotide binding domain, a C-terminal LRR domain and a variable N-terminal effector domain. To date, four key families have been identified, and their N-terminal effector domains define these. The four NLR families are defined as those that express an acid transactivation domain (NLRA), a

baculovirus inhibitor domain (NLRB), a CARD domain (NLRC) or a pyrin domain (NLRP). A subclass of NLRs has been reported and is localised to the mitochondria, capable of inducing NF- κ B and JNK-1 and is included in the nomenclature as the NLRX family. These families are further sub-divided in to individual NLRs expressed in a variety of tissues. Unlike TLRs, NLRs are intracellular receptors wherein they respond to a range of PAMPs as well as host ligands, or danger associated molecular patterns (DAMPs)(Becker and O'Neill, 2007)

1.3.4 NOD receptors, endothelial cells and disease

In contrast to TLRs, NOD function in the vasculature is less explored. Evidence for a function of NOD1 in endothelial cells emerged in 2005. Using a NOD1-siRNA knockdown in endothelial cells, it was shown that inflammatory responses of endothelial cells to the Gram negative bacteria, *Chlamydia pneumoniae* and *Listeria monocytogenes* were NOD1 driven (Opitz et al., 2005; Opitz et al., 2006). As the presence of *Chlamydia pneumoniae* has been reported in atherosclerotic plaques in humans (Kuo et al., 1993), it is possible therefore that NOD1 contributes to vascular inflammation and dysfunction. However, the role of NOD1 in atherosclerosis has not been studied directly. By contrast, our group has shown a clear role for NOD1 in vascular dysfunction, and cardiovascular collapse in sepsis has been demonstrated where effects were seen to be more severe than those induced by the TLR4 agonists LPS (Cartwright et al., 2007). We have gone on to show that human endothelial cells express distinct and discernable TLR4 and NOD1 pathways via separate signalling pathways which can be targeted selectively with pharmacological antagonists (Gatheral et al., 2012). Other NLRs, included NLRP3 have been associated with cardiovascular disease (Düwell et al., 2010; Mallat et al., 2001). The study of NLR biology and function in

cardiovascular cells and tissues thus represents an active area of research that could yield new insights in to disease pathophysiology and new targets for therapy.

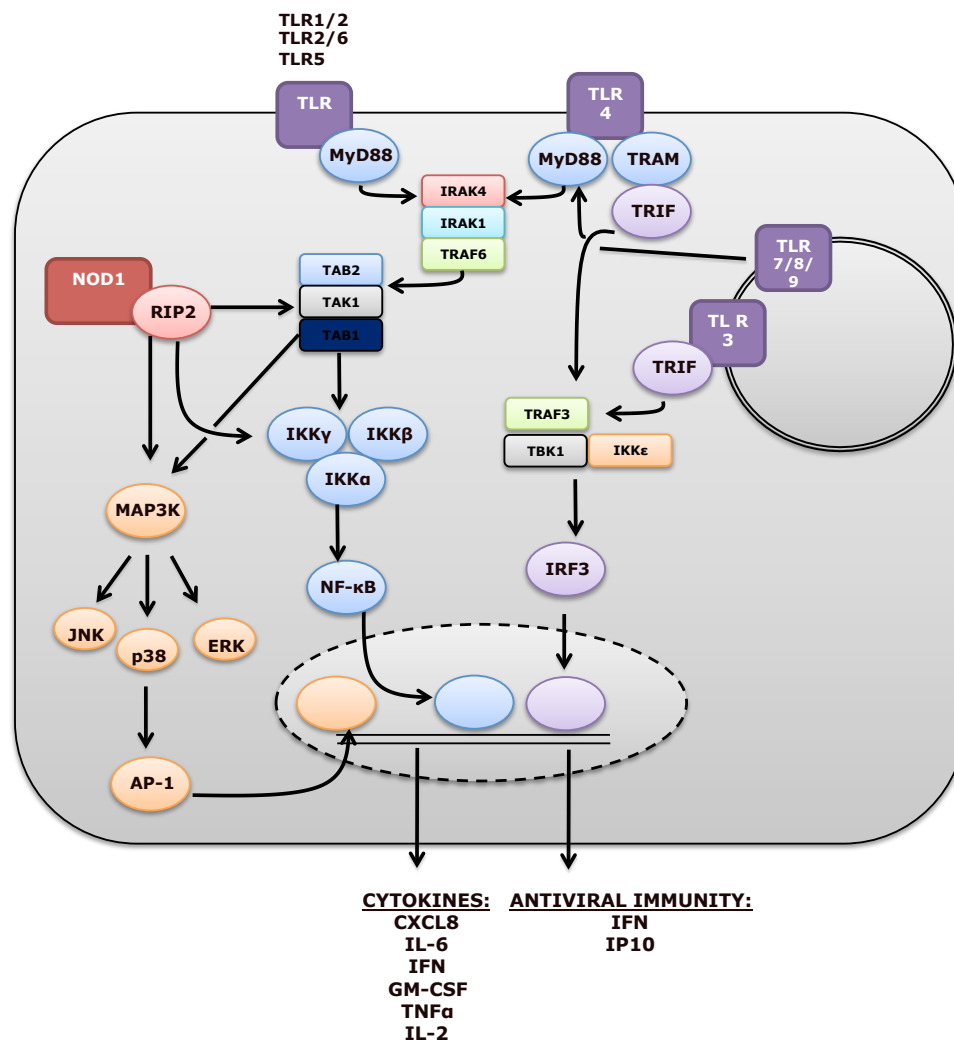


Figure 1.4 Summary of Toll-like receptor (TLR) and nucleotide oligomerisation domain (NOD) signalling. Abbreviations: myeloid differentiation factor-88 (MyD88), Mitogen activated protein kinase-3 (MAP3K), Receptor interacting protein (RIP), Tumour necrosis factor- α (TNF α) receptor associated factor (TRAF)-3/6. Transforming growth factor β activated kinase (TAK), TAK-1 binding protein (TAB), TIR-containing adaptor protein inducing interferon- β (TRIF), TRIF related adaptor molecule (TRAM), Interferon (IFN) regulatory factor (IRF), Nuclear factor kappa B (NF- κ B), C-jun N-terminal kinase (JNK), extracellular signal activated kinase (ERK), Interleukin (IL)-1 receptor associated kinase (IRAK), I κ B kinase (IKK), TAK-binding kinase (TBK), granulocyte colony stimulating factor (GM-CSF).

1.4 STEM CELLS AND REGENERATIVE MEDICINE

Regenerative medicine and stem cell biology are offering significant advances in how we treat disease. Stem cells can now be isolated, engineered *in vitro* and used to regrow and/or repair tissues damaged by disease. These stem cell technologies can also be used to develop *in vitro* assays to test efficacy and toxicity of drugs, this is particularly relevant to the testing of biological drugs designed for a personalised medicine approach. In my PhD thesis I have studied the morphology and function of endothelial cells derived from various stem cell sources. I have gone on to use stem cells in the specific application of bioassays for testing side effects of drugs like the CD28 superagonist TGN1412. I will therefore introduce in some detail the general area of stem cell biology, the specific application and usefulness of this in terms of the endothelium and, finally, the area of human tissue bioassays and associated limitations.

Stem cells are cells that have the capacity to self-renew an unlimited number of times, or differentiate in any number of specialised cell types in the body. Stem cells that can form any cell type in the body are defined as pluripotent whereas cells that form a pre-defined lineage of cells are defined as multipotent. Multipotent progenitors can arise from pluripotent stem cells but this is not always the case. For the purpose of my thesis I will specifically introduce the area of (i) embryonic stem cells, (ii), adult stem cells and (iii) induced pluripotent stem cells.

1.4.1 Early studies on rodent, primate and human embryonic stem cells

The term stem cell was introduced based on analysis of embryo development, where it seemed logical that all cells in the body must 'stem' from a special group of cells. Knowledge on the mechanisms by which stem cells differentiate, and their proliferative potential came from studies on spermatogenesis (Oakberg, 1971). True embryonic stem cells however are obtained from pre-implantation blastocysts. The first successful isolation of stem cells from embryos was performed in mice (Martin, 1981). However, we now have the technology to isolate and study stem cells from human embryos. This was pioneered by James Thomson and colleagues at the University of Wisconsin (Thomson et al., 1998). Indeed, James Thomson was recognised by Time Magazine as '*the man who brought you stem cells*' (Time Magazine, August 20th, 2001). For ethical and technical reasons at the time, early work leading to the ultimate isolation of stem cells from human embryos was carried in non-human primates, specifically two species of rhesus monkey (*Macaca mulatta* and *Callithrix jacchus*). In order to draw conclusions on how closely these primate stem cells might resemble human stem cells, Thomsons group used human embryonal carcinoma cells as comparators (Thomson et al., 1995). Human embryonal carcinoma cells are derived from teratomas and express stage specific embryonic antigen (SSEA)-3, SSEA-4, TRA-1-60 and TRA-1-81 (Andrews et al., 1984). Positive expression of each of these were found in the cells isolated from monkey embryo and allowed for the reasonable conclusion that these stem cells were closely related to human embryonic stem cells. Thus, primate embryonic stem cells were thought to be a superior tool to mouse embryonic stem cells and embryonal carcinoma cells for their closer relationship to human cells, and in the case of embryonal carcinoma cells and stable chromosome number (Thomson et al., 1995).

However, in 1998, pioneering research demonstrated that human embryonic stem cells could be isolated from human embryos surplus to *in vitro* fertilisation programs. Fourteen inner cell masses were isolated from five pre-implantation blastocysts in this study. From these, five human embryonic stem cell lines (hESC) were derived (Thomson et al., 1998). These cells were also shown to express SSEA-3 and 4, TRA-1-60 and TRA-1-81 and fulfilled the criteria set by Thomson in primate embryonic stem cells. These cell lines were subsequently cryopreserved and have been used extensively to study developmental biology, model human disease, applied to *in vitro* assays and organ engineering. Notably, the first clinical trial of hESC differentiated to nerve cells was commenced in 2009 and, after delay, is ongoing. A further clinical trial using hESC-derived retinal pigmented epithelial cells and early data indicates safety of the approach (Schwartz et al., 2012).

1.4.2 Adult stem cells

Knowledge that a population of cells existed in the bone marrow emerged from two ground breaking studies in mice. In 1949 Leon Jacobson demonstrated that mice could be protected from lethal ionising radiation by shielding the femur (Jacobson et al., 1949). Then in 1951 another group demonstrated that intravenous infusions of bone marrow in mice could also protect against radiation (LORENZ et al., 1951). These data suggested that the bone marrow was a source of circulating cells, and it was Barnes and Louitt who first suggested that these effects of femur shielding, were a result of the protection of stem cells in the bone marrow (BARNES and LOUITT, 1955). This provided the first evidence of adult stem cells beyond those involved in spermatogenesis and provided the foundations for cell therapy and the first bone marrow transplants (THOMAS et al., 1959). These adult stem cells circulate in blood and reside in tissues of the body. These cells are typically multipotent, which means

they act as 'progenitors' or 'precursors' to many but not all differentiated cell types. Adult stem cells were in fact the first type of stem cell to be isolated and expanded from peripheral blood of mice in the lab (GOODMAN and HODGSON, 1962). These cells derived from bone marrow and peripheral blood, and in line with data from early bone marrow transplants in humans, have been studied in relation to cancer (Metcalf and Moore, 1970) and can now be isolated and preserved for use in bone marrow transplants and cell therapy for cancer (Goldman et al., 1978).

Indeed, adult stem cell approaches and their role in human physiology continue to be studied and pioneered today. It is now known that many populations of adult stem cells exist and these cells participate in endogenous repair of organs and tissues in the human body. In fact, it is now possible to view many diseases, including cardiovascular disease, as 'diseases of failed repair'. That is to say, patients who develop disease or those at higher risk, have progenitor cells with reduced or damaged capacity to repair tissues (Hill et al., 2003). Whether this is true, and reductions or increases in adult progenitor cells are reactive or causative is unclear. To illustrate this, I will take endothelial progenitor cells (EPCs) as an example which epitomise the current thinking on the role of adult progenitor cells in human biology and disease.

Endothelial Progenitor Cells (EPCs)

EPCs were first defined in 1997 by Asahara et al (Asahara et al., 1997). These cells were initially defined as CD34+, CD133+, VEGFR+ circulating progenitor cells that were involved in vessel homeostasis and post-natal angiogenesis. This marked a substantial paradigm shift in how we think about stem cells and vascular biology. A great number of studies followed that

aimed to further define this novel cell population and these are discussed later in the context of other stem cell derived endothelial cells. An early clinical study on circulating levels of EPCs demonstrated that EPC numbers in the peripheral blood were lower in patients with cardiovascular disease, and that this correlated with severity of disease (Hill et al., 2003). This led to the suggestion that EPCs are involved in a protective endogenous repair system, and if the performance of this repair is reduced, disease can result. In further support of this, Xu et al. illustrated that the endothelial cells found on vein isografts from wild type mice in TIE2-LacZ mice were negative for β -gal staining, and therefore suggested that these were derived from a recipient progenitor cell (Xu et al., 2003). They went on to show that these recipient cells were derived in part from a bone marrow population of cells and that this function was reduced in ApoE(-/-) hyperlipidaemic mice.

In line with the apparent role of reduced adult progenitor cell number in disease, strategies to boost adult progenitor cell populations have been developed. Indeed, there are now reports that the beneficial effects of some key cardiovascular therapies that are already available, such as statins can be attributed to boosting of EPC number (Vasa et al., 2001; Walter et al., 2002).

1.4.3 Induced pluripotent stem cells

HESC have been limited by ethical concerns, their potential to cause immunogenicity and teratoma formation (Knoepfler, 2009). A research goal in the field of stem cell biology therefore has been to overcome this. Indeed, autologous adult progenitor cells provide a way around immunogenicity, as the patient's own cells can be isolated and used. Adult

progenitor stem cells however are less well characterised, particularly in disease settings, and are not fully pluripotent.

In 2007 a group in Japan, led by Prof. Shinya Yamanaka hypothesised that genes known to maintain embryonic stem cell phenotypes could theoretically be used to reprogram somatic cells from adults in to pluripotent phenotypes (Takahashi et al., 2007). The idea of cellular reprogramming was not new as this was based on retro-viral transcription, and had been demonstrated previously by nuclear transfer. This nuclear transfer involved the isolation of a host embryo and transfer of a somatic cell nucleus in to the cells were it assumes an embryonic like state (McLaren, 2000; Wilmut et al., 1997). This strategy formed the basis for the cloning of Dolly the Sheep (BBC News, 22 February 1997). For applications in human tissue engineering however, a more controlled and embryo free approach was required. The idea of reprogramming cells with the intention to gain control of their phenotype *in vitro* was revolutionary. It was known in 2006 that several genes are required for mouse and human embryonic stem cells to maintain the pluripotency. These include the transcription factors, Nanog, Oct3/4 and Sox2. Other genes known to regulate stem cell proliferation were also known and included Stat3, E-Ras, Klf4 and c-myc. In 2007, Yamanaka et al. constructed an assay in which pluripotency could be detected as resistance to the drug G148 (Takahashi et al., 2007). This was achieved by transfecting cells with the G148 resistance gene (β^{geo}) into the promoter region of gene *Fbx15*, which is expressed by mouse embryonic stem cells but not required for pluripotency.

Using this assay, Takahashi et al. demonstrated that introduction of four factors; Oct4, Sox2, klf4 and c-myc into mouse or adult fibroblasts gave rise to a cell population with identical

properties to embryonic stem cells. These cells were termed induced pluripotent stem cells (iPSCs) and have subsequently been shown to be differentiable in to endothelial cells (Homma et al., 2010; Taura et al., 2009), vascular smooth muscle cells, T-lymphocytes, and cardiac myocytes. Other somatic cells have also been used as starting material for iPSCs. These include neuronal cells and another putative progenitor cell derived cell, blood outgrowth endothelial cells (BOEC). BOEC are thought to belong to the family of EPCs and are discussed in detail later. In this study BOEC were reprogrammed to form induced pluripotent stem cells and then differentiated in to induced pluripotent stem cell-derived endothelial cells. The rationale for this was that this kind of approach would overcome the limitations of low cell number of adult progenitors, but will still allow individual progenitor cell biology to be captured by the induced pluripotent stem cells which might go on to be used as therapy. Like adult progenitors therefore, iPSC-derived cells can be used to engineer tissues or organs for individual donors.

Induced pluripotent stem cell technology is not without its problems. Whilst these cells are theoretically superior to other stem cells, the fact these cells have undergone genetic manipulation represents a potential problem. As these cells have been transfected with genes from a retrovirus they may not be safe for use in humans. The retroviral and lentiviral based reprogramming used, whilst efficacious, means that viral genes are permanently integrated into the human genome which poses a risk of mutagenesis and re-activation of re-programming factors. In short, these cell therapy approaches, like embryonic stem cells, could conceivably result in tumours.

Approaches to overcome this have been investigated. It has been shown when neuronal cells are used as starting material for example, that they require fewer transgenes to be

incorporated to achieve an embryonic like state (Kim et al., 2008). This is thought to be a result of higher endogenous levels of c-myc and klf4 in neuronal cells. It is noteworthy however, that neuronal cells are relatively inaccessible compared to fibroblasts, and so this approach rather replaces one limitation with another. Another focus in this area is to develop transgene free approaches to incorporating pluripotency factors in to somatic cells. Transgene free approaches to gene therapy are being developed for a range of gene therapy applications. These include use of transposons such as PiggyBac. Transposon transgenesis is a new technology which involves introduction of naked DNA using common plasmids, which can then be excised, and so provide a safer alternative to using viral transfection protocols. In the case of induced pluripotent stem cell protocols, the circular DNA transposon vector PiggyBac can be loaded with the transcription factors c-myc, Klf4, Oct4 and Sox2 under the control of a doxycycline inducible promoter. This vector (PiggyBac-TET-MKOS) can then be chemically transfected in to somatic cells, resulting in reprogramming and acquisition of a pluripotent stem cell phenotype and the remaining PiggyBac-TET elements removed by addition of PiggyBac-transposons. This results in a population of transgene free induced pluripotent stem cells. Assessment of safety of stem cell therapies, especially those that have undergone manipulation, will be critical to success.

The applications of different stem cells, but requirement for functional characterisation, are illustrated in Figure 1.5.

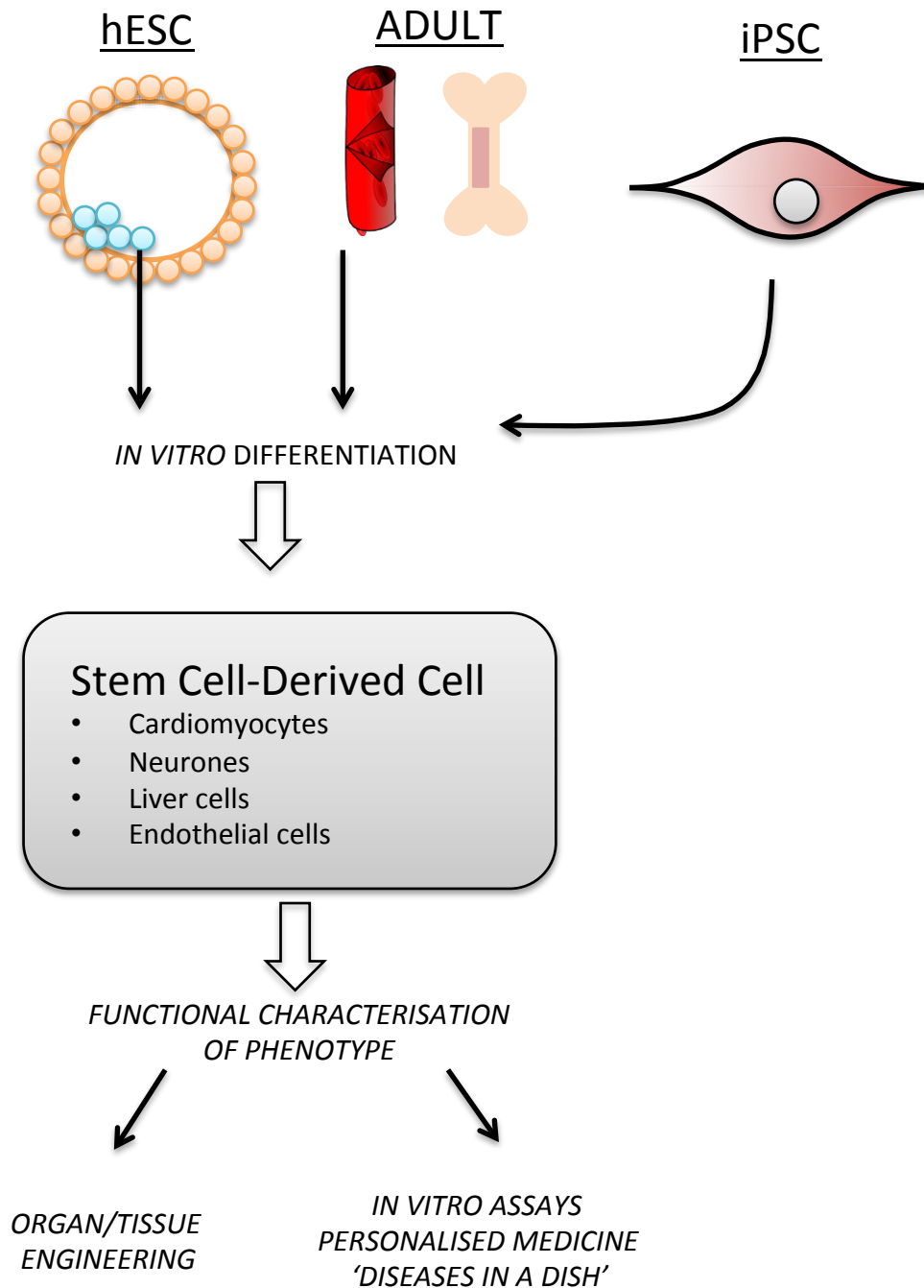


Figure 1.5 Applications of different stem cells and stem cell derived cells in research and therapy. Prior to use however these cells must be characterised for key hallmarks of cell phenotype. Differences in phenotype may be relevant to disease modelling and/or safety of cell therapies. Blood vessel image from pixabay.com.

1.4.4 Building organs and modeling disease in a dish

The concept of building human organs for transplant has been around since the mid-1930s. In a book entitled 'The Culture of Organs' by Alexis Carrel (Figure 1.6) and famed pilot, Charles Lindbergh reported a device that allowed organs to exist outside the body and laid the foundations for the engineering of living organs and tissues in the laboratory. With the advent of stem cell technology, the idea of building organs and tissues in the lab has become a reality.

What do you need to build an organ?

1. A scaffold or platform on which to build complex 3-dimensional structures
2. A source of cells specific for the organ (cardiomyocytes, renal cells etc.)
3. **A means to vascularise the tissue (stem cell derived vascular endothelial cells or endothelial cells from vessels harvested and grown in culture)**

Thus, and as alluded to, healthy endothelial cells will be critical for any and all organ regeneration programs. The need for greater therapeutic options in cardiovascular disease is clear. The British Heart Foundation reports that three people die every day in UK whilst waiting for an organ transplant and even of those receiving donor organs; these are limited by post-surgery complications. The ability to engineer organs and tissues as well as develop therapies to regenerate tissues in the body thus has profound implications. This ability has arisen with the isolation and characterisation of human stem cells.

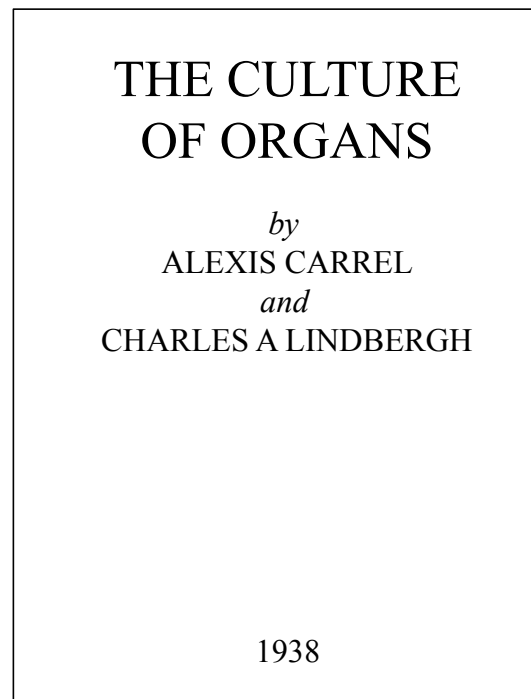


Figure 1. 6. Origins of tissue and organ engineering from articles published in 1930-38. Book cover redrawn from original: www.charleslindbergh.com.

Stem cells can also be used to model diseases ‘in a dish’. With the development of protocols to differentiate iPSC and adult progenitor cells in the lab, we can now derive cells from individual patients. This is particularly important when terminally differentiated cells like neurones or cardiac myocytes are needed. One key example of the power of this approach to research is in motor neuron disease. In a series of studies in 2008 and 2009 (Dimos et al., 2008; Karumbayaram et al., 2009) induced pluripotent stem cells were derived from an 82-year patient with motor neuron disease, differentiated in to motor neurons and shown to recapitulate the disease versus healthy cells. This paved the way for further studies using motor neurons derived from patient stem cells and led to the discovery of a novel pathway (increased transactivase response DNA-binding protein-43 (TDP-43)) involved in motor neuron disease (Serio et al., 2013). Extensive examples of this exist in the literature. In 2013, Kim et al (Kim et al., 2013) used iPSC-derived cardiomyocytes to study arrhythmogenic

right ventricular dysplasia and demonstrated for the first time that handling of metabolism by these cells differed from healthy cells, and so proposed a novel targetable pathway. It is important to note with this information, caution should be taken when considering these as 'autologous' therapies. Autologous therapy might be advantageous in terms of overcoming immunogenicity, but knowing that these cells recapitulate disease states means these cells might not represent optimal stem cell therapies for patients.

Adult stem cell-derived cells have also been employed in disease modelling. As this is a key feature of my PhD, this is discussed later in the context of applications of stem cell derived endothelial cells. The current knowledge on stem cell-derived endothelial cells, examples of their use in therapy and engineering, and *in vitro* research will now be discussed. Indeed, the goal of my PhD has been to build on this in terms of characterisation of these cells and apply them to endothelial cell assays, which, accordingly are discussed in the final section of this introduction.

1.5 STEM CELL DERIVED ENDOTHELIAL CELLS

Endothelial cells can be derived from, human embryonic stem cells (Levenberg et al., 2002), adult progenitors (Ingram et al., 2004) and induced pluripotent stem cells (Taura et al., 2009). As alluded to, these cells have potentially powerful applications for the study of endothelial cell biology; offer new avenues for endothelial cell based therapy; and will be essential for organ regeneration programmes.

1.5.1 Human embryonic stem cell derived endothelial cells (hESC-EC)

hESC-EC were first grown from undifferentiated hESC by Robert Langer's lab in 2002 (Levenberg et al., 2002). This protocol relied on allowing hESC to differentiate spontaneously, which can be achieved by growing hESC on ultra-low attachment cell culture plates. This spontaneous differentiation results in the formation of complex 3-dimensional structures known as embryoid bodies. Human embryoid bodies grown in the presence of vascular growth hormones, including VEGF, express a network of vascular-like channels that express CD31 and VE-cadherin (Levenberg et al., 2002). As the differentiation proceeds, pluripotency genes such as Oct4 subsequently decrease, and this is favourable in terms of reducing risk of tumourgenecity in stem cell therapies. A purer population of endothelial cells was then isolated from embryoid bodies by isolating and expanding a CD31+ population by fluorescence activated cell sorting (FACS). Once isolated, hESC-EC express endothelial cell markers such as CD31 and VE-cadherin (Nourse et al., 2010). In early studies, this kind of characterisation has typically been the extent of the *in vitro* characterisation of these cells as authentic 'endothelial cells' versus endothelial cells from vessels (normally human umbilical veins; HUVEC). A number of papers have demonstrated that hESC-EC form

vascular networks in mice with severe combined immunodeficiency disease (SCID) (Ferreira et al., 2007) when seeded on to biodegradable scaffolds or when injected directly to ischemic tissue including infarcted myocardium (Kraehenbuehl et al., 2011). Others have also shown that hESC-EC align to shear stress (Metallo et al., 2008) but this has not been tested routinely, nor has the ability of hESC-EC to release vasoactive hormones been studied systematically. Thus, whilst hESC-EC have potential applications in therapy and tissue engineering, there is arguably a relative lack of knowledge on critical endothelial cell pathways in these cells. Indeed, protocols have been developed to produce hESC-EC that are suitable for clinical evaluation (Kaupisch et al., 2012), which could be used to prepare cell therapies for large populations of patients, and thorough assessment of endothelial cell phenotype will be critical.

hESC-EC have also been used to study the development of the cardiovascular system. Indeed, elucidation of the differentiation pathway of endothelial cells from progenitors will be essential for success of stem cell endothelial cell based therapies in the future. This is true for all stem cell-derived endothelial cells (embryonic, adult and induced pluripotent derived) and so it may be argued that studies on all different stem cell sources in parallel will be needed for success in this area.

As discussed, characterisation of stem cell derived cells will be essential to success; and of relevance to my PhD, characterisation of stem cell derived endothelial cells. In our group we have previously shown that hESC-EC, unlike endothelial cells from vessels; do not respond to the TLR4 agonist, LPS (Földes et al., 2010). This suggested that hESC-EC might have an

immature innate immune phenotype, and has been further explored and expanded upon in detail in the first two chapters of my PhD.

1.5.2 EPC and blood outgrowth endothelial cells (BOEC)

As discussed, in 1997, it was suggested by Asahara *et al.* that a population of cells exists that are derived from the bone marrow and are capable of differentiating in to endothelial cells and forming new vessels at sites of ischemia (Asahara et al., 1997). However, there has been controversy over the definition of these cells and their role in vascular biology. As a result, no unifying definition of these cells exists and the term EPC in fact encompasses a number of cell types with putative roles in vascular homeostasis and disease. There is some agreement on the nature of ‘early’ versus ‘late’ outgrowth EPCs that can be enriched in cultures using specific isolation protocols. To date, it appears that endothelial progenitor cells can be divided in to two categories.

1. ‘Triple positive circulating EPCs’ which are endothelial cell-like *in vitro* expressing the three specific markers, (i) CD133 and (ii) CD34, expressed on hematopoietic stem cells and vascular cells and (iii) VEGFR2, expressed on endothelial cells; but also express hematopoietic associated markers CD45 and CD14.
2. Blood outgrowth endothelial cells (BOEC), which express the endothelial markers CD31 and VEGFR2 but are negative for hematopoietic markers and the stem cell marker CD133 and behave as mature endothelial cells *in vitro*.

The relationship between these cells is unclear and has been discussed widely in the literature (Hirschi et al., 2008; Ingram et al., 2004; Timmermans et al., 2009). Circulating

EPCs have been shown to arise from a CD45+ hematopoietic stem cell population in the peripheral blood and bone marrow mononuclear cells, but that these cells do not contribute to endothelial cell formation *in vitro* (Case et al., 2007). In order to study the role of circulating EPCs in vascular homeostasis *in vivo* a number of approaches have been taken. Using Id-1 (which is selectively expressed in some circulating EPCs) knockdown in a mouse model of tumor angiogenesis it was suggested that circulating EPCs are involved in angiogenesis (Ciarrocchi et al., 2007). However, as many of the markers used in these approaches are also expressed by mature endothelial cells on vessels and/or in other hematopoietic cells it remains unclear whether the new vessels formed in these *in vivo* models are derived from mature endothelial cells that grow in from adjacent blood vessels or bone marrow derived cells. Despite this, it remains an accepted possibility that these cells contribute to vessel formation *in vivo* in an indirect manner (Rehman et al., 2003; Takakura et al., 2000), and that they are useful biomarkers of cardiovascular function (Hill et al., 2003; Timmermans et al., 2009). At this stage however, circulating EPCs do not appear to represent a useful resource for the study of endothelial cell biology *in vitro*, namely because they display specific differences in morphology and function compared to endothelial cells from mature vessels (Bompais et al., 2004; Timmermans et al., 2009)

BOEC, which are not necessarily circulating EPCs according to the original definition, but differentiated from another putative endothelial progenitor, were first defined by Ingram et al. in 2004 (Ingram et al., 2004) and can be expanded to endothelial cells using well defined protocols (Martin-Ramirez et al., 2012). BOEC, like circulating EPCs, express CD34, CD31, and VEGFR2, but unlike circulating EPC, do not express the hematopoietic markers, CD133, CD14, and CD45 (Timmermans et al., 2009). When expanded *in vitro*, BOEC have

cobblestone morphology and importantly form vessels *in vitro* (Timmermans et al., 2007). The precursor-product relationship between circulating EPC and BOEC has been studied extensively, even in the relatively short period of time since the first description of these cell populations. In 2000, Lin et al. (Lin et al., 2000) showed that endothelial cells expanded from bone marrow derived from patients that had received sex mismatched bone marrow transplants, were donor derived. From this, the authors inferred that these cells were derived from circulating EPC from the bone marrow. It has since been shown however, using a comprehensive *in vitro* approach and fluorescence activated cell sorting (FACS) analysis that BOEC might be derived from a CD45⁻ CD34⁺ population that is distinct from circulating EPC (Timmermans et al., 2007) and this population also likely originates from the bone marrow. On the other hand, BOEC from patients with chronic myelogenous leukemia do not harbor the mutation found in hematopoietic cells in these patients (BCR/ABL1) (Otten et al., 2008), and this suggests that BOEC arise from a clonally distinct progenitor or a source other than the bone marrow. Indeed, others have shown that a CD34⁻ multipotent adult progenitor population is the BOEC-precursor (Reyes et al., 2002) but the protocol used to isolate, and for the definition of, BOEC across these papers is varied. As a result, and because of current technical limitations in protocols to discriminate progenitor cell populations, the exact identity of the BOEC-precursor remains uncertain and part of an ongoing research question. Nonetheless, BOEC, unlike circulating EPC, have great potential applications in the study of endothelial cell and vessel biology. The current dogma for the origin of EPC and BOEC from CD34⁺ cells is shown in Figure 1.7

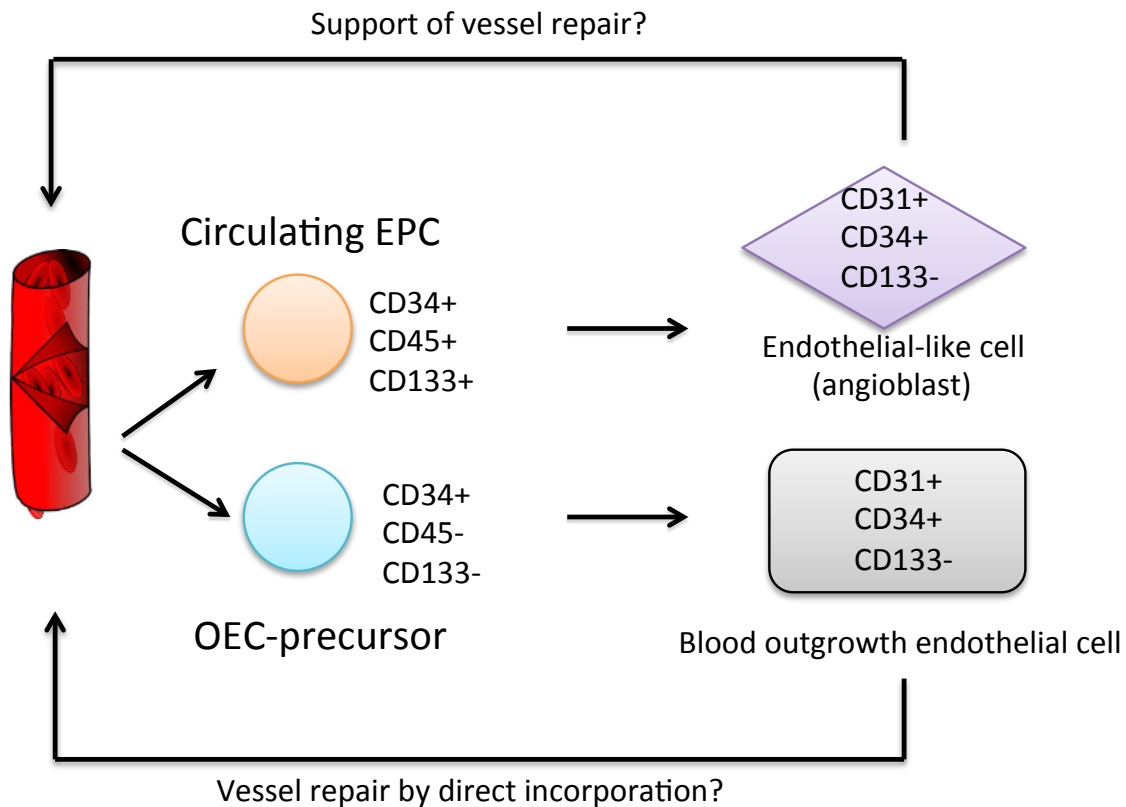


Figure 1.7 Origins and functions of endothelial progenitors cells and outgrowth endothelial (OEC)-precursors. Blood vessel image from pixabay.com.

1.5.3 iPSC-EC

As discussed above, human induced pluripotent stem cells (iPSC), first engineered by Yamanaka in 2007 (Takahashi et al., 2007), are adult human cells that have been re-programmed into a pluripotent phenotype. It was later shown (Taura et al., 2009) that human iPSC can also be differentiated into endothelial cells. Human iPSC-EC have now been studied in pre-clinical models of ischemia and found to be capable of forming vascular networks and increasing blood perfusion of the hind limbs of SCID mice (Rufaihah et al., 2011). iPSC-EC, like circulating EPC and BOEC, also hold the potential for autologous therapy, and in the phenotyping of individual patient endothelial cells. BOEC and iPSC-EC, as described above for other diseases, can therefore be used to construct translational models

of vascular disease, and be used for personalised medicine assays. This critical point forms a basis for my PhD, in that both iPSC and BOEC can potentially be used to phenotype patient vessel biology.

1.5.4 Stem cell derived endothelial cells in therapy

To date, and in terms of stem cell derived endothelial cells, only BOEC and circulating EPC, either as mixed cell or purified populations, have been applied as therapies for humans. The potential for the other types of stem cell derived endothelial cells as therapies has however been considered *in vitro* and *in vivo*.

As a result of the putative role of EPCs in vascular health and vessel formation following the injection of these cells as therapeutic cells has been tested in clinical trials. To date, a search for 'endothelial progenitor cells' on the clinical trials data base (clinicaltrials.gov) yields 232 results and illustrates that therapeutic angiogenesis, using endothelial progenitor cells, is an actively studied potential approach to cardiovascular disease treatment. Equally, endothelial cells from hESC and iPSC also have applications as cell therapies for cardiovascular disease, but these have not yet been tested fully in humans.

In humans, use of bone marrow derived mononuclear cells (BMD-MNCs) has been successful in clinical trials of patients with limb ischemia (Tateishi-Yuyama et al., 2002); the benefits of which have been attributed to the presence of endothelial progenitor cells and pro-angiogenic cytokine secreting haematopoietic cells (Asahara et al., 1997; Rafii and Lyden, 2003; Tateishi-Yuyama et al., 2002). The TOPCARE-AMI trial which begun in 2002 has shown that direct intramyocardial injection of autologous bone marrow derived EPC enhances

myocardial regeneration after acute myocardial infarction (Assmus et al., 2002), and the benefits persist across a 5 year follow up (Leistner et al., 2011). Furthermore, a 2014 Cochane review based on 23 randomised control trials also reported moderate benefit in chronic ischemic heart disease (Fisher et al., 2014). Indeed, whether BMD-MNCs or the peripheral blood mononuclear fraction is the optimal source of cytokine releasing cells and EPCs is unclear (Minamino et al., 2002; Moriya et al., 2009; Tateishi-Yuyama et al., 2002). Finding the optimum formulation of stem cells for therapeutic benefit therefore will be important to determine in the future. One possible limitation in using mixed peripheral blood leukocytes as a source of cells is the low levels of cells in blood of patients with cardiovascular disease. As discussed below, this stimulated interest in pharmacological mobilisation of cells to ensure sufficient EPC presence in the peripheral blood. EPC mobilisation from the bone marrow and recruitment to sites of damage is regulated by cytokines such as VEGF, CXCL12, G-CSF and s-KitL. In 2006, Boyle *et al.* took advantage of knowledge of this axis and used G-CSF to increase the yield of CD34+ EPCs in blood for isolation and subsequent re-injection into 5 patients with chronic ischemic heart disease. Symptoms were improved within the 12 months follow-up period, however this trial lacked a clear control arm. Other trials, using similar mobilisation and isolation strategies have been carried out and report improved left ventricular ejection fraction in a 6-month follow up of patients with acute-myocardial infarction (Li et al., 2007).

As adult progenitor derived cells already exist within the body, pharmacological mobilisation of EPCs and/or BOEC-precursors to the site of injury using pharmacological agents is now an active area of research. In fact, outside the field of endothelial cell biology, mobilisation of stem cells from the bone marrow of cancer patients using drugs such as GM-CSF is part of

standard chemotherapy (Keating, 2011), and as discussed has provided a cornerstone in our understanding of adult progenitor cell biology. Stem cell trafficking and mobilisation is regulated by a cytokine axis involving CXCL12 and CXCR4 (Ceradini et al., 2004; Sugiyama et al., 2006). Interference of this with the CXCR4 antagonist AMD 3100 (Plexifor), which is approved for use in patients with multiple myeloma, results in increased circulating levels of hematopoietic stem cells (Dugan et al., 2010; Keating, 2011).

As alluded to in an earlier section, early evidence suggests that some common drugs used to treat cardiovascular disease may act in part by cardiovascular-stem cell mobilisation. Vesa *et al.* in 2001 found that patients with coronary artery disease, without myocardial infarction, when given atorvastatin had increased circulating CD34+VEGFR2+ EPC (Vasa et al., 2001). Similarly, in a later study, Fadini *et al.* in 2010 (Fadini et al., 2010) found that the dipeptidyl peptidase-4 (DDP4) inhibitor, sitagliptin increased circulating levels of EPC in line with levels of CXCL12, which is normally degraded by DDP4. The potential of cardiovascular stem cell mobilisation was further demonstrated in mice by Smart *et al.* (Smart et al., 2007) who showed that treatment of mouse epicardial explants with thymosin- β 4 resulted in activation of adult epicardial stem cells and differentiation into vascular cells (Smart et al., 2007) that could restore cardiac function in a mouse model of cardiac injury (Smart et al., 2011). Clearly there is a real opportunity for pharmacological innovation in the field of stem cell mobilisation to repair the vasculature and in organ regeneration programs.

Selected populations of EPC have also been considered in clinical trials for the treatment of pulmonary arterial hypertension. Endothelial cell dysfunction is key feature of pulmonary arterial hypertension pathophysiology and the concept of using stem cell derived

endothelial cells to restore this function has proved attractive. In 2007, a randomised placebo control trial in patients with pulmonary arterial hypertension indicated that injection of autologous triple positive EPCs (CD34+, CD133+, VEGFR2+) improved standard clinical parameters (6-min walk test, mean pulmonary artery pressure, pulmonary vascular resistance, and cardiac output) (Wang et al., 2007). The role of EPCs in pulmonary arterial hypertension has also been widely studied. In experimental models of pulmonary arterial hypertension, which typically involves administration of monocrotaline to rats, administration of autologous EPCs resulted in prevention and reversal of monocrotaline induced PAH (Zhao et al., 2005). As this study used fluorescently labeled EPC, the authors showed that lung sections of mice given injections of EPC contained vessel structures that stained positive for the fluorescent label (green fluorescent protein; GFP). This suggests that these cells home to the lung, but it remains unclear if these cells form mature endothelial cells on vessels.

The potential for hESC-EC and human iPSC-EC in human cell therapy remains to be investigated. However, these cell types deserve consideration particularly for patients where EPC function is impaired (Tepper et al., 2002) and autologous therapy and bone marrow or peripheral blood injections are not suitable, and since EPC/BOEC yield is low compared to more pluripotent stem cell sources such as hESC. For the progression of iPSC-EC towards the clinic, these cells require additional questions to be answered before clinical application. As discussed these may include identification of transgene free approaches to reprogramming as well as more knowledge on the cellular reprogramming pathways. The need for greater understanding of these pathways was illustrated by Kane et al. (Kane et al., 2010) who demonstrated that use of reprogramming vectors alone, without transgenes, can result in

somatic cell reprogramming. Understanding these pathways will allow prediction of the fate of these stem cells when injected in humans which will be critical to develop safe cell therapies.

A Cochrane review (2012) (Clifford et al., 2012) in the area of adult stem cell therapy for acute myocardial infarction concluded, on the basis of 33 randomised control trials, that whilst stem cell therapy appears beneficial there is significant heterogeneity in trial design. A more functional approach to assessing the function of stem cell derived endothelial cells for therapy may therefore be important for success. Indeed, this may include the use of assays to assess vascular phenotype and function, which may need to be demonstrated routinely in endothelial – stem cell therapies.

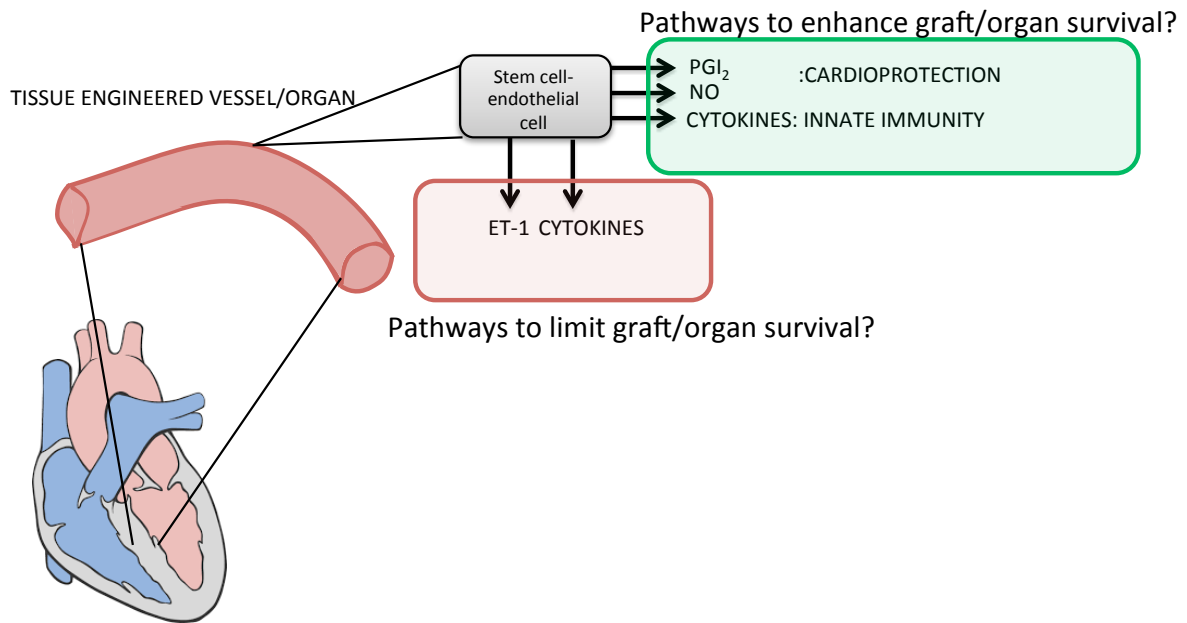
1.5.5 Stem cell derived endothelial cells and organ engineering

A key challenge in organ and tissue engineering is to provide organs with blood flow. The formation of viable and healthy vascular bed to allow transplanted organs to integrate is clearly critical to success in tissue engineering. A number of approaches have been taken to achieve this. In one approach, endothelial cells can be injected directly on to organs. This was illustrated in SCID mice using HUVEC, which when injected into the abdomen wall as part of mix of collagen and fibrin formed vessels (Schechner et al., 2000). The ability of HUVEC to form mature vessels was enhanced when these cells were transfected with the survival gene Bcl-2. This approach however, appears limited by the need to transfect cells with a survival gene which could conceivably result in tumourgenesis. This kind of approach has also not been shown to provide vascularisation of transplanted tissues. In another approach, grafts or tissues can be added without vascular cells but with the ability to release

endothelial cell growth hormones, VEGF and PDGF in order to recruit progenitors from the circulation (Nillesen et al., 2006). Indeed, whilst this approach improved vascularisation, the time for vascular cell growth to occur within the graft may, in reality, be a limitation. A final approach, is to pre-vascularise the tissue *in vitro* prior to transplant (Chen et al., 2009). This approach would allow transplanted tissues to immediately perform as organs and contain healthy cells which have been tested in the lab.

Taking tissue engineered vessel grafts as an example, a 2011 paper suggests that EPCs represent a more suitable source of endothelium than vascular sources since in pigs, vessels re-vascularised with EPCs were more patent than non-endothelialised grafts or grafts coated with mature endothelial cells (Quint et al., 2011). In this study, EPCs remained in the vessel post-implant and integrated with the host vasculature (Quint et al., 2011). Stem cell derived endothelial cells therefore have clear importance in organ and vessel engineering. The essential features of stem cell derived endothelial cells and their applications are summarised in Figure 1.8

ORGAN REGENERATION:



BIOASSAYS:

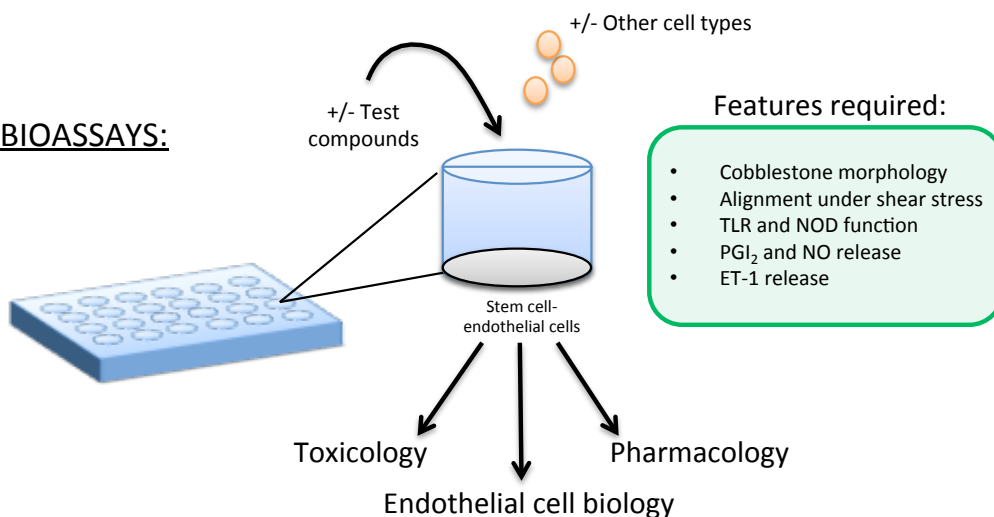


Figure 1.8. Applications and essential features of stem cell derived endothelial cells in 1. Organ engineering and 2. In *in vitro* bioassays to study endothelial cell biology and pharmacology. Prostacyclin (PGI_2), nitric oxide (NO), endothelin (ET-1), Toll-like receptor (TLR), nucleotide oligomerisation domain containing receptor (NOD). Heart image from commons.wikipedia.org: Patrick J. Lynch, medical illustrator; C. Carl Jaffe, MD, cardiologist. <http://creativecommons.org/licenses/by/2.5/>.

1.6 APPLICATIONS OF STEM CELL DERIVED ENDOTHELIAL CELLS *IN VITRO*

Stem cell derived endothelial cells, like other stem cells, can also be used to study endothelial cell biology and pharmacology *in vitro*. In particular, BOEC and iPSC-EC can be used to phenotype individual patient endothelial cells and be used as 'window to the vasculature'. This is especially true for BOEC as these cells are less manipulated *in vitro*.

In line with this, work by Ward et al (Ward et al., 2011) showed that EPCs from patients with coronary artery disease have decreased eNOS function and lacked angiogenic ability compared to EPCs from healthy donors. Further to this, Toshner et al (Toshner et al., 2009) showed evidence of the role of dysfunctional EPCs in PAH. In our group we have also shown that BOEC from patients with PAH have an increased pro-inflammatory phenotype which is relevant to PAH pathophysiology and vascular inflammation (George et al., 2013). Thus, it has become clear that EPCs or BOEC from patients with disease retain phenotypic differences in function that might impact therapeutic success and provide insights in disease pathophysiology.

The idea of using BOEC as an *in vitro* tool to study endothelial cells in disease is now established. Indeed, we have recently shown that BOEC from patients with pulmonary arterial hypertension have an increased inflammatory response to interferons (George et al., 2013). Others have shown that disease phenotype is retained in BOEC from patients with COPD (Paschalaki et al., 2013). Other groups have also combined iPSC technology with this field whereby BOEC are used as a substrate for iPSC which could subsequently be used as translational models of a range of cell types in disease (Geti et al., 2012). Clearly, it will be

important to characterise these cells for hallmarks of cell phenotype, and whether these cells retain a disease phenotype *in vitro*.

Another important potential area for innovation in using stem cell derived endothelial cells is in bioassays of drug efficacy or and/or toxicity. In my thesis I have applied this for the specific use in assays to detect cytokine storm reactions to biological drugs (biologics). The background to this area is discussed below.

1.6.1 Biologics

Biological therapies or biologics, which include antibodies and stem cells, are considered increasingly important for the treatment of human disease, and are set to dominate research efforts of the pharmaceutical industry. It is estimated that, by 2016, seven of the ten top selling drugs will be biological therapies (Malik, 2012). In line with this, the idea of tailoring therapies on a patient-by-patient basis in order to provide personalised medicine is now a key consideration in drug development.

The concept of biologics has been made possible following pioneering work by Kohler and Milstein, which provided technology for the production of mouse monoclonal antibodies against human targets (Köhler and Milstein, 1975). Significant advances since this landmark paper means that it is now possible to engineer humanized and fully human monoclonal antibodies to any target (Lonberg, 2005). This, for the first time, allowed for the development of monoclonal antibodies as therapies that target proteins in disease. An example of this is the use of anti-TNF antibodies in the treatment of rheumatoid arthritis (Feldman et al., 1998).

Importantly, because these medicines are human specific, biological therapies require the use of human tissue-based bioassays for measures of efficacy and safety. The case for the requirement of improved human tissue bioassays was emphasised by the unexpected profound cytokine release caused by the CD28 super agonist, TGN1412 (Suntharalingam et al., 2006).

1.6.2 TGN1412 and the Northwick Park Hospital drug trial disaster

TGN1412 was developed as an anti-CD28 superagonist to treat leukaemia and immune suppression. CD28 is a receptor expressed by T_{helper} cells and provides essential co-stimulation signals for lymphocyte activation and proliferation. Under physiological conditions, CD28 activation alone is not sufficient for cell activation which is dependent on simultaneous activation of CD28 and T-cell receptor/CD3 complexes by major histocompatibility complexes (MHC) on antigen presenting cells.

In the body, the ligands for CD28 include CD80 and CD86, and activation of downstream signalling requires co-stimulation by CD3/TCR complexes. A superagonist of CD28 is defined as any agonist that does not require these additional signals or CD3 to activate T-cells. It is noteworthy however that, the downstream pathway activated by anti-CD28 superagonists, is different to that of standard anti-CD28 antibodies in the presence of co-stimulation (Waibler et al., 2008). Nonetheless, the idea that T-cell activation could be manipulated through CD28 was the premise for the development of anti-CD28 superagonist therapy. The first of these drugs was developed by TeGerno and designated, TGN1412. This drug was developed for the treatment of rheumatoid arthritis and acute lymphoid leukaemia, where

activation of T-cell and IL-2 release where hypothesized to underlie therapeutic benefit in immunosuppressed patients.

The area of biologic therapy, and especially anti-CD28 targeted therapy was severely impacted in 2006. In the phase I clinical trial for TGN1412 six healthy volunteers experienced a profound and a violent systemic response referred to as a 'cytokine storm'. This occurred despite TGN1412 passing the required preclinical safety tests. Importantly, these tests not only included the use of standard laboratory animals and primates but also human T-cells *in vitro* (Stebbing et al., 2007) which were the target cell for this therapy. Whether this could have been predicted, based on what was known about CD28 and CD3 signalling at the time has been widely debated (Horvath et al., 2012; Hünig, 2012).

Cytokine storm describes a cascade of exaggerated inflammatory responses resulting in the release of more than 150 different cytokines and chemokines. The pathways involved in such a reaction are clearly complex and are the product of countless cell interactions, signaling cascades and dynamic interactions between a drug and the immune system. It is thought that T-cells, B-cells and monocytes are the central orchestrators of cytokine release syndrome. Cytokine release syndrome is characterized by rapid increases in the pro-inflammatory cytokines TNF α , IFN γ , IL-6, IL-10 and IL-2 which results in clinical phenotype akin to that of complement cascade activation or sepsis, including fatigue, headache, urticaria, throat swelling, fever, vomiting, hypotension and tachycardia. Whether different inducers of cytokine storm can induce different patterns and levels of cytokine release that correlates with severity however is unknown. A number of biologics are known to cause cytokine release syndrome. In fact, the first biologic licensed for use in humans;

muromonab-CD3 (or OKT3) causes mild to severe cytokine release syndrome in some patients. This reaction manifests as flu-like symptoms and appears associated with increased serum levels of TNF α , IL-2, IL-6 and IFN γ (Bugelski et al., 2009). The propensity for patients to experience cytokine release syndrome also varies from patient to patient (Bugelski et al., 2009) and the basis for this is also under investigation. For example, in patients receiving the mild cytokine storm causing drug, Rituximab, serum cytokine levels and severity of symptoms are greater in those with higher lymphocyte counts (Winkler et al., 1999). Clearly cytokine release syndrome and assays to study this adverse reaction in patient groups or subgroups will be critical to future success.

After the TGN1412 drug trial disaster however, we now know that using simply the target cells is not sufficient to detect cytokine storm responses to biologics. In this regard, the TGN1412 disaster defined an urgent unmet need for improved bioassays to detect cytokine storm and understand CD28 signalling *in vivo*.

A number of key things are critical to point out:

- As these drugs are highly human specific, animal models of efficacy and toxicity might be unsuitable.
- Studying responses in pure target cells does not capture the cell interactions involved in response to biologics, especially those affecting immune or vascular pathways, and are therefore not always adequate.
- As far as possible, assays must reflect the *in vivo* situation and, importantly, capture the cells that are involved in cytokine storm responses (vascular and immune). This may involve a combination of cells in co-culture assays.

- Clearly there is an opportunity for innovation in this area using stem cell derived cells in assays to study cells and human tissues.

1.6.3 Current assays that detect cytokine storm reactions to drugs

A number of assays to detect cytokine storm responses to biologics have been developed and these are described below. To date, there is no consensus on the optimum assay to detect cytokine storm responses.

Antibodies immobilised to plastic

We now know that TGN1412 does not activate peripheral blood mononuclear cells (PBMCs) to release cytokines when antibodies are added in aqueous phase, unless there is an endothelial cell interface present (Findlay et al., 2011b) (discussed in detail below). However, when TGN1412 is immobilised by non-specific binding to a solid phase platform such as plastic it does active PBMCs to release cytokines (Findlay et al., 2010; Findlay et al., 2011b). Whilst, immobilising biologics to plastic provides a simple solution in the case of TGN1412, it might be argued this approach is not representative of the physiological environment nor necessarily capture the cells types that orchestrate cytokine storm responses.

Immobilised antibody assays, are indeed very sensitive to detect TGN1412, but also detect variable cytokine responses (IL-8 (or CXCL8), IL-6 and TNF α) to non-cytokine storm-inducing antibodies such as Tysabri (natalizumab), Avastin (bevacizumab) and Herceptin

(trastuzumab) (Findlay et al., 2011a). It is also likely that the immobilising protocol that works for TGN1412 may not do the same for a different cytokine-storm inducing antibody.

Whole blood

As our group (Bailey et al., 2012) and others (Wolf et al., 2012) have recently described, an alternative approach is to test therapeutic antibodies for cytokine storm responses using undiluted human whole blood. Human whole blood responds readily to TGN1412-like antibodies in a way that does not require, and is not enhanced by, co-culture with endothelial cells (Bailey et al., 2012). However, whole blood assays have recently been criticised (Thorpe et al., 2013a) are less sensitive (Bailey et al., 2012; Thorpe et al., 2013a), do not discriminate between mild and severe cytokine storm inducing therapeutics (Bailey et al., 2012) and thus do not adequately recapitulate the full extent of cytokine storm responses.

HUVEC:PBMC co-culture

Whilst TGN1412 does not activate PBMCs alone, it does activate PBMCs in co-cultures with endothelial cells. The idea of using endothelial cells and PBMCs in co-culture is not new, and is known to be important to capture the vascular-immune cell interactions that take place *in vivo* (Ashida et al., 1981; Hughes et al., 1990; Ward et al., 2009). The advantage of endothelial cell:PBMC co-culture assays for cytokine storm testing over other assays is accepted, as these assays recapitulate the cell interactions occurring in the vasculature (Findlay et al., 2011b). However, to date, endothelial cell: PBMC co-culture assays have relied on using endothelial cells and PBMCs from different donors, typically HUVEC or HAEC from one donor and PBMCs from another. These heterologous cell combinations therefore

represent an inflammatory situation akin to a graft versus host disease phenotype (Huang et al., 1994) which conceivably interferes with responses to biologics.

Clearly, the best type of endothelial cell: PBMC bioassay, where mixed donor immune response could be avoided, would be one that uses cells from the same donor. PBMCs are easy to obtain from blood donated by volunteers, but adult endothelial cells on blood vessels are inaccessible and can only be obtained from tissue removed in surgery or post mortem. In my thesis I applied stem cell approaches to devise a bioassay that allows for endothelial cells and PBMCs from the same donor to be used as cytokine storm detecting bioassays.

Current assays to assess cytokine storm responses to biologics are summarised in Figure 1.9

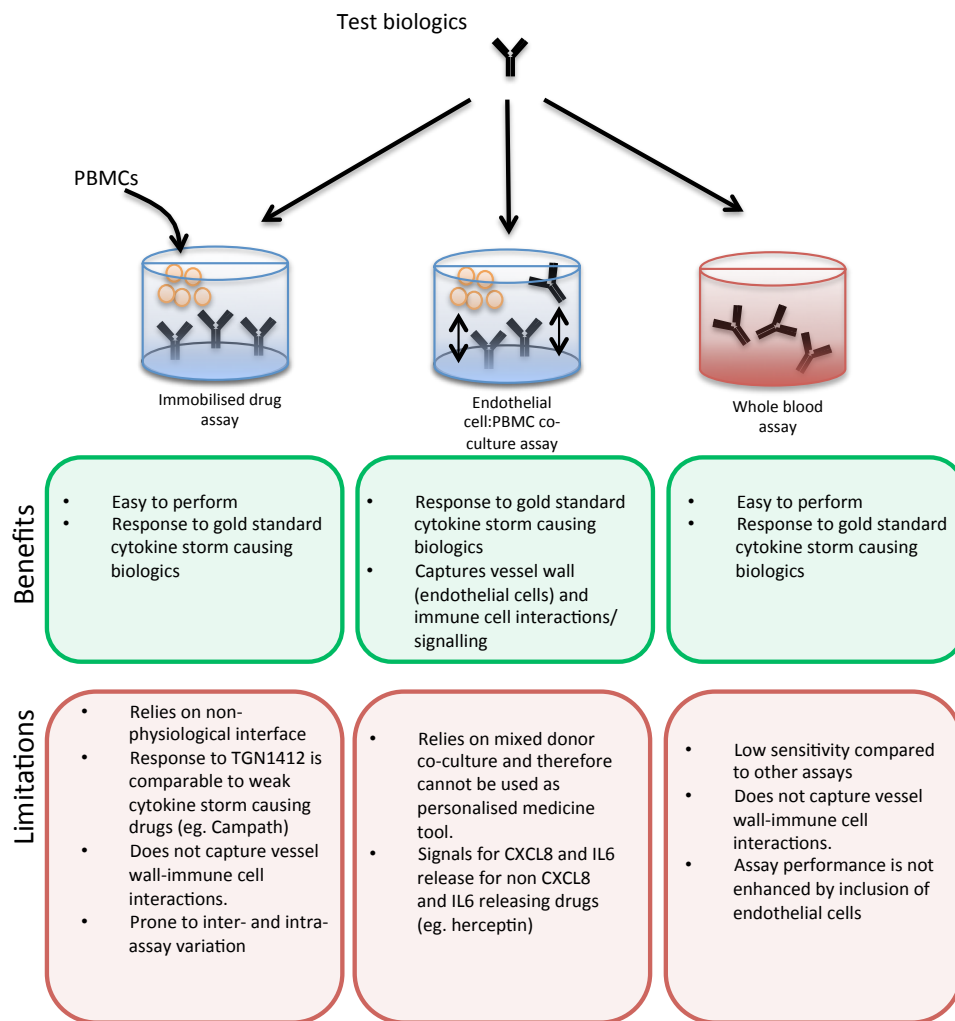


Figure 1.9 Benefits and limitations of current assays used to cytokine storm responses to biologics

1.6.4 Future directions in cytokine storm assays and endothelial:PBMC interactions

As discussed in detail, stem cell biology and research is offering significant advances in how we treat and study disease. These stem cell technologies can also be used to develop complex multi-cell *in vitro* assays to study disease and test therapies for patients (Chapin and Stedman, 2009; Ferreira and Mostajo-Radji, 2013). In the final stages of my PhD, I went on to consider therefore how stem cell derived endothelial cells could be applied in a pharmacological bioassay to detect cytokine storm responses to biologics.

1.7 SUMMARY

Endothelial cells are critical to cardiovascular health. Endothelial cells support the health of cardiovascular system through the release of cardioprotective hormones such as prostacyclin and NO, which rely on the concerted actions of enzymes expressed by endothelial cells. Many of these pathways are regulated by shear stress as a result of the flow of blood through the vessel. Endothelial cells are also critical in innate immunity and respond to pathogens to release cytokines and recruit professional immune cells, ultimately resulting in infection resolution. Pathogen sensing is conferred through the expression of pattern recognition receptors including TLR4 and NOD1.

Endothelial cell dysfunction results in disease and can be driven by aberrant signaling, or loss of function in any one of these pathways. As such, endothelial cell dysfunction is a target for pharmacological therapy with most therapies act either to mimic cardioprotective pathways (prostacyclin or NO) or block cardiotoxic pathways (ET-1).

Stem cells have the capacity to change how we think about and treat disease. Endothelial cells can now be derived from stem cells. Using stem cell derived endothelial cells, a patients own cells can be used to engineer autologous organs as well as be used to recapitulate cardiovascular 'disease in a dish'. Stem cell derived endothelial cells therefore have applications spanning a number of biomedical and clinical research areas. The characterisation of these cells *in vitro* for their ability to align under shear stress, release vasoactive hormones and response to pathogens will be essential for their optimal use in any and all settings.

This forms the premise of my PhD that; characterization of stem cell derived endothelial cells will be essential for their optimal use in therapy and *in vitro* assays. In the later stages of my PhD, I went on to apply stem cell derived endothelial cells in a pharmacological bioassay to detect cytokine storm reactions to biologics. I also used BOEC to phenotype a patient with a homozygous mutation in the cPLA₂ gene (PLA2G4A), which results in complete loss of function.

General hypothesis:

Endothelial cells derived from stem cells function as endothelial cells from vessels and can be used to make bioassays and to study vascular phenotype in patients with disease.

Aims:

1. To characterise endothelial cells derived from different stem cells for their ability to align under shear stress, respond to PAMPs and release vasoactive hormones (prostacyclin and NO) similarly to endothelial cells from vessels.
2. To further study TLR4 and NOD1 pharmacology in hESC-EC, and their response to live Gram-negative bacteria.
3. After selection of an appropriate stem cell derived endothelial cell, to employ these cells in a bioassay to detect cytokine storm responses to biologics
4. To use BOEC to phenotype a patient with a mutation in the gene encoding cPLA_{2α}.

CHAPTER 2: GENERAL METHODS

2.1 MEDIA AND SOLUTIONS

The cell culture media used in my PhD was of particular importance. In order to select for, expand and maintain stem cell derived endothelial cells, endothelial cell media was carefully selected from suppliers and where modifications were made these have been explained in detail.

2.1.1 Mouse embryonic fibroblast conditioned media

Human embryonic stem cells were maintained in their undifferentiated state in mouse embryonic fibroblast (MEF) conditioned medium. The principle of this approach is provided in section 2.2 Cells.

MEF conditioned media is commercially available or can be prepared in-house according to published protocols (<http://www.geron.com/PDF/scprotocols.pdf>). For my PhD, MEF conditioned media was prepared by members of Heart Science, Imperial College London. MEF conditioned media was prepared as described in Brito-Martins et al. (Bruto-Martins et al., 2008) as below:

“After isolation and expansion in culture, MEFs were mitotically inactivated at passage 3. After overnight attachment of 12 million inactive mouse embryonic fibroblasts onto pre-gelatinized T225 flasks in 10% fetal calf serum, medium was replaced with ‘human embryonic stem cell medium’ containing 20% knockout serum replacement, (Life Technologies, UK; Cat no. 10828-028) 1 mM L-glutamine, 10mM non-essential amino acids (1% of stock), 0.1mM β -mercaptoethanol, antibiotics (50U/ml penicillin 50 μ g/ml

streptomycin) and 4ng/ml basic human fibroblast growth factor. Collections of 150 ml of mouse embryonic fibroblast-conditioned medium were made daily for a week. Undifferentiated H7 cells were fed each day with filtered MEF-conditioned medium, which was further supplemented with 8 ng ml⁻¹ basic human fibroblast growth factor. Before subculturing or induction of differentiation, the latter usually 1 week following subculturing, spontaneously differentiated fibroblast-like cells among the ESC colonies were removed by treatment with collagenase for 10 min at 37 °C.”

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2.2.2 Lonza-EGM2 medium

Lonza-EGM2 is supplied in multiple components. Lonza-EBM2 basal medium (Lonza, Belgium; Cat no. CC-3156) and a EGM2 SingleQuat® Growth Factors and Supplements (Lonza, Belgium; Cat no. CC-4176). When combined this provides Lonza-EGM2. The supplements are selected growth factors and hormones to support endothelial cell growth. Details provided by the supplier are shown in Table 2.1.

Lonza EGM2 Bulletkit CC-4176 components
Human endothelial growth factor
Hydrocortisone
Gentamycin Amphotericin-B
10ml Foetal Bovine Serum
Vascular endothelial growth factor
Human fibroblast growth factor-B
R3-insulin growth factor
Ascorbic acid
Heparin (not in Lonza-EGM2-MV)

Table 2.1 List of supplements and growth factors in Lonza-EGM2 SingleQuot supplements CC-4176. Table drawn from information in the product information sheet at lonza.com.

For isolation of blood outgrowth endothelial cells (BOEC), the detailed method for which is described below, media was further supplemented with 10% Hyclone foetal bovine serum (FBS) (Fisher Scientific, Dublin, Ireland; Cat no. HYC-001-330Y and 10703464). Hyclone FBS is an advanced preparation of FBS and supplier information states the following advanced handling steps:

“Defined FBS is filtered through serial 40nm (0.04µm) pore size rated filters, which are the most retentive filters used in commercial FBS production. Defined FBS is the highest quality FBS available and is widely used by those cell culturists who have a concern for viral contaminants and require an extensive biochemical profile. This type of filtration is a practical, cost-competitive method of viral load reduction. Data shows that 40nm filtration

will remove as many as eight logs of viral challenge. This is a dramatic improvement over the current industry standard of using filters with mean pore sizes of 100nm. Studies demonstrate that this filtration regime has no adverse effect on cell growth. Product is of U.S origin”

**Information from product information page at <http://www.webshop.fishersci.com>. Accessed February 2014.*

Most other standard serum preparations are filtered in-house using 0.2µM filters. Upon arrival the Hyclone FBS was heat inactivated to deactivate and destroy any remnants of serum complement proteins that would cause inflammatory activation of cells. As per manufactures instructions 500ml bottles of Hyclone FBS, were thawed out overnight, and heated to 56°C and maintained at 56°C for 30 minutes. Serum was then allowed to cool to room temperature with inversion and mixing of the serum at 10 min intervals.

All experiments on endothelial cells and stem cell derived-endothelial cells, unless otherwise stated in figure legends, were carried out in Lonza-EGM2 with 10% Hyclone FBS. Where possible protocols comparing responses of different cell types to agonists, cells were also run head-to-head in protocols in identical media.

2.2.3 Vasculife® endothelial cell medium

Induced pluripotent stem cell (iPSC) derived endothelial cells (iPSC-EC) were purchased from Cellular Dynamics International (iCell® Endothelial Cells; Cat no. ECC-100-010-001; discussed in a later section) and maintained in a specially formulated ‘Maintenance Medium’. This was prepared by addition of a proprietary iCell® Endothelial Media Supplement (Cellular

Dynamics, California, USA; Cat no. ECM 100-031-001) and all components of a Vasculife® VEGF Medium Complete Kit (Lifeline Cell Technology, Maryland, USA; Cat no. LL-0003), with the exception of FBS as per instructions from Cellular Dynamics, to 500ml of Vasculife® Basal Medium (Lifeline Cell Technology, Maryland, USA; Cat no. LL-0002). Media was stored at 2-8°C for not longer than one month. The contents of the Vasculife® Medium Complete kit (LL-0003) is given in Table 2.2.

Component	Volume/500ml
rh VEGF LifeFactor	0.5 ml
rh EGF LifeFactor	0.5 ml
rh FGF basic LifeFactor	0.5 ml
rh IGF-1 LifeFactor	0.5 ml
Ascorbic Acid LifeFactor	0.5 ml
Hydrocortisone Hemisuccinate LifeFactor	0.5 ml
Heparin Sulfate LifeFactor	0.5 ml
L-Glutamine LifeFactor	10 ml
iCell Endothelial Cells Medium supplement	50 ml

Table 2.2 List of supplements and growth factors in Vasculife® Medium Complete kit LL-0003. Table drawn from information in the product information sheet at cellulardynamics.com.

To ensure consistency in protocols however, iPSC-EC used in experiments in my PhD were plated in Lonza-EGM2 with 10% Hyclone FBS as described above.

2.2.4 Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) medium

DMEM (Cat no. D5546) and RPMI (Cat no. R0883) were purchased from Sigma UK. To both formulations the following standard supplements were added: Non-essential amino acids (Life technologies, UK; Cat no. 11140-050), L-glutamine (25mM), Penicillin-Streptomycin and 10% heat inactivated foetal calf serum (FCS).

2.2.5 Matrix proteins and plate coating

Since in vessels endothelial cells adhere to extracellular matrix proteins, it is important to mimic this when growing endothelial cells in culture. To achieve this, cell culture plates can be coated with matrix proteins including gelatin, fibronectin and collagen. To coat plates with gelatin, gelatin solution (Sigma, UK; Cat no. G-1393) was added to cell culture plates at a concentration of $100\mu\text{g}/\text{cm}^2$ (1% solution in PBS) and plates incubated for 10 minutes at 37°C , 5% CO_2 , followed by aspiration. To coat plates with collagen, type-1 rat tail collagen solution (BD Biosciences, USA; Cat no. 35423) was prepared in 0.02N glacial acetic acid, according to manufacturer's instructions, at a concentration of $50\mu\text{g}/\text{ml}$ and used to pre-coat surfaces used for BOEC isolation and maintenance. Coating was achieved by adding $5.2\mu\text{g}/\text{cm}^2$ (1ml) collagen solution and incubating at 37°C , 5% CO_2 for 1 hour prior to washing three times with 1 x phosphate buffered saline (PBS) without MgCl_2 or CaCl_2 (Sigma, UK; Cat no. 59321C). Fibronectin coating was achieved by addition of fibronectin solution (Invitrogen, UK; Cat no. 33016-015) at a concentration of $3\mu\text{g}/\text{cm}^2$. Details of which matrix proteins were in use for particular protocols are stated in the relevant sections below.

2.2 REAGENTS AND DRUGS

2.2.1 TLR and NOD agonists

As discussed, Toll-like receptors (TLR) and nucleotide oligomerisation domain (NOD) receptors, respond to pathogen associated molecular patterns (PAMPs). Different TLRs and NODs respond to specific PAMPs to result in activation of downstream signalling pathways, which activate innate immune responses. These PAMPs, or mimics thereof, are commercially available and can be used to activate specific TLRs and NOD receptors. The function of particular receptors on cells in culture can therefore be assessed. The agonists of TLR1-9 and NOD1 are defined in Table 2.3

PRR	Pathogen	PAMP	PAMP used in vitro	Expression in Endothelial Cells
TLR1/ TLR2	Gram positive bacteria	Lipopeptides,	Pam3CSK4	+
TLR2	Gram positive bacteria	Lipopeptides,	HKLM	+
TLR6/ TLR2	Gram positive bacteria	Lipopeptides	FSL-1	+
TLR4	Gram negative bacteria	LPS	LPS	+
TLR5	Gram positive and negative flagellated bacteria	Flagellin	Flagellin	+
TLR3	RNA Viruses	dsRNA	Poly(I:C)	+
TLR7	RNA Viruses	ssRNA	Imiquimod	-
TLR8	RNA Viruses	ssRNA	ssRNA40	-
TLR9	DNA Viruses	CpG-DNA	ODN2006	+
NOD1	Gram negative bacteria	Peptidoglycan fragments	C12-iE-DAP and iE-DAP	+

Table 2.3 Agonists and pathogen associated molecular patterns (PAMPs) and their associated receptors. ‘PAMPs used *in vitro*’ were used to activate their specific receptors in cell treatment protocols.

TLR1-9 agonists were purchased as a kit of agonists (Invivogen, UK; Cat no. tlr1-kit1hw). All agonists were prepared from lyophilized powders according manufacturer’s instructions.

Preparation instructions are shown below in Table 2.4

Product	Working concentration	Stock solution concentration	Volume of solvent
Pam3CSK4	0.1 – 1µg/ml	100µg/ml	100µl H ₂ O
HKLM	10 ⁸ cells/ml	10 ¹⁰ cell/ml	100µl H ₂ O
Poly (I:C)	10ng – 10µg/ml	1mg/ml	500µl H ₂ O
Poly (I:C) LMW	30ng – 10µg/ml	1mg/ml	500µl H ₂ O
LPS	10ng – 10µg/ml	100µg/ml	1ml H ₂ O
Flagellin	10ng – 10µg/ml	100µg/ml	100µl H ₂ O
FSL-1	1ng - 1µg/ml	100µg/ml	100µl H ₂ O
Imiquimod	0.25 – 10µg/ml	100µg/ml	250µl H ₂ O
ssRNA40	0.25 – 10µg/ml	100µg/ml	250µl H ₂ O
ODN2006	5µM	500µM	26µl H ₂ O

Table 2.4 Table from Invivogen TLR1-9 agonist kit product information leaflet showing instructions for preparation of stock concentrations. Table drawn from information in the product information sheet at invivogen.com.

The NOD1 agonist γ -D-glutamyl-meso-diamopimelyl-D-alanine (iE-DAP) (Invivogen, USA; Cat no. tlrl-dap) is a commonly used NOD1 agonist and represents the minimal motif recognised by NOD1. In most experiments in my PhD however I have used a form of iE-DAP that has a lipophilic lauroyl group added to its structure (C12-iE-DAP) (Invivogen, USA; Cat no. tlrl-c12dap). This increases membrane permeability, and potency of the iE-DAP moiety on NOD1. The rationale for the use of this more potent agonist in my PhD was to enable comparisons to be made between TLR4 and NOD1, which are expressed by endothelial cells, without the permeability of the cell to iE-DAP being a limiting factor.

2.2.2 Pharmacological antagonists

To study the NOD1 signalling in stem cell derived-endothelial cells in more detail, the rationale for which is given in Chapter 3 and 4, antagonists of key proteins were used.

Inhibitors used were: the dual p38 MAPK and RIP2 inhibitor (SB203580; Merck, Germany), the IKK inhibitor (SC-514; Tocris Bioscience, UK), and the p38 MAPK selective inhibitor (BIRB0796; Axonmedchem, Netherlands). Novel inhibitors of NOD1 (GSK'214) and its associated adapter protein, RIP2 (GSK'214) were a gift from Dr John Bertin and Dr Peter Gough, GSK, Philadelphia, USA. Precise structural details of these drugs are not available but we have previously validated these drugs as inhibitors of NOD1 agonist induced cytokine release in endothelial cells (Gatheral et al., 2012).

To study cytosolic phospholipase A₂ (cPLA₂) in endothelial cells, the rationale for which is given in Chapter 6, the cPLA₂ inhibitor pyrophenone (PYR; Biomol, Germany; Cat no. Cay13294-5) was used.

2.2.3 NOD1 Small interfering (si)RNA

Targeting NOD1 siRNA (Qiagen, UK; Cat no. SI02655450) was used according to manufacturer's instructions. The NOD1 siRNA was supplied as premix, which contains siRNA molecules and proprietary stabilisers. This solution can be added directly to wells of a cell culture plate to give a final concentration of 25nM. The ability of the NOD1 siRNA molecule selected to knockdown NOD1 expression was assessed at the gene level (mRNA expression) and functional level (CXCL8 release in response to NOD1 agonist). Protocols using siRNA and the principle of the approach is discussed in detail in a later section.

AllStars Negative control siRNA which does not target any known mammalian gene was also purchased from Qiagen, UK (Cat no. SI03650318). This was also used according to manufacturer's instructions.

2.2.4 *Haemophilus influenzae*

Haemophilus influenzae: strain code 49247, was purchased from the American Type Culture Collection (ATCC) and provided for my PhD under collaboration with Dr Aurica Telcain, Dr Mike Edwards and Prof. Sebastian Johnston in the Dept. of Respiratory Medicine, Imperial College London. *Haemophilus influenzae* was provided in ATCC® Medium 814: GC Agar/Broth at unknown concentration. *Haemophilus influenzae* from this broth was then seeded on to Columbia Agar/Chocolate Horse Blood plates (Oxoid, UK; Cat no. PB0124A) and incubated overnight at 37°C 5% CO₂. A single colony was then isolated and multiplied further in fresh Columbia Agar/Chocolate Horse Blood plates. From these multiplied cultures, *Haemophilus influenzae* was scraped off and suspended in 2ml Brain Heart Infusion Broth (Oxoid, UK; Cat no. CM1135B) and added to 18ml DMEM with 10% glycerol. This solution was then aliquoted in to 1ml vials and frozen immediately at -80°C. Samples of these vials were expanded on Columbia Agar/Chocolate Horse Blood plates to determine colony forming units (CFU)/ml.

Further dilutions for experiments were made in Lonza-EGM2 medium for use in assays, which are described later. ATCC lists *Haemophilus influenzae* at Biosafety Level 2 which state:

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the current publication of the Biosafety in Microbiological and Biomedical Laboratories from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes for Health.

From: <https://www.lgcstandards-atcc.org/~ps/49247.ashx>. Accessed February 2014.

With this information, *Haemophilus influenzae* when used in the laboratory can be carried out using category II biological safety cabinets, but these cabinets must be dedicated for the handling of pathogenic material and experiments must be carried out in isolation from other non-pathogen related cell culture work. As a result, for experiments using *Haemophilus influenzae* in my PhD, these were carried out in the Dept. of Respiratory Medicine, Imperial College London under collaboration with Prof. Sebastian Johnston. Experiments were carried out with the help and support of Dr. Mike Edwards and Dr. Aurica Telcian in this department.

Haemophilus influenzae, is a Gram-negative bacteria which expresses LPS and Gram-negative bacterial peptidoglycan and is reportedly sensed by TLR4 (Hirano et al., 2009) and NOD1 (Ratner et al., 2007) respectively. In infection protocols, discussed fully below, *Haemophilus influenzae* was used at colony forming unit concentrations of 10^8 - 10^5 for 24hours. In these experiments a 'filter control' was also included. This control represents a bacteria free eluent of conditioned media produced by filtering cultures through a 30-kDa membrane (Ultrafree-0.5 PBTk Centrifugal Filter Unit 30kDa Millipore UFV5BTK00) (Millipore, Gloucestershire, UK). When 10^7 CFU/ml of *Haemophilus influenza* was filtered in this way and added to cells, CXCL8 release was reduced to below basal (Figure 2.1). This

illustrates cell activation by the 10^7 CFU/ml *Haemophilus influenzae* stock was a function of the bacteria and not a broth consistent.

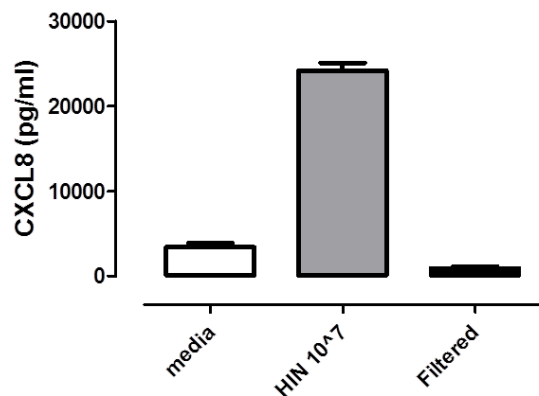


Figure 2.1 Representative data (n=3) of effect of *Haemophilus influenzae* (HIN) broth constituents on CXCL8 release from human umbilical vein endothelial cells (HUVEC).

2.2.5 Therapeutic antibodies

Therapeutic antibodies were selected for testing in cytokine storm assays to include gold-standard cytokine storm inducing drugs as well as clinically used and mild cytokine storm inducing drugs, as well as negative control antibodies that are used clinically but do not cause cytokine storm.

The biologics/therapeutic antibodies, their clinical use and cytokine storm profile in summarised in Table 2.5.

Biologic	Human target	Antibody type	Clinical indication	Cytokine storm profile	Response of current assays
TGN1412	CD28 (superagonist)	humanized	Leukemias; rheumatoid arthritis. WITHDRAWN IN 2006	Severe. Life saving organ support was required in all phase I trial patients	Severe inducer of CXCL8, IL-6, TNF α , IFN, IL-2
ANC.28.1	CD28 (superagonist)	Mouse anti-human	None (TGN1412 model drug)	NA	Severe inducer of CXCL8, IL-6, TNF α , IFN, IL-2
Campath (alumtuzumab)	CD52	humanized	Lymphoid malignancies and prevention of allograft rejection.	Mild. Steroids and antihistamines often co-prescribed	Severe inducer of CXCL8, IL-6 and weak inducer of IFN in some assays. Response in some assays= TGN1412 except for IL-2 and TNF α
Herceptin (trastuzumab)	HER2 (EGFR-2)	humanized	HER2+ breast cancer, HER2+ gastric adenocarcinoma	None	Mild IL-8 and IL-6 induction in some assays
Avastin (bevacizumab)	VEGF	humanized	Metastatic colorectal cancer, non-squamous cell lung carcinoma, metastatic kidney cancer, glioblastoma	None	Mild IL-8 and IL-6 induction in some assays
Arzerra (Ofatumumab)	CD20	human		None	None
OKT3 (Muromonab-CD3)	CD3	Mouse anti-human	Chronic lymphoid leukaemia – refractory to Campath.	Mild to severe. Steroids and antihistamines often co-prescribed	Severe inducer of CXCL8, IL-6, TNF α , IFN and weak induced of IL-2 in some assays

Table 2.5 Summary of characteristic of biologics studies in Chapter 5.

As shown in Table 2.5, antibodies can be chimeric, humanized or fully human. Chimeric antibodies are antibodies that have had the animal Fc region replaced with a human Fc region, examples of which include that anti-TNF α drug Etanercept. Humanised antibodies are distinct from chimeric antibodies as they have gone through additional processing to replace any animal protein with human protein, although the specific complementarity

determining regions which bind the target protein often remain of animal origin. Many antibodies approved for use in therapy are humanized and examples include Campath, Herceptin and Avastin. Technology also exists that enables the production of fully human therapeutic antibodies. Such approaches typically involve using a 'phage display system'. This protocol involves use of gene libraries of specific antibody sequences which when transfected into phage bacteria are transcribed and produced in high quantities. It should be noted that this approach yields only the Fab fragment of the antibody and so requires additional steps, such as those above, to include human Fc proteins to form a full human antibody structure.

2.3 CELLS

2.3.1 Human embryonic stem cells (hESC) and hESC-derived endothelial cells (hESC-EC)

hESC-EC were derived from the H7 cell line of hESC according to a protocol that has been published and developed in the literature (Levenberg et al., 2002; Li et al., 2009). The H7 cell is one of many available hESC lines. Some of these cell lines have normal XY karyotype (H1, H13 and H14 for example) and some have normal XX karyotype (H7 and H9 for example). The genotype and phenotype of these cells in terms of most pluripotency marker expression and telomerase expression does not differ between the cell lines. H7 hESC express stage-specific embryonic antigen (SSEA)-3, SSEA-4, TRA-1-60 and TRA-1-80. Only SSEA-3 expression varies between hESC lines but this is thought to be due to restricted access of the SSEA-3 antibody to the epitope (Thomson et al., 1998). The H7 line of hESC were derived by James Thomson's lab (Thomson et al., 1998) from the pre-implantation blastocyst of fertilized embryos surplus to *in vitro* fertilisation (IVF) procedures. Cells were cultured to the blastocyst stage and the inner cell mass isolated. hESC, like human embryonal carcinoma cells and mouse embryonic stem cells, form all three germ layers when injected in SCID mice. These are endoderm (epithelial cells), mesoderm (endothelial cells, cardiac myocytes, cartilage, bone, smooth muscle) and ectoderm (neuronal epithelium and stratified squamous epithelium).

Experiments using hESC in my PhD were approved by the UK Stem Cell bank and under collaboration agreements with the Geron Corporation (Menlo Park, CA, USA). As hESC undergo spontaneous differentiation in culture, hESC were maintained in MEF conditioned media which suppresses differentiation without affecting proliferation. This occurs as mouse

embryonic fibroblasts secrete as many as 136 proteins that are involved in growth and differentiation (Lim and Bodnar, 2002). The precise proteins involved in maintaining pluripotency remains under investigation.

Undifferentiated cells were maintained in the MEF conditioned media as described above on Matrigel (BD Biosciences UK; Cat no. 356234)-coated 6-well plates (Nunc, Denmark; Cat no 140675). For differentiation of hESC to hESC-EC well developed published protocols were followed. hESC were dissociated into clumps and plated on ultra-low attachment plates (Nunc, Denmark; Cat no: 145383) and incubated in Lonza-EGM2 complete media (see Table 2.1) to allow formation of embryoid bodies. Embryoid bodies are 3D structures that contain endoderm, mesoderm and ectoderm cells, which arise from hESC. After 4 days of spontaneous differentiation and growth, embryoid bodies were re-plated on gelatin-coated 6-well plates (Sigma-Aldrich, St Louis, USA; Cat no. G1393) in Lonza-EGM2. After 13 days cells were stained for the endothelial cell marker CD31 using an AlexaFluor 488 fluorescence dye labelled anti-CD31 antibody (BD Biosciences, Oxford, UK) (see staining protocols below). Cells were sorted using a FACS Aria II cell sorter (BD Biosciences, Oxford, UK) and expanded in gelatin coated-96-well plates (Nunc, Denmark) in Lonza-EGM2 medium for further use. Cells were maintained in Lonza-EGM2 media and grown on gelatin coated 75cm² flasks (Nunc, Denmark; Cat no. 178905). Cells were used between passages 2-8.

The protocol for differentiation of hESC in to hESC-EC is summarised in Figure 2.2

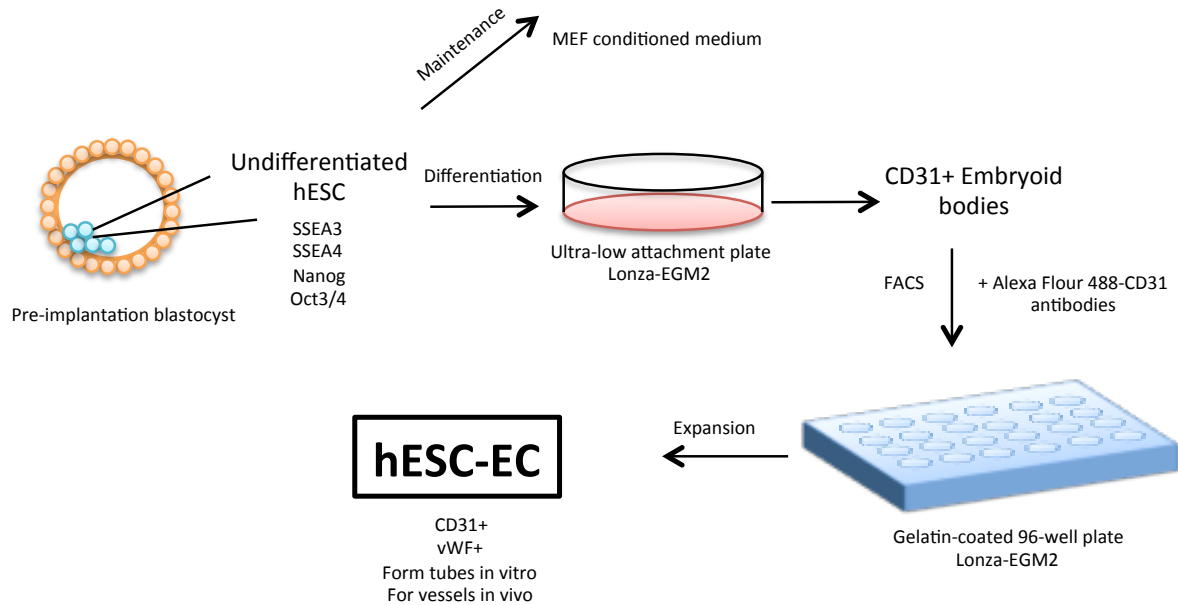


Figure 2.2 Summary of protocol for maintenance of hESC and differentiation in to hESC-EC.

2.3.2 Blood outgrowth endothelial cells (BOEC)

BOEC were isolated according to published protocols (Ingram et al., 2004; Martin-Ramirez et al., 2012; Reed et al., 2011; Starke et al., 2010) with minor modifications. This protocol is discussed in detail under appropriate subheadings below.

Principle of density gradient separation of human blood

BOEC are thought to arise from a CD34+ population of cells and can be isolated from the peripheral blood mononuclear cell (PBMC) fraction using a density gradient approach. The concept of density gradient separation of cells in the blood has been around for decades. Today, density gradient separations are typically carried out using Histopaque® or Ficoll® gradients. Histopaque® and Ficoll® are commercially available preparations of polysucrose

and dextrans that separate to give a solution of different densities when a centrifugal force is applied. When a mixed cell population leukocytes, as in the blood is added these cells localise in the different areas of the Histopaque® or Ficoll® gradient to match the solution density, and so can be separated from blood and studied *in vitro*. Different types of Histopaque® are available which are optimised for the separation and isolation of certain cells. Histopaque® 1077 for example is used for separation of PBMCs from blood. For isolation of BOEC, achieving high yield of PBMCs is important so for these protocols, commercially available Ficoll® gradient tubes that are further enhanced for PBMC isolations were used.

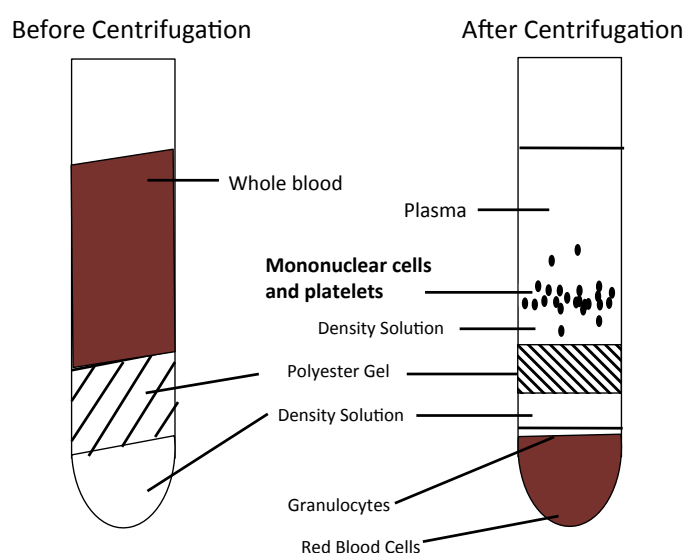


Figure 2.3 Illustration of the layering of PBMCs and other cell fractions in BD® CPT™ Ficoll Vacutainers. Figure drawn from information in the product information sheet at bdbiosciences.com.

Isolation and culture of BOEC

Blood (48ml; 6 x 8ml tubes/patient) was collected into tubes with Ficoll (Thill et al., 2008) (Figure 2.3), from healthy volunteers aged 24-45 and inverted 8 times as per manufacturer's instructions. Tubes were then centrifuged at 1600 RCF for 30 minutes at room temperature with maximum acceleration and braking rates to obtain PBMCs. Tubes were then inverted a further 8 times to allow mixing of the buffy coat (which contains PBMCs and platelets) and plasma fraction. The fractions of blood are shown in Figure 2.3. The contents of 8 tubes was then carefully pooled into a 50ml falcon tube and 10% FBS/PBS added to give a final volume of 50ml. Cells were then centrifuged at 520 RCF for 10 minutes with maximal acceleration and intermediate braking. The supernatant was discarded and pellets resuspended in 10ml of 10% FBS/PBS solution. This process was repeated a further two times giving three washes in total. After the final centrifugation, the pellet was resuspended in 10ml Lonza-EGM2 supplemented with 10% FBS (ThermoFischer, UK; Hyclone). A sample (10 μ l) of the suspension was then diluted in media (90 μ l) and 10 μ l added to a haemocytometer for counting. The counting procedure and the calculation for plating of cells at the appropriate density are shown in Figure 2.4.

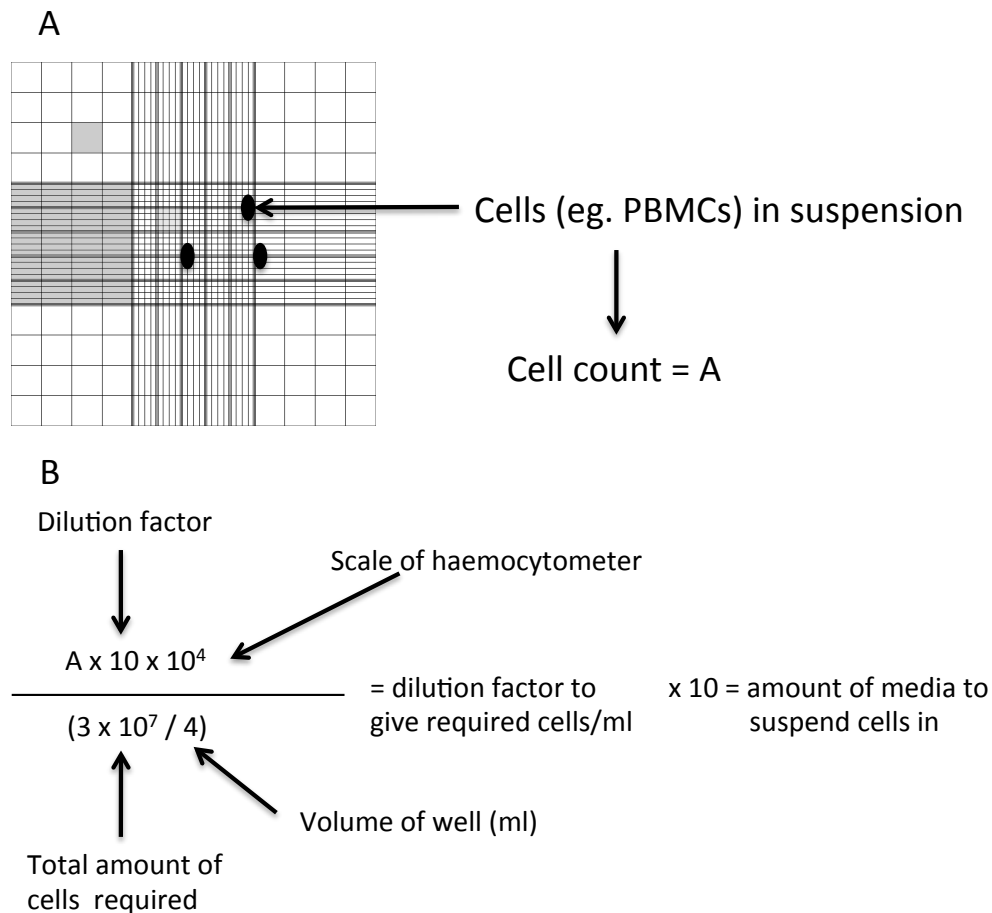


Figure 2.4 (A) Protocol for counting cells on haemocytometer and **(B)** Calculation of dilution of cells to required seeding density. Image of haemocytometer modified from en.wikipedia.com. Attribution: Zephyris at the English Language Wikipedia.

PBMCs were then resuspended in an appropriate amount of Lonza-EGM2 with 10% FBS and distributed across $5.2\mu\text{g}/\text{cm}^2$ collagen coated 6-well plates (Nunc, Denmark) at a density of 3×10^7 cells/well. Plates were incubated at 37°C , 5% CO_2 . After 24 hours media was carefully removed. As the BOEC-precursor is a rare cell and as adherence of this cell to the well is required, media changes were carried out using a P1000 pipette with extreme care and with plates kept flat at all times. Cells were then washed with 1ml Lonza-EGM2 with 10%FBS and plates tipped back and forth once only. This media was then removed, again with extreme care and 4 ml of fresh Lonza-EGM2 10% FBS added to each well. Cells were kept under close observation by light microscopy at all times, and when possible images captured. Within the

next 72 hour and at 24 hour intervals, this process was repeated a further two times. After 4 days media was replaced every 48-56 hours without washing until cobblestone colonies appeared. Colonies of endothelial cells typically emerged between days 5-21.

A summary of donor characteristics of BOEC isolated in my PhD is shown in Table 2.6.

DONOR ID	AGE	SEX	TIME TO COLONY APPEARANCE (days)
EPCA	25	M	20
EPCB	28	F	16 and 21
EPCC	41	M	8 and 12
EPCF	24	F	18
EPCM	-	F	11
EPCQ	26	F	5
EPCS	26	F	19
EPCZ	35	M	16
EPCAG	26	F	13
EPCAK	28	F	7

Table 2.6 List of donor characteristics with successful isolations of BOEC and used in my PhD. – indicates a missing record on this particular isolation.

Once colonies emerged they were allowed to expand for not more than 3-5 days. Colonies were removed by trypsin (Invitrogen, UK; TrypLE® 1x) digest using 2ml trypsin/well. Trypsin was neutralised with 4ml Lonza-EGM2 10% FBS and the 6ml cell/trypsin mix collected in a

50ml falcon tube and centrifuged at 190RCF for 5 minutes at room temperature with maximal acceleration and intermediate brake settings. Cells were then plated on and expanded and maintained on 5.2 μ g/cm² collagen coated 25cm² and/or 75cm² cell culture flasks (Nunc, Denmark) as for all other endothelial cells. Cells were tested for endothelial cell phenotype by morphology using a light microscope and expression of CD31 and/or VE-cadherin expression.

The protocol for the isolation of BOEC from blood is summarised in Figure 2.5

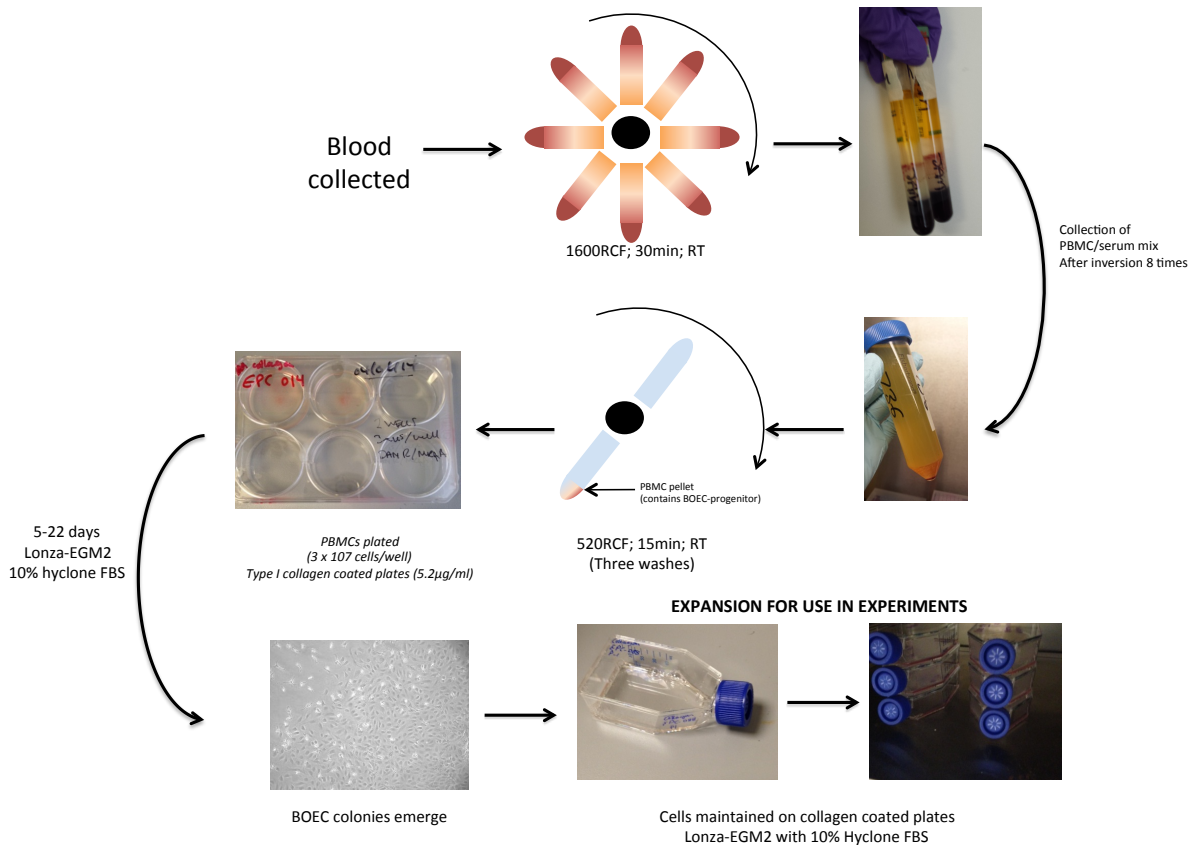


Figure 2.5 Summary of method for the isolation of blood outgrowth endothelial cells (BOEC) from peripheral blood mononuclear cells (PBMCs) for use in experiments. Abbreviations: room temperature (RT), foetal bovine serum (FBS).

Ethics for isolation of BOEC

For the collection of human blood, and the protocol for isolation of BOEC, ethical approval was granted by the Royal Brompton and Harefield Ethics Committee (ethics code: 08/H0708/69). Informed written consent was given by all participants. The consent procedure, and associated patient information sheets and consent forms, were approved by the Royal Brompton and Harefield Ethics Committee. Consent records were maintained as required by the Royal Brompton and Harefield Ethics Committee.

2.3.3 Induced pluripotent stem cell derived-endothelial cells (iPSC-EC)

iPSC-ECs are commercially available and were purchased from Cellular Dynamics international (Colorado USA; Cat no. ECC-100-010-001). Cells were supplied as a cryovial and recovered under manufacturer's instructions. Thus, iPSC-EC were cultured on fibronectin (Invitrogen, UK; Cat no. 33016-015) ($3\mu\text{g}/\text{cm}^2$) coated 75cm^2 cell culture flasks. Cells were maintained in Vasculife® VEGF Medium Complete Kit as indicated in the media and solutions section above.

Method for the production of iPSC-EC

As discussed in Chapter 1, iPSC are derived from somatic cells (fibroblasts) that have been reprogrammed to a 'pluripotent' state using retroviral or lentiviral packaged pluripotency genes (Takahashi et al., 2007). The method for differentiating iPSC into a iPSC-EC is in fact identical to that of the method for isolation of hESC-EC from hESC. The ability of iPSC to form iPSC-EC *in vitro* was first demonstrated by the same group to develop to reprogramming technology in 2009 (Taura et al., 2009). As the iPSC-EC used in my PhD (iCell® endothelial cells) were purchased from company, some data on the characterisation was available from the company and is show in Figure 2.6. Information supplied by the company states that these cells are derived from human foetal lung fibroblasts. Due to restrictions applied by the company I was not able to obtain the precise starting cell line used to generate these cells and so, for control experiments, I used human lung fibroblasts (HLFs) grown from tissue supplied surplus to clinical requirement from surgeries at the Royal Brompton Hospital, detailed below.

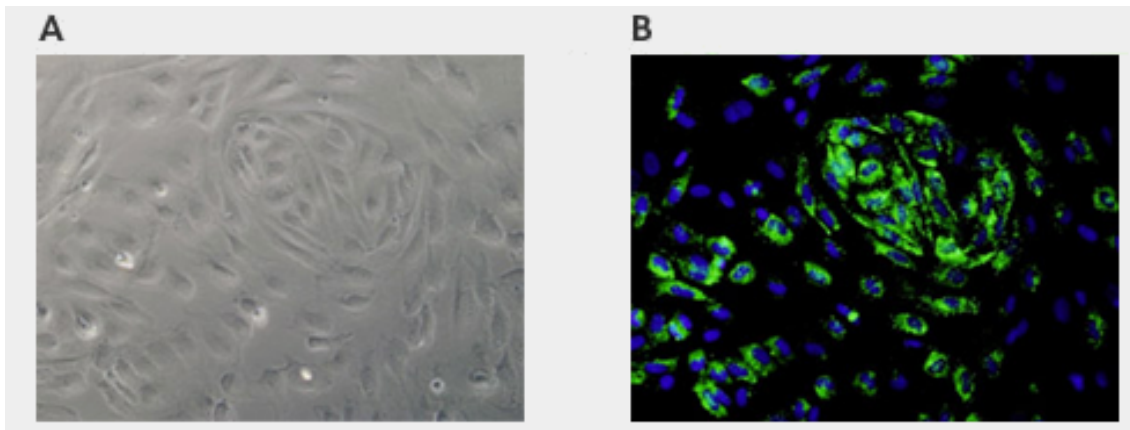


Figure 2.6 Representative of images of induced pluripotent stem cell derived endothelial cell (iPSC-EC) characterisation. **(A)** Cobblestone morphology and **(B)** von-willebrand factor expression of iCell endothelial cells purchased from Cellular Dynamics (Cat no. ECC-100-010-001).

Image is from iCell® endothelial cell product information sheet:
<http://www.cellulardynamics.com/>. With permission.

2.3.4 Isolation and culture HLFs

As a control for the iPSC-EC HLFs were used. HLFs isolated from human lung tissue as described previously (Maher et al., 2010) from histologically normal peripheral lung from lung cancer resection surgeries carried out at the Royal Brompton Hospital under a blanket ethics agreement. Cells were grown in supplemented DMEM with 10% FBS, and were passaged every 3–4 days. All experiments were performed from passage 3–10

2.3.5 Endothelial cells from vessels

Human umbilical vein endothelial cells (HUVEC) used throughout my PhD were a gift from Caroline Wheeler-Jones (Royal Veterinary College, London) and were maintained in Lonza-EGM2 medium. Cells isolated from fresh umbilical veins and used at passage 2 on arrival and used for experiments between passages 2–8.

Human lung microvascular endothelial cells (HMVEC), and human aortic endothelial cells (HAEC) were purchased from Lonza or Promocell as cryopreserved cells. Cells were grown out and maintained in Lonza-EGM2 media and grown on 1% gelatinised (Sigma, USA) 75cm² flasks. Cells were used between passages 2-8.

2.4 SHEAR STRESS MODELLING *IN VITRO*

Why model shear stress in vitro?

As discussed in Chapter 1, endothelial cells on vessels are exposed to physical forces and respond by aligning in the direction of flow. Shear stress in the vessel can be multidirectional/oscillatory or unidirectional/laminar and defines endothelial cell morphology (Meyer et al., 2009; Potter et al., 2011). In areas of unidirectional/laminar shear stress endothelial cells appear elongated and aligned whereas in areas of multidirectional flow they typically have a cobblestone appearance (Potter et al., 2011). Endothelial cells are very sensitive to their biomechanical environment and shear stress is known to affect gene expression and the development of atherosclerosis (Suo et al., 2007; Warboys et al., 2010). As such, the study of these pathways is important and was the focus of the PhD project of my colleague Dr. Claire Potter. To test the ability of endothelial cells to assume an aligned and elongated phenotype I employed this model and this is described below.

Modelling shear stress on the orbital shaker

Endothelial cells (hESC-EC, BOEC or HAEC) were plated on gelatin-coated 6-well plates (as described above) at a density of 100,000 cells/well and grown to confluence. Time to reach confluence in these conditions was typically 4 days in 2ml of Lonza-EGM2 with 10% FBS with a media change on the second day. Once confluent, media was replaced (2ml) and cells were either placed on a PS-300 orbital shaker (Grant Instruments, UK) or grown under normal static conditions, both at 37°C, 5% CO₂. The shaker was set to 150 RPM. The movement of the shaker results in a wave of media that travels around the well resulting in a complex pattern of shear applied with uni-directional/laminar shear stress towards the edge of the well, and non-directional/oscillatory shear at the centre. In this model

therefore, cells at the centre and edge of the well are analogous to those in the lesser and greater curvature of the aortic arch respectively. After application of shear stress for 4 days, cells were washed and fixed in 4% paraformaldehyde for staining and imaging. Alignment of cells was visualised by light microscopy using a Nikon microscope (Nikon, Japan; Eclipse TS100 with a Nikon VR ISO3200 camera attachment). In some experiments, cells were also stained for endothelial cell markers CD31 and/or VE-cadherin. Methods for staining and imaging are given in a later section. Figure 2.7 summarises the pattern of shear stress in a 6-well plate well on the orbital shaker.

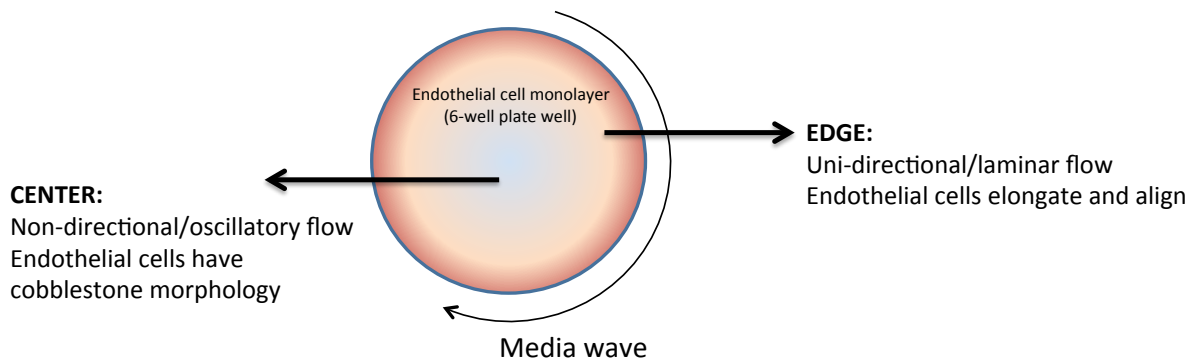


Figure 2.7 Features of shear stress forces at different locations in the well of a 6-well plate on the orbital shaker.

2.5 CELL TREATMENTS AND PHARMACOLOGICAL ASSAYS

2.5.1 Plating cells for experiments

For all experiments, unless otherwise stated in figure legends, stem cell derived endothelial cells or endothelial cells from vessels were plated on gelatin-coated plates (as described above) at a density of 7,000 cells/well in 96-well plates. Cell counts were determined as shown in Figure 2.4. Plating at this density ensures that the cells are added at sub-confluent levels, allowed to adhere and proliferate to form endothelial cell monolayers. As endothelial cells on vessels are constantly exposed to serum proteins and proliferation is inhibited by contact, serum starvation was not carried out for any protocols.

2.5.2 Activation of TLR and NOD receptors on endothelial cells

Treatment protocol

Cells were plated as described above. TLR1-9 agonists were prepared from stock solutions as described above. Supernatants from confluent cells on a 96-well plate were discarded and 180ul of fresh Lonza-EGM2 added followed by 20ul of drugs at 10x concentrated stocks to provide the desired final concentration in the well. Concentrations were selected based on supplier information or from extensive data on the potency and efficacy of these agonists from our group. Final concentrations of agonists were: heat killed *Listeria monocytogenes* (HKLM) (TLR2 agonist; 10^7 cells/ml), Pam₃CSK4 (TLR1/2 agonist; 1µg/ml), FSL-1 (TLR2/6 agonist; 1µg/ml), PolyIC (TLR3 agonist; 10µg/ml), LPS (TLR4 agonist; 1µg/ml), ST-FLA (*Staphylococcus aureus* derived flagellin) (TLR5 agonist; 100ng/ml), IMI (imiquimod) (TLR7 agonist; 1µg/ml), ssRNA40 (TLR8 agonist; 1µg/ml, ODN2006 (TLR9 agonist; 5µM). In most protocols IL-1β (1ng/ml) was also added to activate MyD88 and NF-κB signalling

independently of TLRs. In later protocols where cells were treated with the NOD1 agonists (iE-DAP; 1µg/ml or C12-iE-DAP; 10µg/ml), or *Haemophilus influenzae* (10^5 - 10^8 CFU/ml), LPS and IL-1β were again included to ensure cell responses were consistent across protocols and isolations of cells. All dilutions of drugs were carried out in media in Lonza-EGM2 medium. After 24 hours supernatants were collected and stored at -20°C for analysis. In some experiments, cells were treated for 1 hour, supernatants collected, and cells fixed for imaging and measurement of NF-κB nuclear translocation. Details of the protocols for this analysis are discussed below.

Principle of the assay

As TLRs activate innate immune pathways that culminate on defined transcription factors to cause non-specific inflammation, the function of TLRs and NODs can be assessed via measurement of ubiquitous markers *in vitro*. CXCL8 is released in response to NF-κB activation and is a ubiquitous marker of inflammatory activation. CXCL8 has been used in immunological and pharmacological assays *in vitro* for decades (Paul-Clark et al., 2012), and is easy to measure by simple immunoassay (discussed later). CXCL8 is also a critical mediator of innate immune responses and disease, and is increased following infection, sepsis, and chronic inflammatory disease. On the other hand, activation of TRIF and IRF transcription factors results in release of interferon-related protein-10 (IP10), which can be used as marker of TRIF activation in response to agonists.

Including IL-1β in these protocols indexes the function of MyD88 signalling in these cells. That is to say, a lack of response to any particular TLR agonist which signals through MyD88 but in cells that respond to IL-1β, suggests that the 'lacking component' is at the level of the

receptor. Knowing this is also useful as whether the effects of pathway inhibition of specific proteins and/or TLRs or NODs are specific can be determined by comparing responses to IL-1 β . This is discussed later in sections on the principles of assays for NOD1 knockdown and inhibition.

2.5.3 Infection assay

Treatment protocol

Cells were plated as described above. *Haemophilus influenzae* was prepared from a 10⁹ CFU/ml stock and diluted in Lonza-EGM2 medium to give final concentrations of 10⁸-10⁵ CFU/ml. Once suspended in media at 10x concentrated stocks, bacteria were added in 20ul aliquots to 180ul of media in the 96-well plate. Cells were also treated with LPS (1 μ g/ml), C12-iE-DAP (10 μ g/ml) and IL-1 β (0.01-0.1ng/ml). After 24 hours supernatants were collected and stored at -20°C for analysis.

Principle of the assay

As discussed, Gram-negative bacteria are sensed by two key PRR pathways: TLR4, which is the receptor for LPS, and NOD1, which is the receptor for peptidoglycan moieties. In order to test the potential of endothelial cells (hESC-EC and HUVEC for these protocols) to respond to whole Gram-negative bacteria, cells were infected with *Haemophilus influenzae*. As with other types of Gram-negative bacteria, *Haemophilus influenzae* is reportedly sensed by cells via TLR4 and/or NOD1 PRR pathways (Hirano et al., 2009; Ratner et al., 2007). The relative contribution of TLR4 and NOD1 was then assessed using HUVEC, hESC-EC (which are TLR4-deficient), NOD1 targeting siRNA, and pharmacological inhibitors of NOD1 and RIP2.

2.5.4 siRNA knockdown of NOD1

Treatment Protocol

To achieve knockdown of NOD1 at the gene level, an siRNA knockdown approach was taken. Cells were plated as described above. Supernatants were removed and 188µl of fresh Lonza-EGM2 was added. Importantly, the antibiotic (gentamicin-amphotericin-B) was excluded from this media as this; according to the siRNA manufacturer's (Qiagen, UK) instructions will interfere with the interaction of the siRNA with the cell DNA. Accordingly, 12ul of NOD1 siRNA premix or negative control were added to the plates according to manufacturer's instructions. After 24 hours or 48 hours supernatants were discarded. Cells were then either treated with Tri-reagent to lyse cells for isolation of RNA, or fresh media added and C12-iE-DAP, IL-1 β or *Haempophilus influenzae* as described above.

Principle of the assay

siRNAs are double stranded, small interfering RNA molecules of 20-25 base pairs in length, and were first developed as biomedical research tools in 2001 (Elbashir et al., 2001). Prior to this, it was known that small double-stranded RNAs (dsRNA) could induce mRNA degradation in *Drosophila* cells. These dsRNA molecules bind specific homologous sequences of DNA and result in the activation of a cascade events that results in degradation of the target mRNA. This allows the transcription of specific genes in cells to be 'knocked -down' and the effects studied at the gene level and functional level. As this regulation involves an evolutionarily conserved pathway, accordingly, this pathway represents a physiological post-transcriptional gene silencing function in human cells. This pathway involves two key proteins, RISC and DICER. Once activated, these proteins remove double stranded RNA and so 'knockdown' expression of the target gene.

Validation of the assay

In any assay using siRNA to knockdown a receptor, in this case NOD1, it is important to validate the assay in a number of ways:

1. That expression of the target gene is reduced versus a housekeeping gene. This can be measured post-transfection with the siRNA using real time-PCR (see measurements section below). In this case NOD1 mRNA expression.
2. That response to a known specific agonist is reduced. In this case, NOD1 agonist induced CXCL8 release.
3. That response to an agonist, known to cause similar responses *in vitro*, but via a different receptor or pathway is unaffected. In this case, IL-1 β which induces NF- κ B and CXCL8 release independently of NOD1.
4. And the response is not a result of loss of cell viability, which can be assessed using commercially available assays, and in my PhD, this was the Alamarblue® assay (discussed in the measurements section below)

Indeed, all of the measurements must be measured against a non-targeting siRNA molecule in order to account for non-specific effects induced by the addition of RNA in to cells. The protocol for, and pathways involved in siRNA-mediated knockdown of NOD1 are summarised in Figure 2.8

2.5.5 Pharmacological inhibition protocols for NOD1 and downstream signalling pathways for NOD1 and TLR4

Pharmacological antagonists of NOD1 and associated signalling pathways are available and are highlighted in the drugs and reagents section above. Cells were plated as described.

Supernatants were then removed and 160µl of fresh media was added. As these inhibitors were prepared in DMSO, with a final concentration of 0.1% DMSO, cells in controls wells were treated with media containing 0.1% DMSO as vehicle. Inhibitors used for protocols to determine the contribution of NOD1 to C12-iE-DAP and *Haemophilus influenzae* induced responses were added as pre-treatments for 30 minutes (NOD1 inhibitor; GSK1219217A (GSK'217); 300nM; RIP2 inhibitor; GSK2576214A (GSK'214); 300nM). In some experiments, inhibitors of IKK (SC-514; 0.01-1µM), RIP2/p38 MAPK (SB203580; 0.01-1µM) and the selective p38 MAPK (BIRB0976; 0.1-10µM) were added in identical protocols. As with other treatment protocols, drugs were added from 10x concentrated stocks to give a final volume of 200µl in the well of the 96-well plate. Plates were then incubated for 30 minutes, followed by the addition of C12-iE-DAP (10µg/ml), *Haemophilus influenzae* (10⁷ CFU/ml), LPS (1µg/ml) or IL-1β (0.01-1ng/ml) for 24 hours.

Protocols for inhibition of NOD1 pathways using siRNA and the pharmacological approaches described are summarised in Figure 2.8

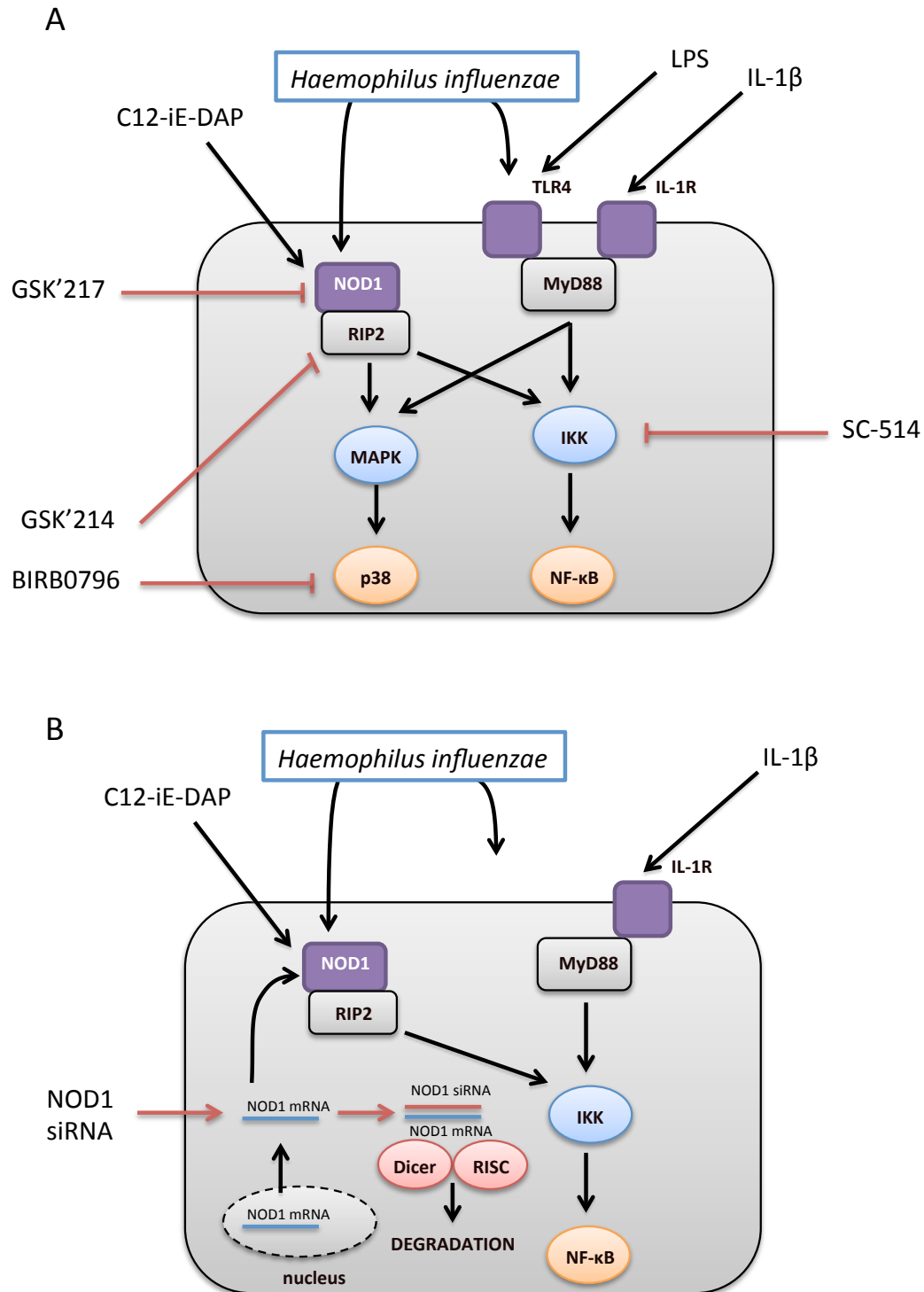


Figure 2.8 Summary of pathways activated and inhibited by protocols employing **(A)** pharmacological inhibitors and **(B)** NOD1 targeting siRNA to study NOD1 signalling in human embryonic stem cell-derived endothelial cells (hESC-EC).

Pharmacological inhibition protocols to study cPLA₂ in endothelial cells

In protocols to determine the contribution of cPLA₂ versus iPLA₂ to prostacyclin release by endothelial cells, a cPLA₂ inhibitor (pyrrophenone) was used. For these protocols, cells were plated on 96-well plates as described, and in addition on 48 well plates at a density of 20,000 cells/well. Supernatants were collected and fresh media added with or without IL-1 β (1ng/ml) to activate cyclooxygenase (COX). After 24 hours, supernatants were collected and stored at -20°C for analysis. For 96-well plates only, an inhibitor of cPLA₂ (Pyrophenone; PYR; 0.001-0.1 μ M) or vehicle (0.1% DMSO) was added. IL-1 β or vehicle (Lonza-EGM2) was replaced in the required wells. After 30 minutes supernatants were collected and stored for analysis. The cPLA₂ inhibitor, with and without IL-1 β was then replaced followed by addition of three prostacyclin release inducing agents of the Ca²⁺ ionophore (A23187; 50nM), arachidonic acid (AA; 30nM) or thrombin (1unit/ml). Cells were incubated for 30 minutes and supernatants collected and stored at -20°C for analysis. For the 48-well protocols, inducers of prostacyclin release were added as described above, cells were incubated for 30 minutes and supernatants collected and stored at -80°C for lipidomics analysis (discussed below). Cell monolayers on 48-well plates were also lysed and collected for DNA/RNA extraction.

In further experiments to assess the contribution of cPLA₂ versus iPLA₂ to prostacyclin production by endothelial cells from vessels, mouse aortic rings were employed. Isolation of aortic rings was carried out by my colleague Dr. Nick Kirkby. Aortic rings were isolated from CO₂-euthanised mice (male, BALB/c, 10 weeks; n=6), and incubated for 1 hour at 37°C. Mouse aortic rings were then incubated for 30 minutes with the cPLA₂ inhibitor, pyrrophenone (PYR; 0.1-10 μ M), or the iPLA₂ inhibitor bromoenol lactone (BEL; 0.1-10 μ M) or

vehicle (0.1% DMSO) as above. Mouse aortic rings do not respond robustly to thrombin, so for these experiments, prostacyclin release was stimulated for 30 minutes with acetylcholine (ACh; 1 μ M; saline), which also acts via a G_q-protein coupled receptor signalling pathway to activate PLA₂ pathways in a similar fashion to thrombin. Supernatants were collected and stored at -80°C for analysis.

cPLA₂-deficient patient

To further study the contribution of cPLA₂ to prostacyclin production from endothelial cells I was able to take advantage of a rare mutation in a pair of siblings with a frameshift mutation in the cPLA_{2 α} gene (PLA2G4A). This mutation results in a decrease in prostaglandin production and circulating levels of prostacyclin (Brooke et al., 2012). As BOEC are derived from adult stem cells, I have employed these cells to phenotype the endothelial cells of this patient in chapter 6. This accords with the idea that adult stem cells represent powerful translational research tools and can be used to study 'disease in a dish'. Blood was taken from the patient at Barts and the London Hospital and BOEC isolated as described in detail above. These cells were then treated with and without IL-1 β for 24 hours, media removed and replaced with media for 30 min containing agents to activate PLA₂ (A23187 or thrombin) or supply substrate (arachidonic acid) directly. Using these cells and the Ca²⁺ ionophore A23187 and the G_q agonist thrombin allowed me to determine whether these pathways act through cPLA₂, treating the patient BOEC as 'knockout' human cells. Arachidonic acid was included, as in other protocols, to bypass the requirement for PLA₂ and ensure that cells were able to produce prostacyclin via COX. This serves to support any findings as being a result of inhibition or loss of cPLA₂ rather the inhibition or loss of other downstream proteins required for prostacyclin release. For experiments using cPLA₂-

deficient BOEC, supernatants were collected and frozen at -80°C for analysis of prostacyclin release (using 6-ketoPGF_{1 α} by ELISA, see below) and by mass spectrometry for a full lipidomics analysis (discussed in measurement section below).

The pathways activated and inhibited to assess the contribution of cPLA₂ to prostacyclin release in BOEC from healthy and a cPLA₂-deficient patient are summarised in Figure 2.9

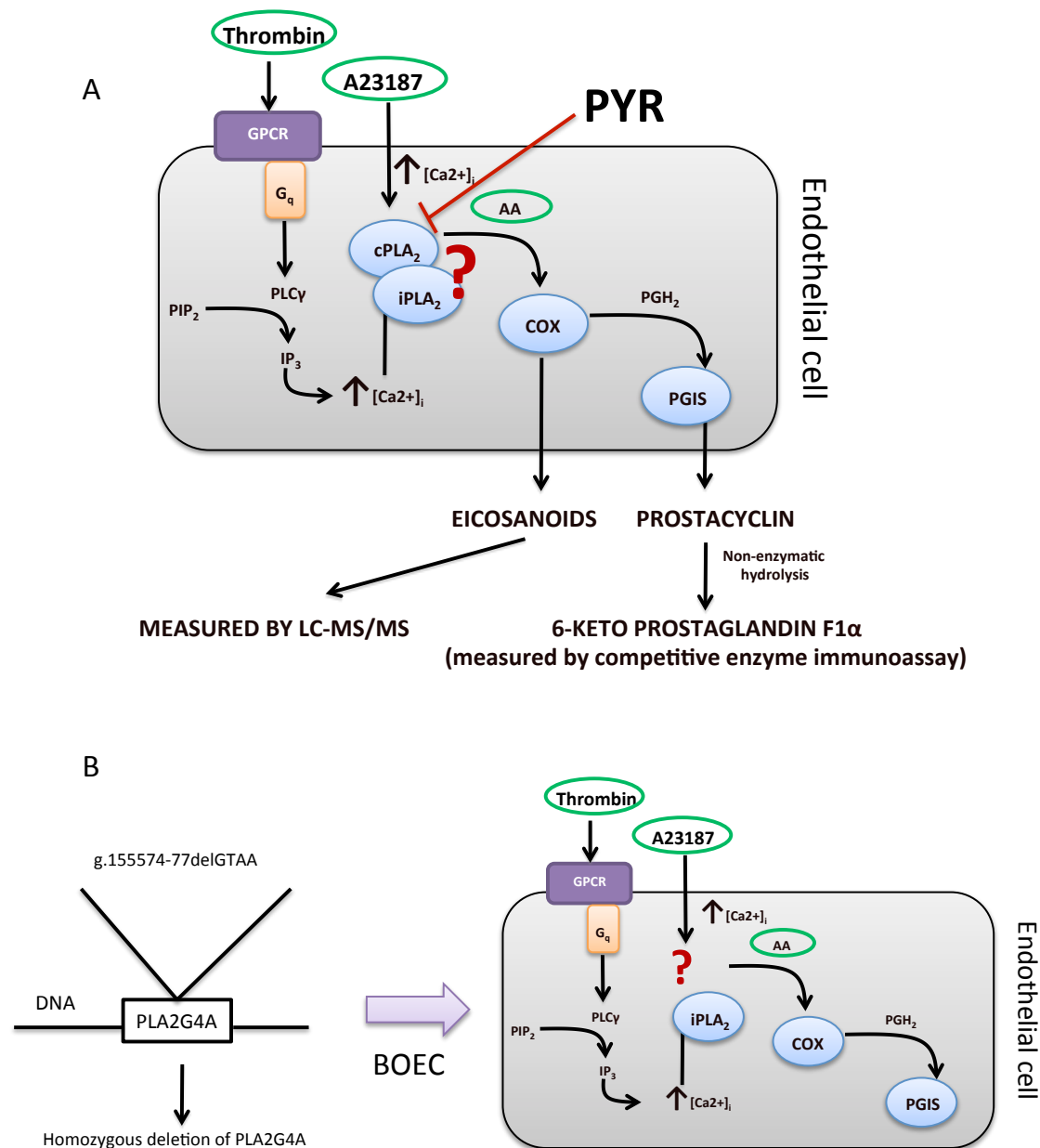


Figure 2.9 (A) Summary of pathways activated and inhibited in protocols to determine the contribution of cPLA₂ to prostacyclin release from endothelial cells. **(B)** Blood outgrowth endothelial cells (BOEC) were also grown from a patient with a homozygous mutation in the PLA2G4A gene which results in loss of cPLA₂ function. The impact on endothelial cells is unknown and was studied. Agonists added in the protocol are circled in green. Abbreviations: G-protein coupled receptor (GPCR), phospholipase A₂ (PLA₂), phospholipase C_γ (PLC_γ), phosphatidylinositol 4,5-bisphosphate (PIP₂), inositol 1,4,5-trisphosphate (IP₃), cyclooxygenase (COX), arachidonic acid (AA), prostacyclin synthase (PGIS), prostaglandin H₂ (PGH₂), Liquid chromatography tandem mass spectrometry (LC-MS/MS), pyrrophenone (PYR)

2.5.6 Autologous BOEC:PBMC co-culture protocols

To allow co-culture of endothelial cells and PBMCs from the same donor, BOEC were grown from blood as described and plated on gelatin coated 96-well plates. Once confluent, PBMCs from the same donors from which the BOEC were isolated were recalled and PBMCs isolated using a Histopaque® gradient approach. PBMCs were resuspended in a 50:50 mix of Lonza-EGM2 (BOEC medium) and RPMI (PBMC medium) with 10% FBS. PBMCs or 50:50 media alone was then added to wells with and without BOEC from the same donor. Media was also added to BOEC alone giving rise to three conditions: BOEC monoculture, PBMC monoculture, BOEC:PBMC co-culture. Indeed, endothelial cell:PBMC co-culture is not new (Ashida et al., 1981; Bailey et al., 2012; Stebbings et al., 2007; Ward et al., 2009) and is valuable since they represent some of the key cell interactions in the vessel that modulate inflammatory responses. However, this approach using BOEC, allows for the first time, endothelial cells and PBMCs from the same donor to be grown in co-culture. This assay was filed for a patent in March 2013 on which I am a 'contributor to the work of the invention'.

BOEC:PBMC co-cultures and monocultures of each cell type alone were then treated with biologics/therapeutic antibodies: anti-CD28-superagonist ANC28 (10µg/ml), the anti-CD52 drug Campath (10µg/ml), the anti-HER2 drug Herceptin (10µg/ml), the anti-VEGF drug Avastin (10µg/ml) or Arzerra (10µg/ml). These concentrations were selected based on those run in key assays in the literature and are relevant therapeutic doses. Cells were also treated with PAMPS and cytokines: the TLR4 agonist LPS (1µg/ml), the TLR2 agonist Pam₃CSK4 (1µg/ml), the NOD1 agonist C12-iE-DAP (10µg/ml), and the cytokines TNFα (10ng/ml) or IL-1β (1ng/ml). After 24 hours supernatants were collected and samples stored at -20°C for analysis.

The protocol for this assay is summarised in Figure 2.10. As the development, validation and application of this assay formed a key aspect of my PhD, this is discussed in detail in Chapters 5 and 7.

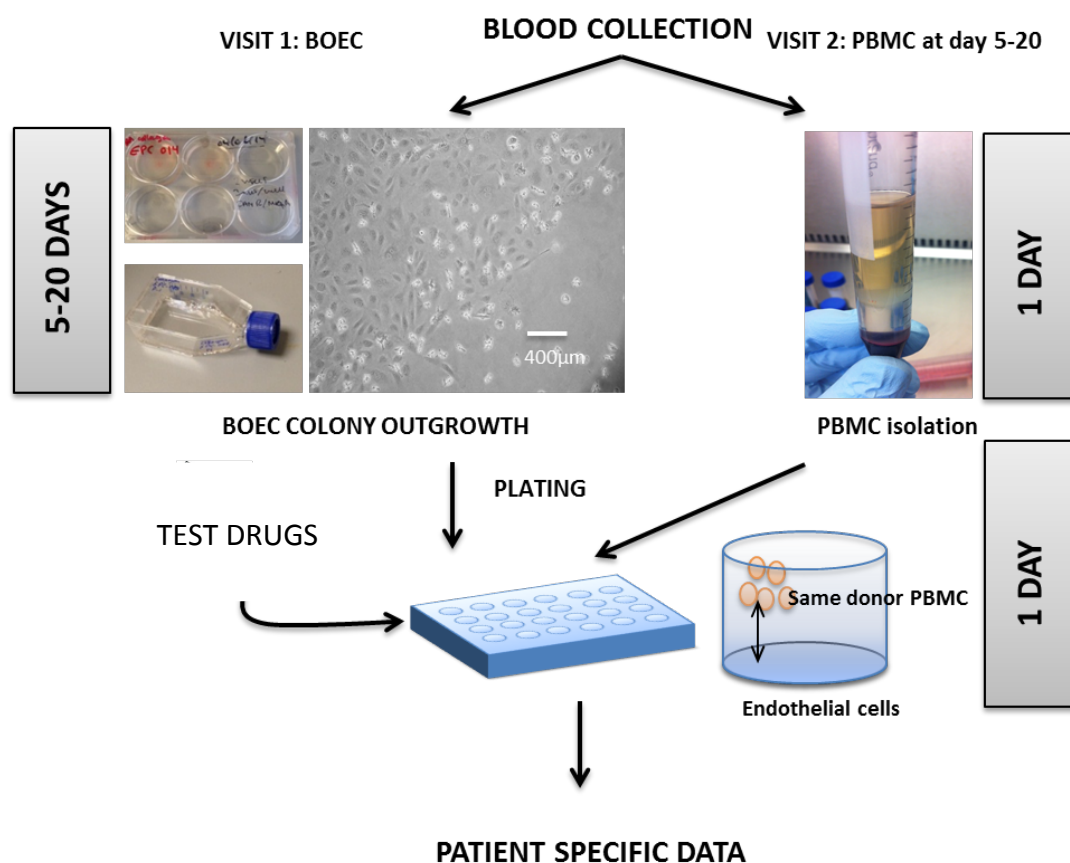


Figure 2.10 Schematic of protocol for set up of same donor blood outgrowth endothelial cell (BOEC): Peripheral blood mononuclear cell (PBMC) co-culture assays. This assay has been filed for a patent with application number PCT/EP2014/055695.

2.6 STAINING AND IMAGING

Antibodies for immunofluorescence staining of endothelial cells are summarised in Table 2.7

For all staining protocols, cell monolayers were washed three times with PBS and fixed immediately in 4% para-formaldehyde (PFA) for 10 minutes at room temperature. Plates were then washed three times with PBS at 5 minute intervals. Plates were permeabilized with 0.2% Triton X-100 for 10 minutes and blocked with 4% FBS in PBS for 1 h at room temperature.

Epitope	Species	Order information	Dilution	Secondary antibody
TRA-1-60	Mouse anti-human	Millipore, UK; Cat no. MAB4360)	1 : 100 in 3% BSA in PBS	Alexa Fluor® rabbit anti-mouse 488
NF-κB (p65 subunit)	Rabbit anti-human	Santa Cruz Biotech, Germany; Cat no. sc-372	1:100 in 3% BSA/ PBS	Alexa Fluor® goat anti-rabbit 546
VE-cadherin	Goat anti-human	Santa Cruz Biotech, Germany; Cat no. sc-6458	1:250 in 3% BSA/ PBS	Alexa Fluor® donkey anti-goat 594
CD31	Mouse anti-human	Biolenged, UK; Cat no. 303110	1:100 in 3% BSA/ PBS	NA (antibody is conjugated to 488 Alex Fluor dye)
COX-1	Rabbit anti-human	Cayman Chemical, UK; Cat no: 160107	1:250 in 3% BSA/ PBS	Alexa Fluor® goat anti-rabbit 546

Table 2.7 List of antibodies, dilutions and secondary antibodies used to stain endothelial cell monolayers. NB. All secondary antibodies were diluted 1:400 in 3% BSA/PBS

Primary antibodies were added in 3% bovine serum albumin (BSA) in PBS and plates were incubated for 1 hour at room temperature. Except for CD31 which is tagged with a 488-Alexa Fluor molecule, an appropriate secondary antibody-Alexa Fluor probe (Table 2.7) was added in 3% BSA/PBS for 45 min at room temperature. For this incubation, and for all further stages, plates were protected from light. Cell nuclei were stained with the nuclear dye, 4',6-diamidino-2-phenylindole (DAPI) (10µg/ml). Plates were then stored in PBS at -4°C prior to imaging.

Images were acquired using a Cellomics® VTi HCS Arrayscan (camera make/model: Arrayscan 12bit dynamic range high resolution thermo-cooled with a Zeiss Plan Neurofluor 10 x objective lens) (Thermo Fisher, Pittsburgh, USA). The Cellomics® Arrayscan is a platform that uniquely allows the spatial and temporal distribution of fluorescence to be quantified in 96-well plates. This includes nucleus and cytosolic levels of fluorescence, which allows nuclear translocation of transcription factors to be quantified. This is discussed below.

2.7 MEASUREMENTS

2.7.1 ELISAs

Protocol

CXCL8 and ET-1 were measured using a simple enzyme linked immunosorbent assay (ELISA). Specific ELISAs were purchased from R and D Systems (CXCL8; Cat no. DY208; ET-1; Cat no. SET100). ELISAs were carried out according to manufacturers instructions. Briefly, specific 'capture' antibodies specific to CXCL8 were added to 96-well high binding half plates (Greiner bio-one, UK; Cat no. 675061) and incubated at room temperature overnight (CXCL8). For ET-1 ELISAs, plates are supplied pre-coated with ET-1 primary antibodies from the company. After all incubations plates were washed three times in 0.5% Tween/trizma buffered saline (TBS). Supernatants of cells treated in the protocols described above were thawed from frozen stocks at room temperature. Supernatants were diluted in assay buffer (reagent diluent) for the specific assays (CXCL8 reagent diluent; 0.1% BSA in 0.5% Tween/TBS at pH 7.2). ET-1 reagent diluent was supplied prepared by the manufacturer as an 18ml solution of buffered protein in a mix of proprietary stabilisers. Dilutions were determined using in-house knowledge of expected basal levels, and/or dilutions were adjusted and assays repeated as required. For CXCL8 ELISA, plates were incubated at room temperature for 1 hour in 1% BSA/PBS to block non-specific binding prior to addition of standards or samples. Standard concentrations of CXCL8 and ET-1 were prepared according to manufacturer's instructions. Example standard curves for CXCL8 and ET-1 ELISAs are shown in Figure 2.11.

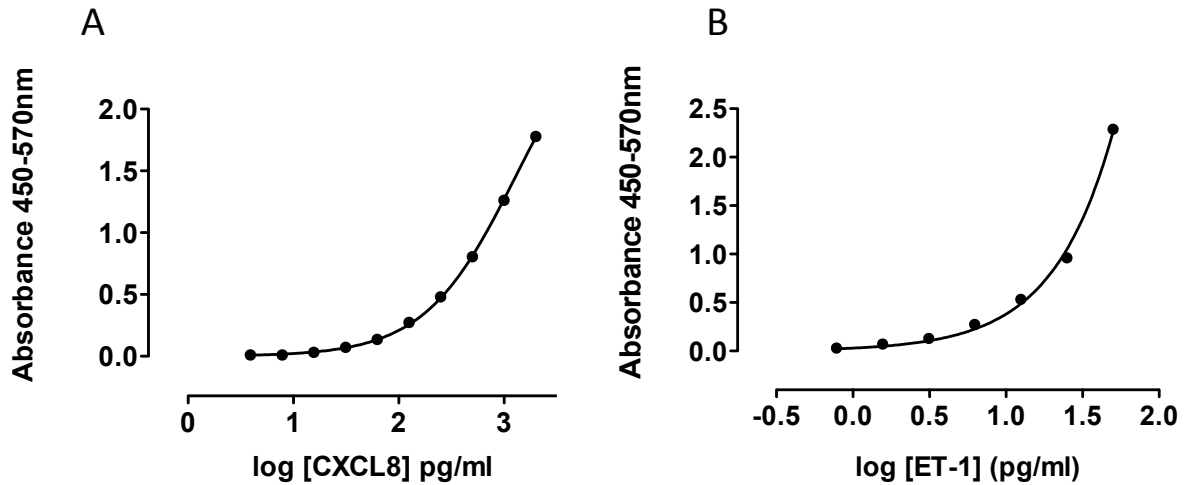


Figure 2.11 Examples of standard curves for **(A)** CXCL8 and **(B)** ET-1 ELISAs

Samples or standards were then added to the plate, covered with adhesive strip and incubated at room temperature (2 hours for CXCL8 and 1 hour for ET-1). Samples or standards were then aspirated from wells and plates washed three times in wash buffer (0.5% Tween/PBS). Plates were inverted and blotted on paper towels to ensure wells were dry. Specific secondary ‘detection’ antibodies attached to a conjugate (biotin for CXCL8 and proprietary conjugate for ET-1) were then added to all wells and plates incubated at room temperature (2 hours for CXCL8 and 3 hours for ET-1). Wells were aspirated and washed three times in wash buffer as above. Horseradish peroxidase (HRP)-streptavidin (50µl) was then added and plates incubated at room temperature for 20 minutes. Wells were then aspirated and washed as above. A substrate solution was then prepared by adding substrate A to substrate B in a 1:1 ratio and added (50µl) to the plate. Plates were incubated and protected from light for 20 minutes. To stop the reaction 25µl of 2N H₂SO₄ was added and optical density was determined using a microplate reader (Dyne, Madellan Biosciences) with absorbance readings at 450-570nm.

Principle of the assay

ELISA technology was first developed in 1977 as an immunoassay to quantify the amount of unknown compound or 'anlyate' in a sample. Such measurement could be made in blood, serum and cell culture supernatants. ELISAs work on the principle of capture and detection of the anylate. Anylate can be captured using primary antibodies bound to the surface of 96-well plates, which bind and 'capture' a target anylate. This can be then be detected using secondary antibodies, also targeted at the anylate but conjugated to a binding protein such as biotin. An enzyme attached to strepavidin, typically HRP is then added which binds to the biotin. In fact, up to four molecules of streptavidin can tag biotinylated antibodies and so this step also serves to amplify to signal. At this stage therefore, the amount of enzyme is proportional to the amount of anylate. An appropriate substrate solution is then added, typically, 3,3',5,5'-tetramethylbenzidine (TMB) which is a chromogenic substrate that donates protons for the reduction of hydrogen peroxide to water by HRP. This causes a colour change from colourless to blue, were the intensity of the signal is proportional to HRP activity. Finally, an acid solution, typically H_2SO_4 is added to stop the reaction and results in blue-to-yellow colour change, which can be read on a spectrophotometer. In order to relate the colour intensity to a concentration of anylate, standard curves of known concentration of anylate are prepared for each assay. This allows the absorbance readings of unknown wells to be interpolated and provides quantitative data.

2.7.2 Measurement of cytokines and chemokines by MSD

Protocol

Human pro-inflammatory 9-Plex MULTISPOT 96-well -10 spot MSD plates (N05007A-1 and 9-plex MULTISPOT 96-well -10 spot MSD plates (K15001C-2) were purchased from MSD (Gaithersburg, Maryland, USA). Assays were carried out according to manufacturer's instructions.

Briefly, an 8-point standard curve (2500-0.61pg/ml; 4 fold dilutions) was prepared using supplier 'calibrator blend', which contains known concentrations of 9 analytes (9 cytokines or 9 chemokines). Samples were diluted 1:10 in Lonza-EGM2 medium. Following 30 minutes incubation of plates at room temperature with 'diluent 2', samples or standards (25µl) were added to each well. Plates were incubated for 2 hours at room temperature with vigorous shaking (500 RPM). Plates were then washed 3 times in ELISA wash buffer and blotted to ensure plates were dry. Detection antibody solution (25µl) was then added to each well and plates incubated for a further 2 hours with vigorous shaking (500RPM). Plates were then washed three times as above and 150µl of 2 X read buffer (supplier provided) was added to each well. Plates were read using an MSC Sector Imager 2400 and analysed using MSD Discovery® Workbench software. Cytokines analysed were IL-2, CXCL8, IL-12p70, IL-1β, GM-CSF, IFNγ, IL-6, IL-10. Chemokines analysed were eotaxin, MCP-1, MIP-1β, eotaxin-3, MCP-4, TARC, IP-10 and MDC.

Principle of the assay

MSD plates multispot plates uniquely allow for the measurement of multiple readouts in a sample. They work on a similar principle to ELISA but rather than plates being coated in a

single antibody, they are coated with spots containing immobilised capture antibodies to a target analyte (in this case a cytokine or chemokine). These capture antibodies are attached to a working electrode surface. Once samples or standards have been added and analytes bound to capture antibodies an MSD-SULFO-TAG™ detection antibody is added. These SULFO-TAG™ antibodies are activated by voltage stimulate which is applied to the MSD Sector Imager. The luminescence emitted by each spot, measured separately, affords the measurement of the 9-analytes (in this case cytokines or chemokines). The principle of the assay as depicted in the manufacturers handbook is shown in Figure 2.12 and an example standard curve is shown in Figure 2.13.

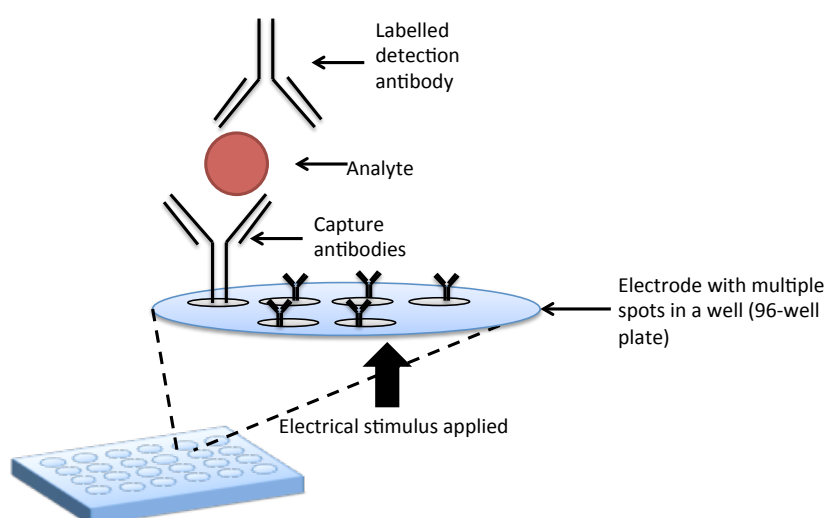


Figure 2.12 Principal of multi-spot electro-chemoluminescence. Figure drawn from information in the product information sheet at meso-scale.com.

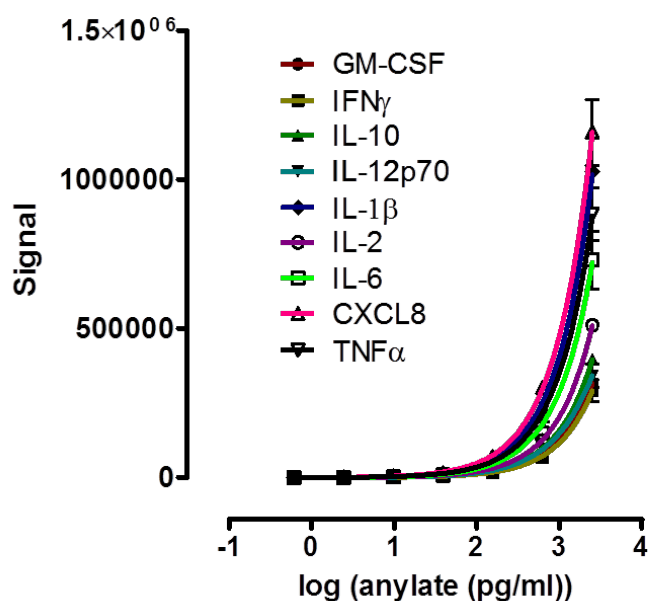


Figure 2.13 Example standard curves for MSD multiplex 9-spot cytokine assay.

2.7.3 Measurement of 6-keto PGF_{1 α} by inverse binding-ELISA

Protocol

Prostacyclin release was measured as its breakdown product 6-keto PGF_{1 α} , which is formed by spontaneous non-enzymatic hydrolysis of prostacyclin (Figure 2.9). For this a specific inverse binding ELISA was used (Cayman Chemical, UK; Cat no. 515211). Buffers were prepared according to manufacturer's instructions. Supplier 10X EIA buffer was diluted to 1X supplier buffer using ultrapure water. 6-keto PGF_{1 α} acetylcholinesterase (AChE) Tracer and EIA Antiserum were reconstituted in EIA buffer according to manufacturer's instructions.

Wash buffer for these assays was prepared using supplier 'wash buffer' concentrate and made with 0.05% supplier Polysorbate 20. Samples were diluted 1:2 or 1:5 in Lonza-EGM2 according to supplier recommendation for dilution of tissue culture samples. A standard curve (1000-1.6pg/ml) was prepared according to manufacturer's instructions. Samples,

standards (50µl) and assay controls were added to the plate, followed by addition of tracer (50µl) and antiserum (50µl) according to manufacturer's instructions. Plates were then incubated at 4°C for 18 hours. Plates were aspirated and washed five times with wash buffer followed by addition of the Ellman's reagent provided (details are given below). After 2-3 hours optical density was read at 420nm. Data was recorded when B₀ wells which contains supplier buffer, tracer and antiserum only, were in the range of 0.3-1.0 absorbance units.

Principle of the assay

Inverse binding assays are similar to ELISAs described above, but in these assays, anylates must compete for the binding sites of antibodies with a specific fluorescent tagged or 'tracer' ligand for the antibody. As a result, the more anylate there is in a sample, the lower the signal (fluorescence or absorbance) as more of the labelled form is competed off the binding site.

In this inverse binding assay for 6-keto PGF_{1α}, the plates are coated with a mouse anti-rabbit antibody which captures anti-6-keto PGF_{1α} in the supplier antiserum. Once bound, these act as a receptor for either free 6-keto PGF_{1α} in the sample or standard, or can be bound by 'tracer', and tracer and 6-keto PGF_{1α} in the sample must compete for the binding site. The 'tracer' contains molecules of PGF_{1α} covalently attached to AChE. AChE catalyses the conversion of acetylthiocholine to thiolcholine, which in turn undergoes non-enzymatic hydrolysis to liberate 5-thio-nitrobenzoic acid which absorbs light at 412nm. An example standard curve for this assay is shown in Figure 2.14.

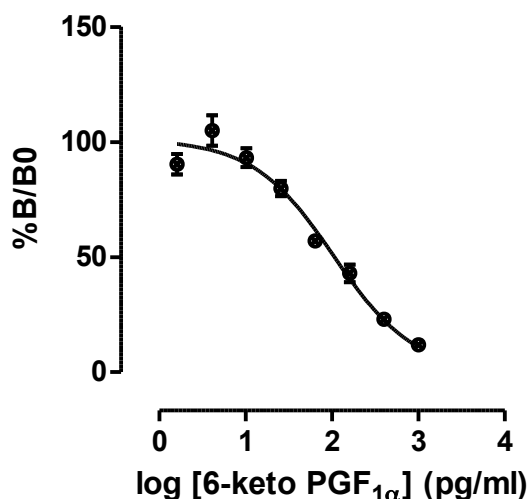


Figure 2.14 Example of standard curve for the Cayman Chemical inverse binding immunoassay for 6-keto PGF_{1α}.

2.7.4 Measurement of NF-κB nuclear translocation

Protocol

To quantify translocation of NF-κB to the nucleus, and thereby activation of cell signalling, a compartment analysis bio-application (Thermo Scientific, USA) attached a Cellomics® VTi HCS Arrayscan was used. Wells of 96-well plates treated with TLR, NOD agonists or IL-1β for 1 hour, and stained with primary antibodies for NF-κB as described, were imaged in PBS. The algorithm applied identifies objects (cells) based on DAPI staining and applies a 2μM border (ring) around the edge of the nucleus. This ring is used to quantify staining in the cytosol of uniformly distributed cytosolic transcription factors and has been applied by others (Ding et al., 1998). This allows for nuclear intensity/cell to be calculated as the difference between nuclear intensity and cytosolic intensity for each cell. Data was acquired from 1000 cells/well and treated wells were normalised to control (100%). The application of the algorithm to a representative image of endothelial cells is shown in Figure 2.15.

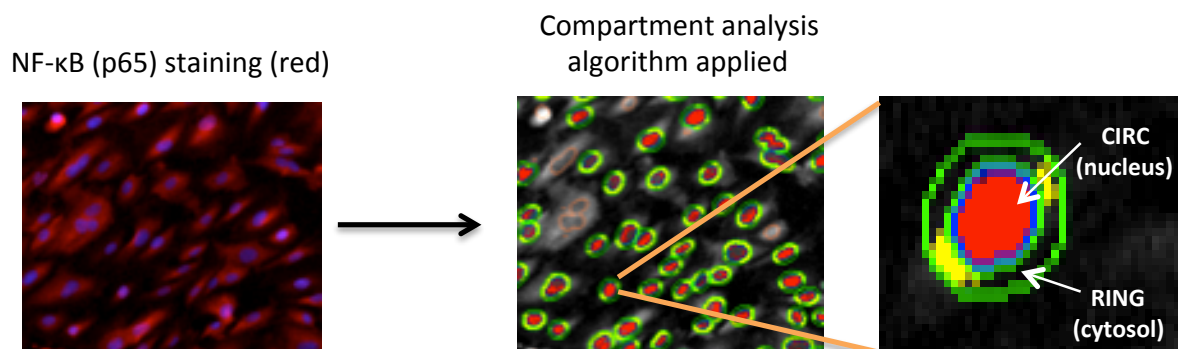


Figure 2.15 Application of the ThermoFisher compartment analysis algorithm to determine nuclear intensity of NF-κB staining in individual cells.

Principle of the assay

The release of cytokines such as CXCL8 can be driven by activation of transcription factors including NF-κB. These transcription factors reside in the cytosol in an inactive form and often sequestered by an inhibitor or retention protein. Once activated, these transcription factors translocate to the nucleus and bind response elements that activate genes, which ultimately result in a response (for example, cytokine release). Nuclear translocation is therefore an early event in cellular responses and can be visualised and measured using staining protocols as described.

2.7.5 Measurement of eicosanoids by LC-MS/MS

Protocol

To consider a more complete profile of eicosanoids released by BOECs, supernatants were also analysed using a liquid chromatography – tandem mass spectrometry (LC-MS/MS) ‘lipidomics’ approach. These protocols were carried out at the National Institute of Environmental Health Sciences, USA, under collaboration with Matthew Edin, and with the help of my colleague Dr. Nick Kirkby. This allows for analysis of a wide range of biologically

active lipid mediators within a small sample volume (Kirkby et al., 2013; Masoodi and Nicolaou, 2006). Prostanoids were extracted and analysed as previously described (Edin et al., 2011; Newman et al., 2002). Indeed, this kind of omics approach will be important to study broad ranges of cytokine release, lipid release, gene transcription changes in personalised medicine assays.

Briefly, samples were spiked with PGE₂-d₄, 10(11)-epoxyheptadecanoic acid and 10(11)-dihydroxynonadecanoic acid internal standards then mixed with 0.1 volumes for 1% acetic acid (v/v) in 50% methanol. Samples were then pre-extracted using Oasis HLB C18 3ml columns, and then further separated by high pressure liquid chromatography with Phenomenex Luna C18(2) columns. Extracts were analysed using negative ion electrospray ionization tandem mass spectrometry in triplicate using an MDS Sciex API 3000 instrument with Applied Biosystems TurbolonSpray source. Lipid mediator abundance was quantified from peak areas of characteristic fragments and normalised to the extraction efficiency of internal standards.

Principle of the assay

LC-MS/MS allows for the quantitative analysis of large numbers of cell metabolites, proteins, and in this case eicosanoids. These experiments can be performed using targeted or untargeted analysis wherein structures of lipids being analysed in sample are known or unknown respectively. MS works on the principle that when a complex structure is ionised and accelerated through an electromagnet, unique patterns of deflection emerge as result of the mass of the ionised species. These can be detected and the relative intensity of different anylates based on mass-to-charge ratio can be plotted. In LC-MS/MS this samples

are first separated by mass using liquid chromatography before being fed in to an ion source. Ionisation in most modern mass spectrometers is achieved by electrospray ionisation which prevents fragmentation of the analyte prior to separation based on mass. In LC-MS/MS this process is coupled with a second round of mass spectrometry in which fragmentation of the analyte occurs and these unique fragment patterns can be used to determine precise chemical structures. In targeted LC-MS/MS this is advantageous as the fragmentation pattern of particular analytes is unique to the target analyte and so can be used to quantify large numbers of molecules in complex fluids including cell culture supernatants and serum.

2.7.6 Measurement cell viability

Cell viability following incubations was determined using either the commercially available AlamarBlue® assay or based on cell counts. For cell counts, fixed cells were stained with 10µg/ml DAPI and imaged using the Cellomics® VTI HCS ArrayScanner. Data for cell counts are expressed as average cells/field based on 49 fields.

2.7.7 Cell proliferation

In some protocols, run as part of a collaboration with the National Institute of Biological Standards and Control (NIBSC), proliferation assays were carried out by NIBSC using an in-house H³-thymidine incorporation assay (Stebbins et al., 2007).

2.7.8 Statistical analysis

Unless otherwise stated data is the mean $\pm$ SEM for n-experiments where n represents separate treatments. Unless otherwise stated all experiments were carried out on cells of at least 3 separate donors or, as for cell lines, on at least 3 separate isolations with separately prepared drugs. Data was analysed using appropriate tests and GraphPad Prism software with specific tests defined in individual figure legends. Statistical significance was taken to be achieved where $p < 0.05$. In some experiments, data was analysed using a principle component analysis, which is described in detail in Chapter 5.

In some experiments, where valuable drugs were being used, I chose to prioritise experimental replicates over biological replicates, whereby some experiments were run with single treatment wells but at least three times. This approach captures both biological variation (due to drug preparations and pipetting errors) and experimental variation (due to different donor cells and those at different passage). This is consistent with the directive of Cumming et al (Cumming et al., 2007).

Technical replicates, which are defined as repeat measurements from one cell isolation (well) were not carried out during this thesis, except in the capacity of standard curves required for individual assays as per manufacturer instructions.

CHAPTER 3: CHARACTERISATION OF STEM CELL DERIVED ENDOTHELIAL CELLS

3.1 RATIONALE

Stem cell derived endothelial cells have a growing number of applications within clinical medicine and biomedical research. Endothelial cells can now be derived from a number of distinct stem cell populations giving rise to: human embryonic stem cell (hESC) derived endothelial cells (hESC-EC), blood outgrowth endothelial cells (BOEC) (derived from blood progenitors) and induced pluripotent stem cell derived endothelial cells (iPSC-EC). In order for these stem cell derived endothelial cells to be used optimally it is important that they are fully assessed for the cardinal hallmarks of endothelial cell biology and phenotype. Previous characterisation of these cells has typically been restricted to endothelial cell marker expression; however, a key limitation of this approach is that many of these markers overlap across several cell phenotypes (Hirschi et al., 2008; Reed et al., 2012).

Thus, the initial aim of my PhD was to derive and/or acquire these three key types of stem cell derived endothelial cells and then use previously established protocols to characterise them according to their ability to:

1. Express typical endothelial cell cobblestone morphology when grown under static culture conditions (Potter et al., 2011; Potter et al., 2012) and to align under directional (laminar) shear stress by assuming the associated elongated phenotype found in endothelial cells on vessels (Potter et al., 2011).
2. Given that endothelial cells on vessels are critical for innate immune responses; to sense pathogen associated molecular patterns (PAMPs) by releasing cytokines.
3. To release two key endothelial cell hormones, endothelin (ET)-1 and prostacyclin which are essential regulators of endothelial cell and vessel biology.

3.2 SUMMARY OF METHODS

A full description of the methods used in this chapter is given in Chapter 2 and are summarised below.

3.2.1 Isolation and culture of hESC-EC

Differentiation of hESCs (H7 cell line) into hESC-EC was carried out as described previously (Földes et al., 2010). Briefly, cells were dissociated into clumps and plated on ultra-low attachment plates (Nunc, Denmark) with Lonza-EGM2 to allow formation of embryoid bodies. After 4 days embryoid bodies were re-plated on 1% gelatinised (Sigma, USA) 6-well plates in Lonza-EGM2. After 13 days clusters were dissociated and stained for the endothelial cell marker CD31 using an AlexaFluor 488 fluorescence dye labelled anti-CD31 antibody (BD Biosciences, UK). Cells were sorted using a FACS Aria II cell sorter (BD Biosciences, UK) and expanded in Lonza-EGM-2 medium for further use. Cells were maintained in Lonza-EGM2 media and grown on 1% gelatinised (Sigma, USA) 75cm² flasks. Cells were used between passages 2-8.

3.2.2 Isolation and culture of BOEC

BOEC were isolated as described in chapter 2. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from blood and expanded on tissue culture plates for up to 22 days in Lonza-EGM2 with 10% fetal bovine serum (FBS) on 5.2µg/cm² collagen coated plates. Typically between 5 and 21 days in culture, endothelial cell colonies emerged and were expanded for use in experiments. Cells were maintained in Lonza-EGM2 media with 10%

FBS. Cells were maintained in Lonza-EGM2 media and grown on collagen coated (Sigma, USA) 75cm² flasks. Cells were used between passages 2-8.

3.2.3 Acquisition and maintenance of iPSC-EC

iPSC-EC used in my PhD were purchased from Cellular Dynamic International (Madison, USA). Cells were maintained in Lonza-EGM2 (see general methods) on fibronectin (Invitrogen, California, USA) coated 75cm² flasks (Nunc, Denmark) according to manufacturer's instructions.

3.2.4 Isolation and culture of human lung fibroblasts (HLF)

HLFs were isolated from human lung tissue as described previously (Maher et al., 2010) from histologically normal peripheral lung from lung cancer resection. Cells were grown in supplemented DMEM, and were passaged every 3–4 days. All experiments were performed from passage 3–10

3.2.5 Endothelial cells from vessels

Human umbilical vein endothelial cells (HUVEC) used throughout my PhD were a gift from Caroline Wheeler-Jones (Royal Veterinary College, London) and were maintained in Lonza-EGM2 medium. Cells were at passage 2 on arrival and used for experiments between passages 2-8.

Human lung microvascular endothelial cells (HMVEC), and human aortic endothelial cells (HAEC) were purchased from Lonza or Promocell as cryopreserved cells. Cells were grown

out and maintained in Lonza-EGM2 media and grown on 1% gelatinised (Sigma, USA) 75cm² flasks. Cells were used between passages 2-8.

3.2.6 Cell staining

All cells were stained for the endothelial cell markers CD31 and/or VE-cadherin. Cells were also stained for COX-1, which is expressed by endothelial cells. Undifferentiated hESC were also stained for the pluripotency marker TRA-1-60 (Wright and Andrews, 2009). In all cell staining protocols, cells were cultured for 4 days, unless stated otherwise in figure legends, and media was discarded or collected for analysis where required. Cell monolayers were then washed three times in PBS and fixed for 10 minutes in 4% paraformaldehyde. After fixing, cell membranes were permeated using 0.2% Triton-X100 and blocked with 4% FBS/PBS. Cells were then stained with specific antibodies as required, followed by secondary staining with Alexa Fluor® conjugated secondary antibodies. Cell nuclei were stained with 10µg/ml of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Details of specific primary and secondary antibodies combinations used are given in chapter 2. Images were acquired using either a Nikon inverted fluorescence microscope, a confocal laser scanning microscope or a Cellomics® VTI HCS ArrayScanner. Acquisition settings for individual protocols are stated in figure legends. Images were acquired at x10 magnification at room temperature with PBS as the imaging medium.

3.2.7 Assessment of the ability of stem cell derived endothelial cells to align under shear stress

Endothelial cell alignment under shear stress was determined using a model previously defined by our group and developed by my colleague Claire Potter (Potter et al., 2011). Briefly, cells were cultured on standard 1% gelatinised 6-well plates (Nunc, Denmark) and placed on an orbital shaker. The movement of the shaker results in a wave of media that oscillates around the well resulting in a complex pattern of shear applied with directional (laminar) shear towards the edge of the well, and non-directional (turbulent) shear at the centre. Alignment of cells was visualised by light microscopy and by fluorescence imaging of cells stained with appropriate markers as described above in 3.2.6.

3.2.8 Treatment protocols and responses to PAMPs

Cells were plated on 1% gelatinised 96-well plates (Nunc, Denmark) and grown to confluence. All cells were plated and incubated throughout experiments in Lonza-EGM2 media with 10% FBS unless otherwise stated. Cells were seeded at a density of 7,000 cells/well. For assessment of responses to PAMPs, cells were treated with agonists of Toll like receptors (TLR)1-9 or nucleotide oligomerisation domain receptor-1 NOD1 (see figure legends and Table 2.3 in chapter 2; all from Invitrogen, UK) for 1 or 24 hours. Where possible responses of cells of different origins were compared directly in the same media and treated under identical conditions. At 24 hours, supernatants were collected and stored at -20°C for analysis. Cells were also treated IL-1 β (R & D Systems, Abingdon, UK) for 1 or 24 hours, and in some experiments, with interferons (IFN) (R and D Systems) for 24 hours.

3.2.9 Measurement of CXCL8, IP10, ET-1 and prostacyclin

CXCL8, IP10, ET-1 and prostacyclin (measured as its breakdown product 6-keto-PGF_{1α}) were measured in supernatants by specific ELISA according to manufacturer's instructions; details are defined in Chapter 2. Optical density was determined using a microplate reader (Dyne, Madellan Biosciences) with absorbance readings at wavelengths according to manufacturer's instructions

3.2.10 Measurement of NF-κB nuclear translocation

For measurement of translocation of NF-κB, cells were incubated with NF-κB-p65 (human) primary antibodies raised in rabbit (Santa Cruz Biotechnology, UK) for 1 hour at room temperature followed by secondary staining with AlexaFluor® 546 anti-rabbit antibodies raised in goat (Invitrogen, UK) for 45 minutes at room temperature. Cells were washed three times between incubations with PBS at 5 minute intervals. Cell nuclei were stained with 10µg/ml DAPI. Plates were imaged using a Cellomics® VTi HCS Arrayscanner. Some wells were treated with secondary antibody only. Images were acquired at x10 magnification at room temperature with PBS as imaging medium.

3.2.11 Measurement of cell viability

Cell viability following incubations was determined using either the commercially available AlamarBlue® assay or based on cell counts. For cell counts, cells were stained with 10µg/ml 4',6-diamidino-2-phenylindole (DAPI) or Hoescht nuclear stains (both Invitrogen, UK) and imaged using the Cellomics® VTi HCS Arrayscanner. Data are expressed as average cells/field based on 49 fields.

3.3 RESULTS

3.3.1 Basic morphology and endothelial cell markers associated with stem cell derived endothelial cells

hESC-EC

hESCs had a colony morphology typical of undifferentiated stem cells (Figure 3.1A) and expressed the pluripotency marker TRA-1-60 (Figure 3.1B). When cells were grown in Lonza-EGM2 on ultra-low attachment plates and allowed to spontaneously differentiate approximately 0.1% of the total population expressed the endothelial cell marker, CD31, indicating a small population of endothelial cells were present; these were isolated and sorted from the mixed cell population by FACS (Figure 3.2A). These cells, now assumed to be endothelial cells were defined as hESC-EC. HESC-EC were plated and expanded using Lonza-EGM2 and continued to express CD31 in culture (Figure 3.2B). hESC-EC also expressed the endothelial cell enzyme, COX-1 (Figure 3.2B). Notably however, hESC-EC did not have cobblestone morphology typical of endothelial cells (Jaffe et al., 1973b) and appeared elongated (Figure 3.2B).

BOEC

BOEC were grown from PBMC fraction of human blood as previously described (Martin-Ramirez et al., 2012; Starke et al., 2010; Starke et al., 2013). When PBMCs were plated on collagen coated 6-well plates, colonies of endothelial cells typically emerged between days 5 and 22 (Figure 3.3; day 11 colony shown). Once the colony had appeared to stop expanding, typically 4 days after emergence, cells were expanded to confluence in 25cm² cell culture flasks and, unlike hESC-EC but like endothelial cells from vessels, had typical endothelial cell

cobblestone morphology when grown under static culture conditions (Figure 3.3 and 3.4). The same was true for BOEC expanded in 75cm² (Figure 3.3) cell culture flasks and this morphology was maintained throughout use in experiments up to at least passage 8. BOEC expressed CD31 and another endothelial cell marker, VE-cadherin, as well as COX-1 (Figure 3.4).

iPSC-EC

iPSC-EC were purchased from Cellular Dynamics International (Colorado, USA). Cells arrived as a cryovial and when expanded these cells were characterised as having endothelial cell morphology and expressed CD31, VE-cadherin and COX-1 (Figure 3.5A). These cells were originally derived from human foetal lung fibroblasts using a reprogrammed strategy similar to that in the original seminal work on these cells (Takahashi et al., 2007; Taura et al., 2009; Yu et al., 2007). Thus, in order to validate these cells, head to head protocols between human lung fibroblasts (as controls) and iPSC-EC were run for CD31 expression. As mentioned, iPSC-EC expressed CD31 but human lung fibroblasts did not (Figure 3.5B), indicating that iPSC-EC had 'acquired' an endothelial cell phenotype through the reprogramming protocol (Takahashi et al., 2007; Yu et al., 2007). Both iPSC-EC and HLF expressed COX-1 (Figure 3.5)

3.3.2 Response of stem cell derived and vessel endothelial cells to shear stress

As with endothelial cells grown from the vasculature (in this case HAEC) endothelial cells from blood progenitors (BOEC) were cobblestone in appearance when grown under static culture conditions (Figure 3.6A) or when exposed to non-directional/turbulent shear stress (Figure 3.6B; center). As with HAEC, BOEC aligned under conditions of directional shear

stress (Figure 3.6B; edge). By contrast, as mentioned above, endothelial cells from embryonic stem cells (hESC-EC) had a different morphology and were elongated (rather than cobblestone) when cultured under static conditions (Figure 3.6) or under conditions of non-directional shear stress (Figure 3.6B; center), and as a consequence, any 'alignment' in response to directional shear stress was not detectable (Figure 3.6B; edge). Due to limitations in cells I was not able to study the effects of shear stress on iPSC-EC. This is discussed in the limitations section of this chapter.

3.3.3 Responses of stem cell derived endothelial cells to inflammatory stimuli: PAMPs and cytokines

Endothelial cells are critical sensors of bacteria and viruses and signal to professional immune cells to orchestrate inflammatory reactions (Mitchell et al., 2007; Opitz et al., 2009). As discussed in chapter 1, endothelial cells on vessels fulfil their role in innate immunity through expression of pattern recognition receptors (PRRs) including TLRs and NOD receptors, which respond to PAMPs (Gatheral et al., 2012; Opitz et al., 2005). Endothelial cells also respond to IL-1 β , which activates cells independently of PRRs, but via the shared adapter protein MyD88. In protocols to determine the ability of stem cell derived endothelial cells to respond to PAMPs, comparisons were again made with endothelial cells from the vasculature (either HMVEC or HUVEC). In this chapter the well-studied pro-inflammatory cytokine/chemokine CXCL8 was measured as a generic readout of cell activation. In some experiments cell activation was additionally confirmed at the level of NF- κ B nuclear translocation. The release of a larger range of pro-inflammatory cytokines from endothelial cells was also considered in experiments that feature in chapter 4.

All the cell types tested responded to IL-1 β to release increased CXCL8 (Figure 3.7) and translocation of NF- κ B (Figure 3.8). All endothelial cells tested, except for hESC-EC responded to LPS (Figure 3.7). Notably, whilst HUVEC released CXCL8 at levels similar to BOEC, HUVEC tended to show weaker signals for NF- κ B translocation at this time point. This is discussed in more detail in the general discussion. Importantly, hESC-EC did not respond to any of the PAMPs tested (Table 3.1). By contrast, BOEC, iPSC-EC, HMVEC or HUVEC responded to PAMPs against TLR2 and TLR3 (Table 3.1). All endothelial cells tested, including hESC-EC, responded to the NOD1 agonist C12-iE-DAP or to IL-1 β , with increased CXCL8 (Figure 3.7) and nuclear translocation of NF κ B (Figure 3.8). The relative consequence of lack of TLR4, but replete NOD1, responses in the immune function to live Gram-negative bacteria in hESC-EC is addressed in detail in the subsequent chapter (Chapter 4).

Cell viability was not affected by any treatments in these protocols (Table 3.2 and 3.3).

3.3.4 Release of vasoactive hormones by stem cell derived endothelial cells

As discussed, endothelial cells on vessels release prostacyclin, which is cardioprotective, and ET-1, which is associated with cardiovascular disease (Mitchell et al., 2008; Mombouli and Vanhoutte, 1999). In culture, endothelial cells can be activated to release prostacyclin, which is measured as its breakdown product, 6-keto-PGF1 α . Under basal conditions in culture, endothelial cells also release high levels of ET-1 (Stewart et al., 1990). These are specific features of endothelial cells and were therefore used to test further the phenotype of the different stem cell derived endothelial cells.

ET-1

BOEC and iPSC-EC released comparable levels of ET-1 to endothelial cells from vessels including HMVEC, HUVEC and HAEC (Figure 3.9). hESC-EC and HLFs released undetectable levels of ET-1 under basal conditions (Figure 3.9).

Prostacyclin

Under control culture conditions, or after stimulation with IL-1 β , prostacyclin release was detected from each cell type tested with the following rank order magnitude BOEC>HMVEC $\geq$ iPSC-EC>hESC-EC (Figure 3.10)

3.4 SUMMARY

It this chapter I have shown that:

- Where tested, BOEC and iPSC-EC where more morphologically similar than hESC-EC to endothelial cells from vessels.
- hESC-EC, unlike BOEC and endothelial cell from vessels do not align under shear stress, and have an elongated phenotype under static conditions
- hESC-EC, unlike BOEC, HUVEC, HMVEC and iPSC-EC, did not respond to the TLR4 ligand, LPS, to release CXCL8.
- hESC-EC, BOEC, iPSC-EC and endothelial cells from vessels do respond to the NOD1 agonist, C12-iE-DAP to release CXCL8.
- hESC-EC released lower levels of both ET-1 and prostacyclin compared to BOEC, iPSC-EC and endothelial cells from vessels.

The premise of this chapter was that the assessment of stem cell derived endothelial cells in terms of morphology, response to shear stress, innate immunity and vasoactive hormone release is important for their full characterisation as relevant to 'authentic' endothelial cells from blood vessels.

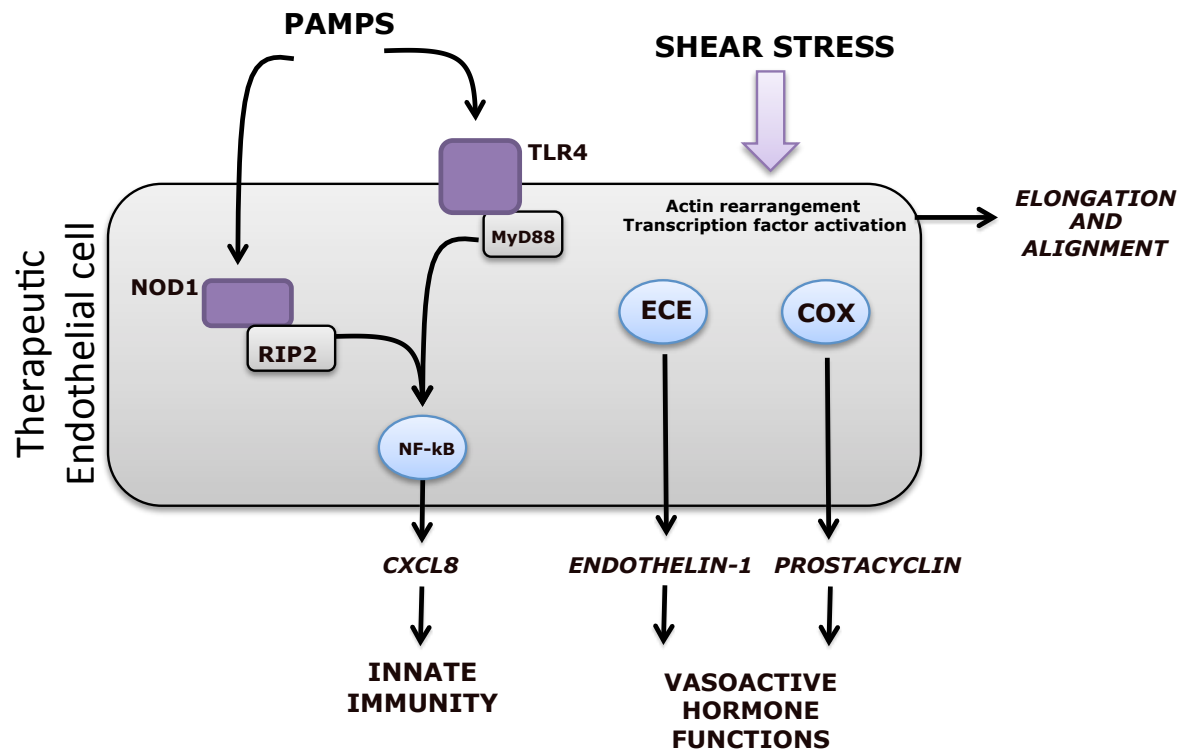
During the course of my PhD, some studies have demonstrated the ability of BOEC to respond to shear stress and function as autologous vascular grafts (Ensley et al., 2012), and the importance of studying shear stress pathways in more detail in these cells has been noted (Glynn and Hinds, 2013). This forms the basis of ongoing work in our research group.

Unlike hESC-EC, both BOEC and iPSC-EC aligned under shear stress, responded to TLR ligands and released high levels of ET-1 and prostacyclin. These findings made both of these stem cell derived endothelial cells suitable for further study in bioassays. In my thesis I opted to take forward BOEC, which I went on to apply in *in vitro* assays described in chapter 5 and 6. This was principally because BOEC are more accessible and less manipulated than iPSC-EC.

As hESC-EC did respond to agonists of NOD1, in the next chapter I have specifically addressed how the function of NOD1 in these cells relates to their ability to sense live bacteria.

CELL TYPE	ALIGNMENT UNDER SHEAR STRESS	TLR4 function	NOD1 function	ET-1 release	Prostacyclin release
ENDOTHELIAL CELLS ON VESSELS	+	+	+	+	++
hESC-EC	- (already elongated)	-	+	-	+
BOEC	+	+	+	+	+++
iPSC-EC	+	+	+	+	++

Summary Figure 1. Summary of endothelial cell functions in stem cell derived endothelial cells determined in Chapter 3. +++ and + etc. indicates rank order for cells releasing prostacyclin. Release data is 24h release under basal conditions for ET-1 and following incubation with IL-1 β (1ng/ml) for prostacyclin release. – for release data indicates undetectable release



Summary Figure 2. Endothelial cells being used in therapy or as research tools will require the key functions and pathways shown. 1. Innate immunity through pattern recognition receptors (TLR4 and NOD1). 2. Response to shear stress to form an aligned phenotype. 3. Release of vasoactive hormones (endothelin-1 and prostacyclin) which are synthesised by key enzymes in endothelial cells (ECE; endothelin converting enzyme and COX; cyclooxygenase). Nitric oxide release is also important feature of endothelial cells but is more difficult to study in human cells and has not been addressed in protocols of Chapter 3. Abbreviations: Toll-like receptor (TLR), nucleotide oligomerisation domain containing receptor (NOD), Receptor interacting protein-2 (RIP2), pathogen associated molecular patterns (PAMPs)

3.5 LIMITATIONS

The data presented in this chapter are limited in a number of ways, which are outlined and discussed below:

A notable limitation of this chapter is that I was not able to demonstrate directly that the h7 hESC line used to generate hESC-EC used in my PhD expressed a larger panel of pluripotency markers, including SSEA3, Oct4, c-myc. These markers are used widely to confirm a pluripotent phenotype and have been shown in this cell type by others (Thomson et al., 1998). My colleague Dr. Gabor Foldes also demonstrated in pilot work that this cell line expresses SSEA4 and high levels of Oct4 mRNA. Information on the characterisation of the pluripotent phenotype of these cells is also available via the University of Wisconsin Biobank where these cells were first isolated and are commercially available for use in research.

It is also noteworthy that the yield of hESC-EC (0.1%) generated from the differentiation protocol was low. Whilst this was sufficient to allow cells to be expanded for use in experiments this will be important to address when scaling up these cells for wider application. Developing this protocol further however was outside the scope of my PhD.

Whilst every effort was made to run all protocols to characterise the stem cell-derived endothelial in direct parallel, this was not always possible. As a result, whilst all protocols were suitably and carefully controlled some protocols were run with small differences. This limitation occurred as a result of cells being available at different times and protocols being developed and modified during the course of this project. However, I believe that these

relatively small differences did not have a major impact on the conclusions/summary points shown above.

It should also be noted that in experiments where staining was carried out using antibodies that I was not able to test the effect of isotype controls for these antibodies. This would have added to the certainty as to the specificity of the staining on endothelial cells. An additional approach to testing for specificity would include the addition of a blocking peptide that represents the original peptide sequence to which the antibody was raised. To compensate for this limitation however, I was able to show that the antibody used to stain for CD31, which is expressed by endothelial cells, did not result in positive staining in human lung fibroblasts, which do not express CD31 (Figure 3.5). Taken together, this suggests that the CD31 staining by endothelial cells in my PhD was specific to endothelial cells.

Another limitation is that I was not always able to perform sufficient numbers of experiments to use statistically testing and extra n-values for some protocols would have increased the robustness of the data. However, this being said, data presented was clear, consistent and, as in all protocols, included appropriate controls where necessary.

A key finding of this chapter was that hESC-EC do not have TLR4 function. I have exclusively used cells derived from the H7 cell line to demonstrate this. This is clearly an important limitation and it would therefore be interesting to look if this 'no TLR4' phenotype is preserved across other hESC-line derived endothelial cells. Going forward, it might be equally important to consider the phenotype of hESC-EC derived using subtly different protocols, including those that have been developed for large scale production of cell

therapies. Notably, others in the group showed that undifferentiated hESCs derived from two separate lines are devoid of TLR4 function (Földes et al., 2010) and this may add weight to the findings in this particular hESC-EC line.

Finally, in order to establish the parallels of endothelial cells from stem cell origins with those from vessels it is important to test their functionality in vascular response models; the key ones being thrombosis and vasomotor control. This is a limitation of this chapter and was unfortunately beyond the scope of my thesis to establish the required protocols.

3.6 FIGURES

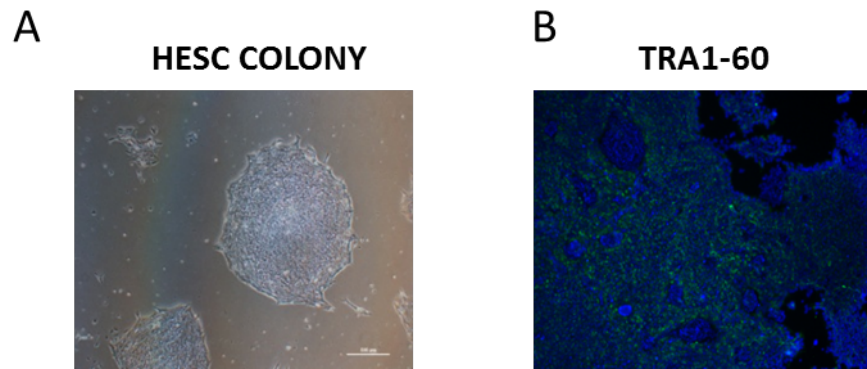
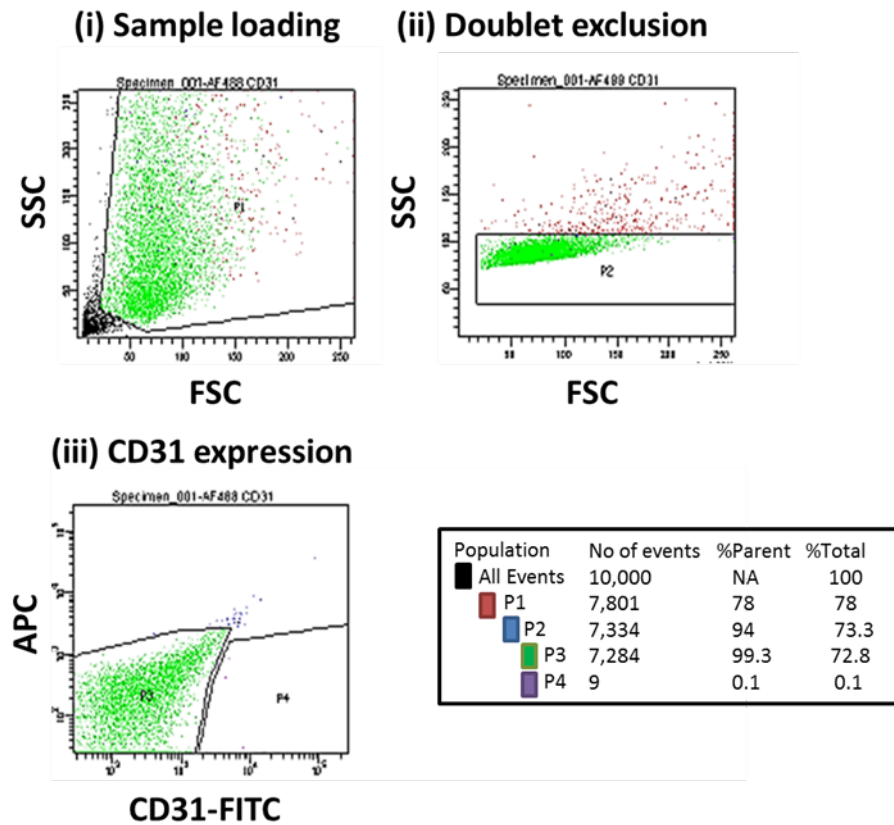


Figure 3.1 Images of undifferentiated human embryonic stem cells (hESC). **(A)** Morphology (Foldes et al., 2010: CC-BY licence) and **(B)** pluripotency (TRA-1-60; green) marker expression in hESC. For panel A images were acquired using a light microscope at 10x magnification and for panel B using an inverted fluorescence microscope at 10x magnification. Cell nuclei were stained with 10 μ g/ml DAPI (blue).

A



B

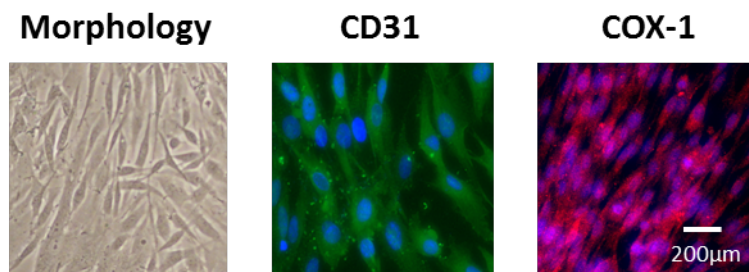
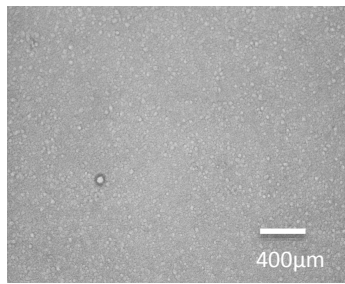
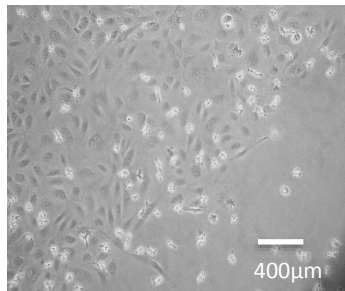


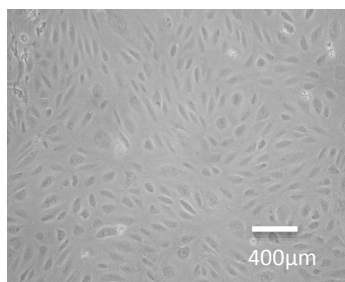
Figure 3.2 Isolation and characterisation of human embryonic stem cell derived endothelial cells (hESC-EC) from embryoid bodies. **(A)** Contour plots for separation of CD31+ cells from embryoid bodies; (i) forward scatter (FSC) and side scatter (SSC) of total sample (P1; red), (ii) FSC and SSC of population (P)-1 showing doublet exclusion, (iii) CD31 expression of P2 (blue) to give P3 (green) (CD31-) and P4 (pink) (CD31+). P4 which was selected and plated on 1% gelatinized coated plates for expansion. **(B)** Morphology of 15 day expanded hESC-EC, CD31 (green; Foldes et al., 2010: CC-BY licence) and COX-1 (red) expression in cells (left to right). Cell nuclei are stained with 10µg/ml DAPI (blue). Images for morphology were acquired using a light microscope, for CD31 using an inverted fluorescence microscope, and for COX-1 using a Cellomics® VTi HCS Arrayscanner. All images are at 10 x magnifications with scale bars indicated.



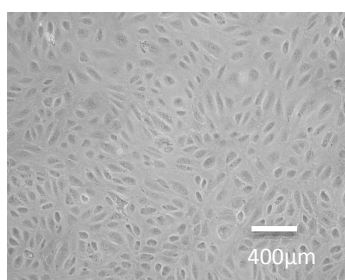
Day: 1
Plate: 6-well
Coating: Collagen (5.2µg/cm²)
Cells: PBMC fraction
Passage: NA



Day: 11
Plate: 6-well
Coating: Collagen (5.2µg/cm²)
Cells: BOEC colony
Passage: NA



Day: 16
Plate: T25
Coating: Collagen (5.2µg/cm²)
Cells: confluent BOEC
Passage: 1



Day: 19
Plate: T75
Coating: Collagen (5.2µg/cm²)
Cells: confluent BOEC
Passage: 2

Figure 3.3 Isolation of blood outgrowth endothelial cells (BOEC) from peripheral blood mononuclear cells (PBMCs). At day 1, PBMCs were plated at 3×10^7 cells/well in 4ml Lonza-EGM2 media with 10% FBS. Colonies emerged between day 7 and 20 (day 11 in the example above). Cells were then expanded to 25cm² and then 75cm² flasks for use in experiments at passage 3 or cryopreservation. Images were captured using a light microscope with a Nikon VR ISO3200 camera attachment. All images are at 10x magnification with scale bars indicated.

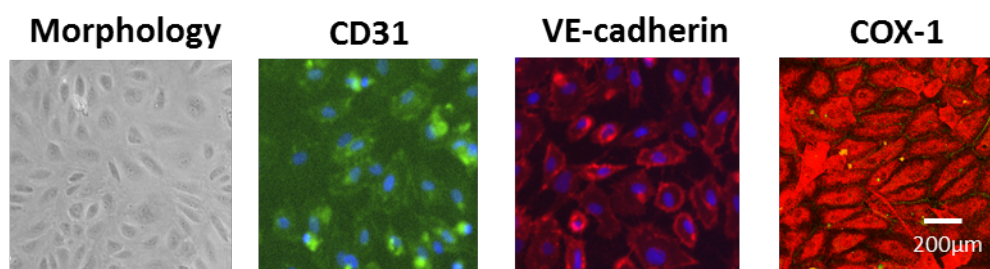
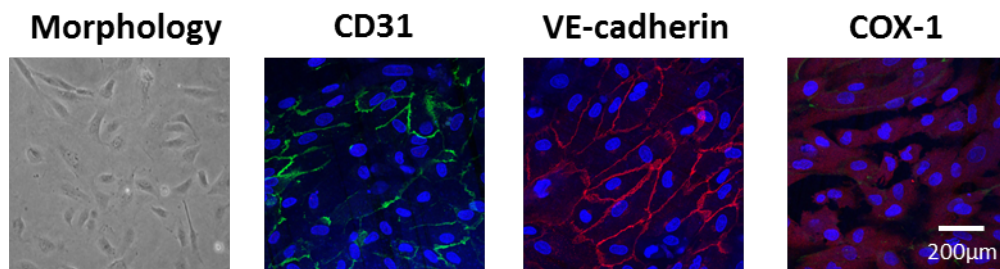


Figure 3.4 Characterisation of blood outgrowth endothelial cells (BOECs) in culture. Morphology of BOEC imaged by light microscope with a Nikon VR ISO3200 camera attachment and CD31 (green), VE-cadherin (red) and COX-1 (red) expression in BOECs (left to right). Cell nuclei are stained with 10µg/ml DAPI (blue). Images for CD31 and VE-cadherin were acquired using a Cellomics HCS VTi arrays scanner and for COX-1 using a confocal microscope. All images are at 10x magnification with scale bar indicated for the COX-1 image which is true for all images.

A; iPSC-EC



B; HLF

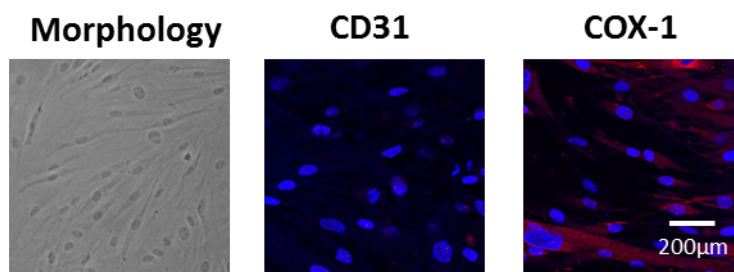


Figure 3.5 Characterisation of induced pluripotent stem cell derived endothelial cells (iPSC-ECs) versus human lung fibroblasts (HLF). **(A)** Morphology of, and CD31 (green), VE-cadherin (red) and COX-1 (red) expression in iPSC-ECs. Cell nuclei were stained with 10µg/ml DAPI (blue). **(B)** Morphology of, and CD31 (green) and COX-1 (red) expression in, HLF. Images for morphology were acquired using a light microscope with a Nikon VR ISO3200 camera attachment, for CD31, VE-cadherin and COX-1 using a confocal microscope. All images are cropped equally from images at 10x magnification. All images were captured at 10x magnification with scale bar indicated for the COX-1 image which is true for all images.

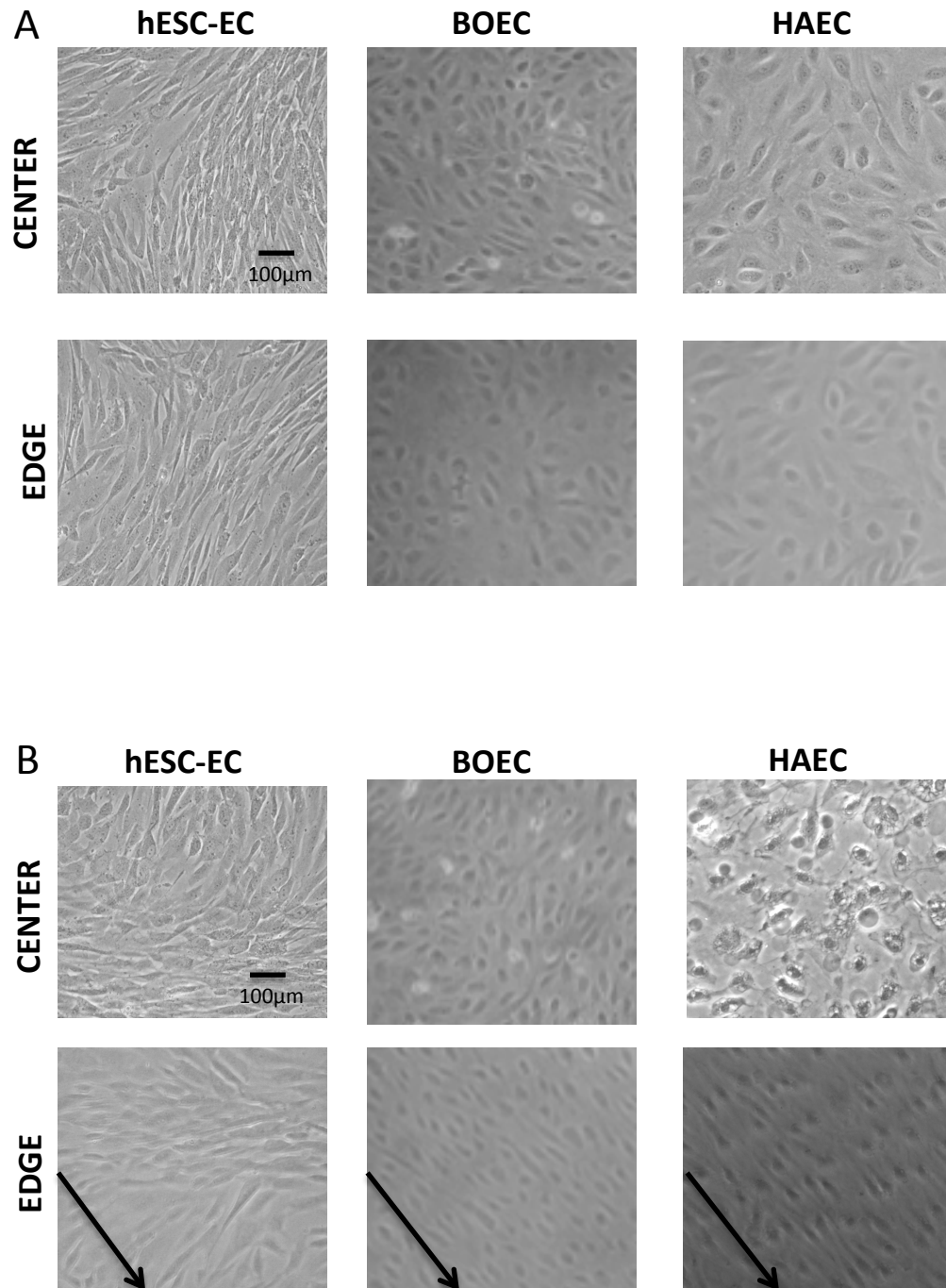


Figure 3.6 Responses of different stem cell derived-endothelial cells to shear stress. Human embryonic stem cell derived endothelial cells (hESC-EC), blood outgrowth endothelial cells (BOEC), and human aortic endothelial cells (HAEC) (left to right) under **(A)** static conditions and **(B)** after 4 days of shear stress. Images were taken at the edge of the well, where shear stress is unidirectional and cells align, and at the center of the well where shear stress had no preferred direction. Images shown are at 10x magnification with scale bars shown on hESC-EC centre images and are true for all images. Black arrows on shear plate edge images indicate the direction of flow.

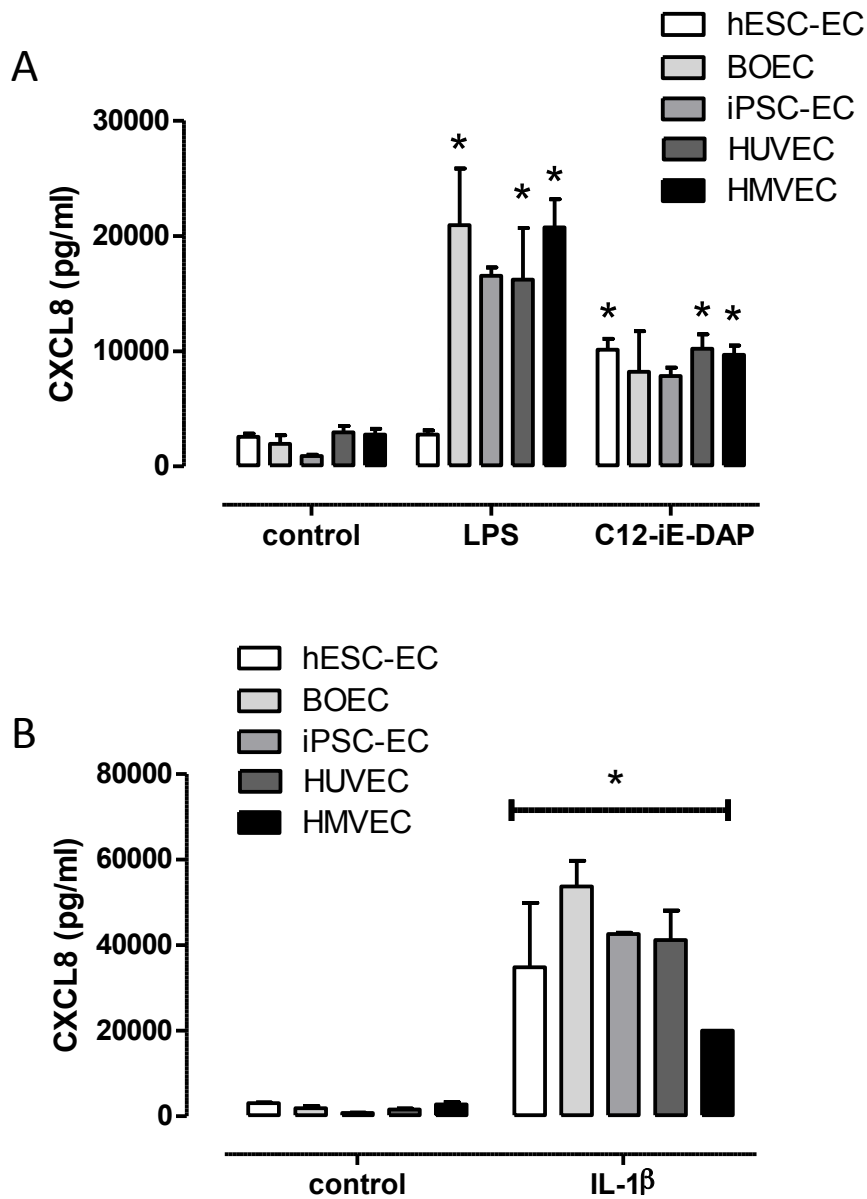


Figure 3.7 CXCL8 release from different stem cell derived endothelial cells in response to **(A)** TLR4 and NOD1 agonists and **(B)** IL-1 β stimulation. Human embryonic stem cell derived endothelial cells (hESC-EC) (n=8), blood outgrowth endothelial cells (BOEC) (n=8), human lung induced pluripotent stem cell derived endothelial cells (iPSC-EC) (n=2 from one pilot experiment), human umbilical vein endothelial cells (HUVEC) (n=8) and human microvascular endothelial cells (HMVEC) (n=6) were treated for 24h with LPS (TLR4 agonist; 1 μ g/ml) or C12-iE-DAP (NOD1 agonist; 10 μ g/ml) or IL-1 β (1ng/ml). For iPSC-EC, n=2 from one pilot experiment. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test for each cell type (*p<0.05). Analysis was not performed on data from iPSC-EC.

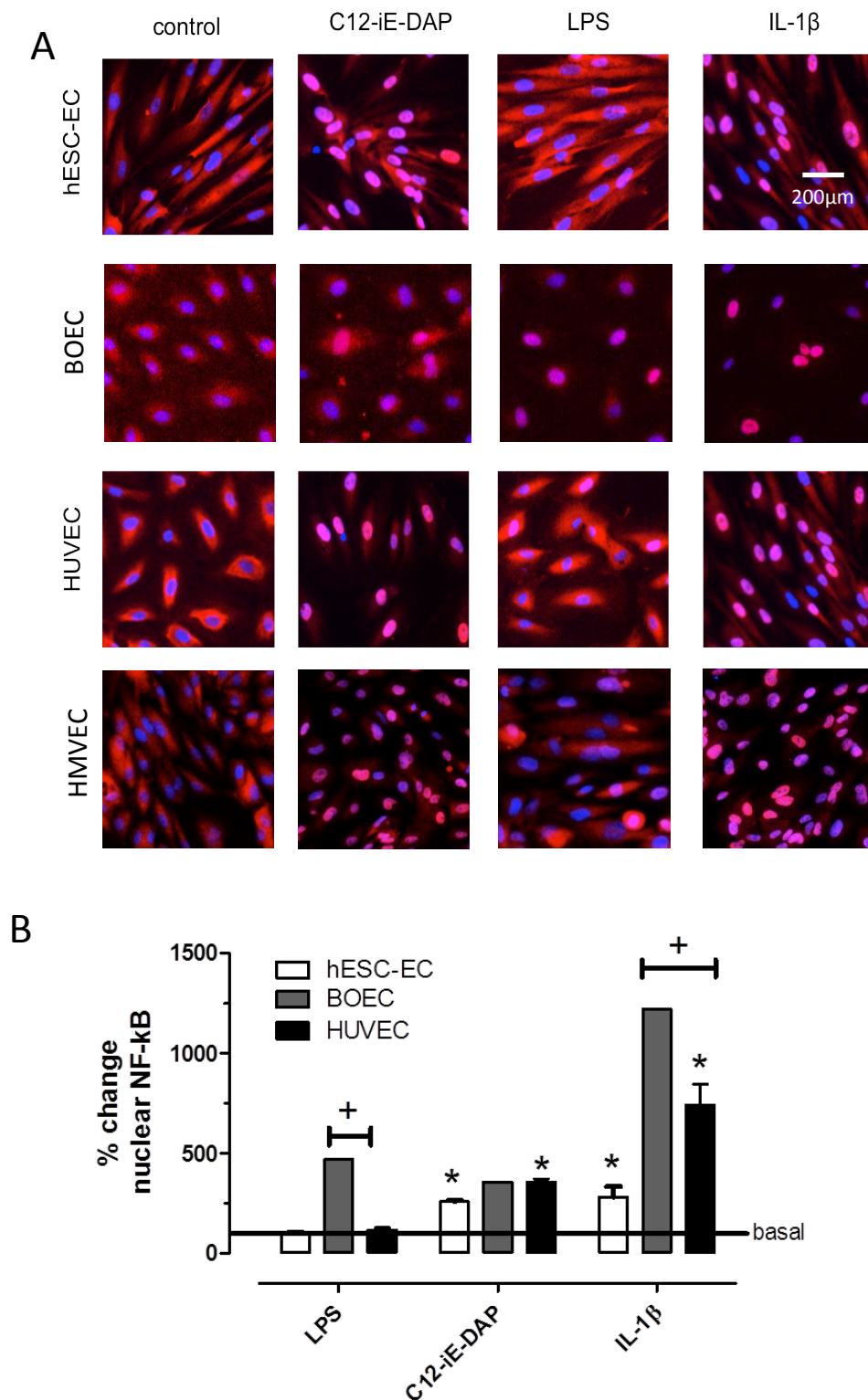


Figure 3.8 Response of stem cell derived endothelial cells to pathogen associated molecular patterns (PAMPs) in terms of NF- κ B nuclear translocation. **(A)** Representative immunocytochemistry images of human embryonic stem cell derived endothelial cells (hESC-EC), blood outgrowth endothelial cells (BOEC), human umbilical vein endothelial cells (HUVEC) and human lung microvascular endothelial cells (HMVEC) stained for NF- κ B following 1h treatment with C12-iE-DAP (NOD1 agonist; 10 μ g/ml), LPS (TLR4 agonist;

1 μ g/ml) or IL-1 β (1ng/ml). Nuclei were stained with 10 μ g/ml DAPI. Images were acquired using a Cellomics VTi HCS Arrayscanner with a Carl Zeiss microscope. Images were acquired at 10 x magnifications with scale bars indicated. **(B)** Quantification of NF- κ B nuclear translocation in hESC-EC (n=6), BOEC (n=1 from one pilot experiment) and HUVEC (n=6) in response to the above treatments. Data are normalised to 1000 cells/well and normalised to control (100%). Data are mean (%control) $\pm$ SEM. Statistical significance was determined by one-sample t-test (*p<0.05). Statistical testing was not carried out BOEC. Statistical significance between cells was determined by two-way ANOVA followed by Bonferroni's post-test (+p<0.05)

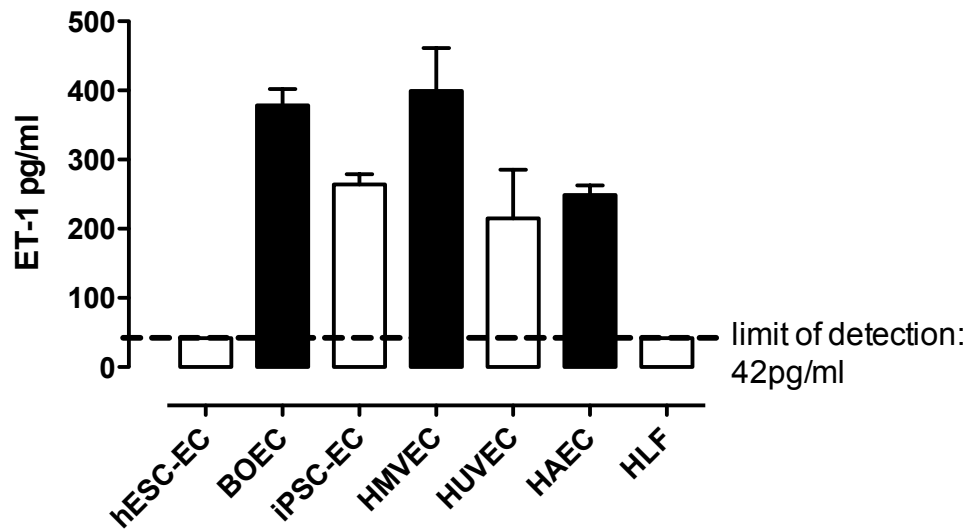


Figure 3.9 Endothelin-1 (ET-1) release from different stem cell derived endothelial cells versus endothelial cells from vessels and human lung fibroblasts (HLF). ET-1 release from human embryonic stem cell derived cells (hESC-EC; n=8), blood outgrowth endothelial cells (BOEC;), induced pluripotent stem cell derived endothelial cells (iPSC-EC; n=2 from one pilot experiment), human lung microvascular endothelial cells (HMVEC; n=4), human umbilical vein endothelial cells (HUVEC; n=6), human aortic endothelial cells (HAEC; n=3 from 1 experiment) or HLF (n=2). Cells were grown in similar conditions and basal ET-1 release measured after 24h in culture.

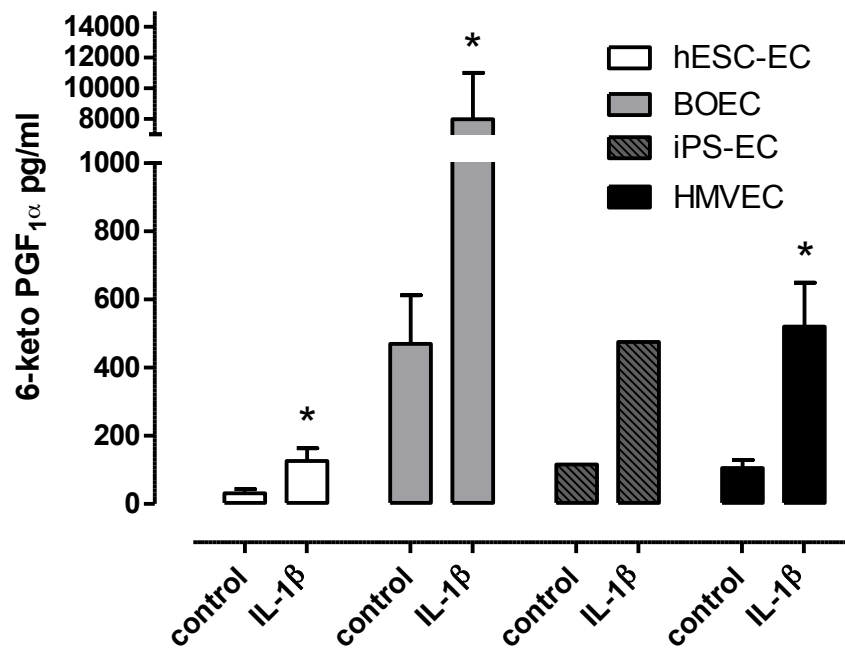


Figure 3.10 Prostacyclin release from different stem cell derived endothelial cells versus human lung microvascular endothelial cells (HMVEC). Human embryonic stem cell derived cells (hESC-EC; n=5), blood outgrowth endothelial cells (BOEC; n=9), induced pluripotent stem cell derived endothelial cells (iPSC-EC; n=2 from one pilot experiment), human lung microvascular endothelial cells (HMVEC; n=5). Prostacyclin release was measured as 6-keto PGF_{1α} following 24h incubation +/- IL-1β (1ng/ml). Data are mean ± SEM. Statistical significance for the effect of IL-1β on prostacyclin release was determined by students t-test (*p<0.05).

	hESC-EC	BOEC	iPSC-EC	HMVEC
Control	1796.9 ± 430.8	1955.1 ± 499.9	779.5 ± 106.5	3476.7 ± 608.8
HKLM	2310.3 ± 622.9	2675.5 ± 918.0	1315.5 ± 173.1	3817.4 ± 879.3
Pam3CSK4	1733.4 ± 549.1	7436.1 ± 3769.0	7334.3 ± 357.4	3745.7 ± 813.3
FSL-1	3078.0 ± 1013.0	8151.0 ± 3036.0	14682.4 ± 1044.5	2992.1 ± 620.8
PolyIC	1262.6 ± 403.6	14851.7 ± 3543.7	2016.3 ± 264.9	18871.0 ± 3281.5
LPS	2125.8 ± 815.2	283011.4 ± 6367.2	15077.7 ± 775.4	46331.2 ± 16032.6
ST-FLA	1265.8 ± 1013.0	4477.5 ± 1039.9	9804.4 ± 2827.5	3104.2 ± 1064.6
Imiquimod	1173.4 ± 380.6	2302.4 ± 546.7	2059.5 ± 44.2	2561.4 ± 439.4
ssRNA40	1803.9 ± 692.4	2188.8 ± 704.9	1751.9 ± 175.3	3506.3 ± 1094.9
ODN2006	1356.9 ± 591.3	2090.2 ± 511.1	1468.0 ± 108.6	3871.4 ± 1648.6
IL-1β	17447.9 ± 7271.2 *	40586.3 ± 5556.3 *	42624.0 ± 274.3 *	13833.9 ± 887.7 *

Table 3.1 Effect of TLR agonists and IL-1β on CXCL8 release from different stem cell derived endothelial cells and human lung microvascular endothelial cells (HMVEC). Human embryonic stem cell derived endothelial cells (hESC-EC), blood outgrowth endothelial cells (BOEC), human lung induced pluripotent stem cell derived endothelial cells (iPSC-EC) and human microvascular endothelial cells (HMVEC) were treated for 24h with/without HKLM (TLR2 agonist; 10⁷ cells/ml), Pam₃CSK4 (TLR1/2 agonist; 1μg/ml), FSL-1 (TLR2/6 agonist; 1μg/ml), PolyIC (TLR3 agonist; 10μg/ml), LPS (TLR4 agonist; 1μg/ml), ST-FLA (Staphylococcus aureus derived flagellin) (TLR5 agonist; 100ng/ml), IMI (imiquimod) (TLR7 agonist; 1μg/ml), ssRNA40 (TLR8 agonist; 1μg/ml, ODN2006 (TLR9 agonist; 5μM) or IL-1β (1ng/ml). Data are mean ± SEM (hESC-EC n= 8-10; BOEC n= 14; iPSC-EC n=2 from one pilot study; HMVEC n=4). Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test (*p<0.05). Analysis was not carried out on iPSC-EC.

	hESC-EC	BOEC	iPSC-EC	HMVEC
Control	0.430 ± 0.041	0.268 ± 0.033	0.275 ± 0.019	0.313 ± 0.021
HKLM	0.415 ± 0.045	0.256 ± 0.043	0.243 ± 0.016	0.248 ± 0.060
Pam3CSK4	0.398 ± 0.046	0.235 ± 0.051	0.241 ± 0.013	0.160 ± 0.001
FSL-1	0.396 ± 0.053	0.277 ± 0.041	0.280 ± 0.046	0.207 ± 0.002
PolyIC	0.441 ± 0.043	0.302 ± 0.003	0.236 ± 0.017	0.300 ± 0.000
LPS	0.398 ± 0.046	0.230 ± 0.053	0.245 ± 0.020	0.205 ± 0.025
ST-FLA	0.436 ± 0.036	0.231 ± 0.051	0.238 ± 0.010	0.292 ± 0.019
Imiquimod	0.460 ± 0.073	0.228 ± 0.053	0.273 ± 0.033	0.299 ± 0.02
ssRNA40	0.444 ± 0.045	0.264 ± 0.046	0.348 ± 0.043	0.172 ± 0.000
ODN2006	0.446 ± 0.052	0.276 ± 0.047	0.276 ± 0.005	0.353 ± 0.000
IL-1β	0.414 ± 0.052	0.242 ± 0.05	0.290 ± 0.015	0.207 ± 0.002

Table 3.2 Effect of TLR agonists and IL-1β on cell viability of different stem cell derived endothelial cells and human lung microvascular endothelial cells (HMVEC). Human embryonic stem cell derived endothelial cells (hESC-EC), blood outgrowth endothelial cells (BOEC), human lung induced pluripotent stem cell derived endothelial cells (iPSC-EC) and human microvascular endothelial cells (HMVEC) were treated for 24h with/without HKLM (TLR2 agonist; 107 cells/ml), Pam₃CSK4 (TLR1/2 agonist; 1μg/ml), FSL-1 (TLR2/6 agonist; 1μg/ml), PolyIC (TLR3 agonist; 10μg/ml), LPS (TLR4 agonist; 1μg/ml), ST-FLA (Staphylococcus aureus derived flagellin) (TLR5 agonist; 100ng/ml), IMI (imiquimod) (TLR7 agonist; 1μg/ml), ssRNA40 (TLR8 agonist; 1μg/ml, ODN2006 (TLR9 agonist; 5μM) or IL-1β (1ng/ml). Data are mean ± SEM (hESC-EC n= 6; BOEC n= 6; iPSC-EC n=2 from one pilot study; HMVEC n=2). All data are from experiments on cells with representative CXCL8 profiles in response to treatments.

	cell number		alamarBlue®	
	hESC-EC	HUVEC	BOEC	iPSC-EC
Control	310.3 ± 77.5	155.1 ± 30.3	0.432 ± 0.076	0.271 ± 0.011
LPS	307.0 ± 77.9	96.1 ± 18.2	0.375 ± 0.052	0.241 ± 0.009
C12-iE-DAP	276.4 ± 68.5	128.7 ± 25.8	0.409 ± 0.072	0.258 ± 0.021

Table 3.3 Effect of TLR4 and NOD1 agonists on cell viability of different stem cell derived endothelial cells. Cells were treated for 24h with LPS (TLR4 agonist; 1µg/ml) or C12-iE-DAP (NOD1 agonist; 10µg/ml). Cell viability of human embryonic stem cell derived endothelial cells (hESC-EC) and human umbilical vein endothelial cells (HUVEC) was determined using a Cellomics VTi HCS Arrayscanner and results are expressed as the average number of cells per field of view. Cell viability of blood outgrowth endothelial cells (BOEC), and human induced pluripotent stem cell derived endothelial cells (iPSC-EC) was determined using alamarBlue and results are expressed as absorbance at 570nm-630nm. Data are mean ± SEM (hESC-EC and HUVEC n=6; BOEC n=6; iPSC-EC n=2 from one pilot experiment).

CHAPTER 4: RESPONSES OF HESC-EC TO *HAEMOPHILUS* *INFLUENZAE* AND ROLE OF NOD1 RECEPTORS

4.1 RATIONALE

In the previous chapter I showed that unlike endothelial cells from other sources, human embryonic stem cell derived endothelial cells (hESC-EC) shared some characteristics of endothelial cells, in that they express markers, but do not respond to the Toll-like receptor 4 (TLR4) agonist LPS. These findings have important implications, which could mean that hESC-EC are either (i) superior to other endothelial cells as therapies because TLR4 is involved in atherosclerosis or (ii) carry increased side effects because without TLR4 the cells would be immuno-deficient. Thus, in this chapter I have followed up specifically on the implications of TLR4 deficiency in hESC-EC.

In Chapter 3 I used CXCL8 as a generic marker of cell activation. However, LPS induces a range of pro-inflammatory cytokines. In order to be sure that the effects seen in hESC-EC were not due to a specific dysfunction in TLR4/CXCL8 signalling, here I have repeated experiments and measured a full range of 9 separate pro-inflammatory cytokines in cells stimulated with LPS.

In Chapter 3 I also found that hESC-EC respond to nucleotide oligomerisation domain receptor (NOD1) agonists. TLR4 and NOD1 are the principal pattern recognition receptors (PRR) expressed by endothelial cells that sense Gram-negative bacteria (Gatheral et al., 2012; Opitz et al., 2009). In this chapter I have investigated the impact of the absence of TLR4 in hESC-EC on the ability of the cells to respond to live Gram-negative bacteria. This is further explored by addressing the role of NOD1 in these responses and whether NOD1 can accommodate sensing of Gram-negative bacteria by hESC-EC.

The specific questions addressed in this chapter are:

1. Is the inability of hESC-EC to release CXCL8 in response to LPS recapitulated when a full range of pro-inflammatory cytokines is measured?
2. How do hESC-EC respond to the live Gram-negative bacteria compared to endothelial cells from vessels (HUVEC; which have both TLR4 and NOD functions)?
3. What is the contribution of NOD1 to the sensing of live Gram-negative bacteria by hESC-EC?

4.2 SUMMARY OF METHODS

A full description of the methods used in this chapter is given in Chapter 2 and are summarised below.

4.2.1 Cell culture and treatments

hESC-EC and human umbilical vein endothelial cells (HUVEC) were cultured as described in Chapter 2 and 3, and plated on 96-well plates (Nunc, Denmark) at a seeding density of 7,000 cells/well in Lonza-EGM2 media. Once confluent, cells were treated with reagents, drugs or live Gram negative *Haemophilus influenzae* bacteria for either 1 or 24 hours before media was collected and cells fixed for imaging, lysed for extraction of RNA or treated with Alamarblue for measurement of cell viability. Drugs used included LPS (TLR4 agonist), iE-DAP (NOD1 agonist), C12-iE-DAP (NOD1 agonist), GSK2576214A (GSK'214; RIP2 inhibitor; a gift from GSK), GSK1219217A (GSK'217; NOD1 inhibitor; a gift from GSK), SC-514 (IKK inhibitor), SB203580 (RIP2/p38 inhibitor), BIRB0796 (p38 inhibitor). In these experiments, cells were incubated with inhibitors for 30 minutes prior to addition of agonists and/or bacteria.

For experiments with bacteria, cells were incubated with live *Haemophilus influenzae* (ATTC strain 49247) at colony forming units (CFU) of 10^8 - 10^5 per ml for 24 hours. *Haemophilus influenzae* comes from the supplier in bacteria support media. In some experiments bacteria-free samples of this media were prepared by filtering the broth and used as experimental 'vehicle' controls (see Figure 2.1; chapter 2). In later experiments cells were pre-incubated with and without NOD1 targeting siRNA or NOD1 and RIP2 antagonists as described below.

4.2.2 Quantitative real time PCR (qRT-PCR) for TLR4 and NOD1 expression

TLR4 expression was measured using a commercially available focused array (Gene Array PAHS-058, SABiosciences, UK) and NOD1 was measured using specific primers (TaqMan Chemistry, Applied Biosystems, UK) for qRT-PCR. All assays were carried out according to manufactures instructions. For TLR4, data were normalised to the mean of 5 housekeeping genes included in the array (B2M, HPRT1, RPL13A, GAPDH, ACTB) and for NOD1 to the house keeping gene, GAPDH. The PCR was performed with ABI 5700 (Applied Biosystems, USA) and Rotor-Gene 3000 (Corbett Research, UK) RT-PCR instruments. Relative gene expression was determined by the $\Delta\Delta C_t$ method where fold changes is calculated as $= 2^{-\Delta\Delta C_t}$.

4.2.3 Measurement of pro-inflammatory cytokines using a Meso Scale discovery (MSD) platform

Levels of 9 pro inflammatory cytokines were measured using '*human pro-inflammatory 9-Plex MULTISPOT 96-well -10 spot*' purchased from MSD (Gaithersburg, Maryland, USA). Samples were diluted 1:10 in assay buffer and assays carried out according to manufacturer's instructions. MSD plates were read using an MSC Sector Imager 2400 and analysed using MSD Discovery® Workbench software. Cytokines analysed were TNF α , IL-2, CXCL8, IL-12p70, IL-1 β , GM-CSF, IFN γ , IL-6, IL-10.

4.2.4 Small interfering-RNA (siRNA) mediated knockdown of NOD1

For NOD1 siRNA knockdown protocols, targeting NOD1 siRNA (Qiagen, UK) was used according to manufacturer's instructions. Cells were plated 24 hours before transfection. Scrambled non-targeting siRNA (Qiagen, UK) was used as negative control. Following 48

hours of transfection cells were either lysed for extraction of RNA or fresh media added with treatments as above. After 24 hours supernatants were collected for analysis.

4.2.5 Measurement of CXCL8

CXCL8 was measured by ELISA (R and D Systems) according to manufacturer's instructions. Optical density was determined using a microplate reader (Dyne, Madellan Biosciences) with absorbance readings at 450-570nm.

4.2.6 Measurement of cell viability

Cell viability following incubations was determined using either the commercially available AlamarBlue® assay (Invitrogen, UK) or based on cell counts. For cell counts, cells were stained with 10µg/ml 4',6-diamidino-2-phenylindole (DAPI) and imaged using the Cellomics VTi HCS Arrayscan (Thermofisher, USA). Data are expressed as average cells/field based on 49 fields. Unless otherwise stated none of the treatments greatly affected cell viability (Table 4.1).

4.3. RESULTS

4.3.1 Effect of the TLR4 agonist LPS versus NOD1 agonists on release of a range of cytokines from hESC-EC

As seen in chapter 3, the NOD1 agonist C12-iE-DAP, as well as iE-DAP, induced CXCL8 release from either HUVEC or hESC-EC whilst the TLR4 agonist LPS released CXCL8 from HUVEC only (Figure 4.1). In order to be sure this response was not limited to CXCL8 8 other cytokines were measured in conditioned media from these cells. As with CXCL8, hESC-EC and HUVEC responded to the NOD1 agonist C12-iE-DAP to release GM-CSF, IL-12p70, IL-2, IL-6, IL1 β and TNF α . HUVEC, but not hESC-EC, also tended to release GM-CSF, IFN γ , IL-12p70, IL-2, IL-6 and TNF α in response to treatment with LPS (Figure 4.2 to 4.9).

4.3.2 Relative expression of TLR4 and NOD1 in hESC-EC versus HUVEC

In line with what was seen for cytokine release, hESC-EC expressed lower levels of TLR4 than HUVEC whereas levels of NOD1 gene were comparable between the two cell types (Figure 4.10).

4.3.3 Responses of hESC-EC to *Haemophilus influenzae*

The experiments above confirm that hESC-EC have no TLR4 function, and that this is explained by reduced receptor expression. As discussed, Gram-negative bacteria are sensed by two key pattern recognition receptor (PRR) pathways; TLR4 and NOD1. As with other types of Gram-negative bacteria, *Haemophilus influenzae* is reportedly sensed by cells via TLR4 and/or NOD1 pathways (Hirano et al., 2009; Ratner et al., 2007). I therefore performed experiments where cells were infected with live bacteria to establish the impact of a

dysfunctional TLR4 pathway on the sensing of Gram-negative bacteria. As with other protocols, hESC-EC did not respond to LPS, but did respond to C12-iEDAP, whilst HUVEC responded to both (Figure 4.11A). hESC-EC and HUVEC both responded to infection with the Gram-negative bacteria *Haemophilus influenzae* by releasing CXCL8 (Figure 4.11B). The potency of *Haemophilus influenzae* was similar in both cell types. *Haemophilus influenzae* in control media incubated for 24 hours without cells did not interfere with the CXCL8 ELISA.

4.3.4 Role of NOD1 in responses of hESC-EC to HIN to release CXCL8

In order to establish the role of NOD1 receptors in the activation of hESC-EC by *Haemophilus influenzae*, both a molecular approach-using gene knock down, and a pharmacological approach using selective inhibitors of NOD1 and RIP2 was employed.

NOD1 receptors can be knocked down using conventional targeting siRNA (see general methods and above). As expected, incubation of hESC-EC with NOD targeting siRNA for 48 hour resulted in knockdown of NOD1 gene expression (Figure 4.12A). Knockdown of NOD1 receptors at the gene level also reduced responses of hESC-EC to the NOD1 agonist C12-iEDAP and, importantly, also to *Haemophilus influenzae* infection (Figure 4.12B). Responses to IL-1 β were not affected by NOD1 siRNA targeted knockdown indicating that the loss of response to *Haemophilus influenzae* by hESC-EC was specific to NOD1 (Figure 4.13).

To further examine the role of NOD1 and corroborate these findings, pharmacological assays were employed. In early experiments characterising the signalling pathways involved in NOD1 agonist induced CXCL8 release by hESC-EC, I found selective inhibition of C12-iEDAP responses with SB203580 which inhibits RIP2 and p38, with only mild inhibitory effects

with BIRB0796, which inhibits p38 (Table 4.2). Responses to both C12-iE-DAP and IL-1 β were inhibited by SC-514 which inhibits NF- κ B mediated responses. These observations suggested that NOD1 responses in hESC-EC, as in other cell types, are mediated by RIP2. In order to test this further, I used novel inhibitors of NOD1 and its associated adapter protein RIP2 developed by GSK, which have previously been used and validated by our group (Gatheral et al., 2012). We have previously shown using endothelial cells from vessels that the NOD1 antagonist GSK'217 or the RIP2 inhibitor GSK'214 inhibit CXCL8 release induced by NOD1 agonists, without affecting TLR4 responses (Gatheral et al., 2012). In line with this, GSK'217 or GSK'214 reduced CXCL8 release (Figure 4.14) as well as NF- κ B nuclear translocation in hESC-EC stimulated with C12-iE-DAP (Table 4.3). Importantly, CXCL8 release from *Haemophilus influenzae* infected hESC-EC was also reduced by NOD1 and RIP2 inhibition (Figure 4.14). CXCL8 levels from IL-1 β treated hESC-EC were not affected by the NOD1 (GSK'217) and RIP2 (GSK'214) inhibitors (Figure 4.15).

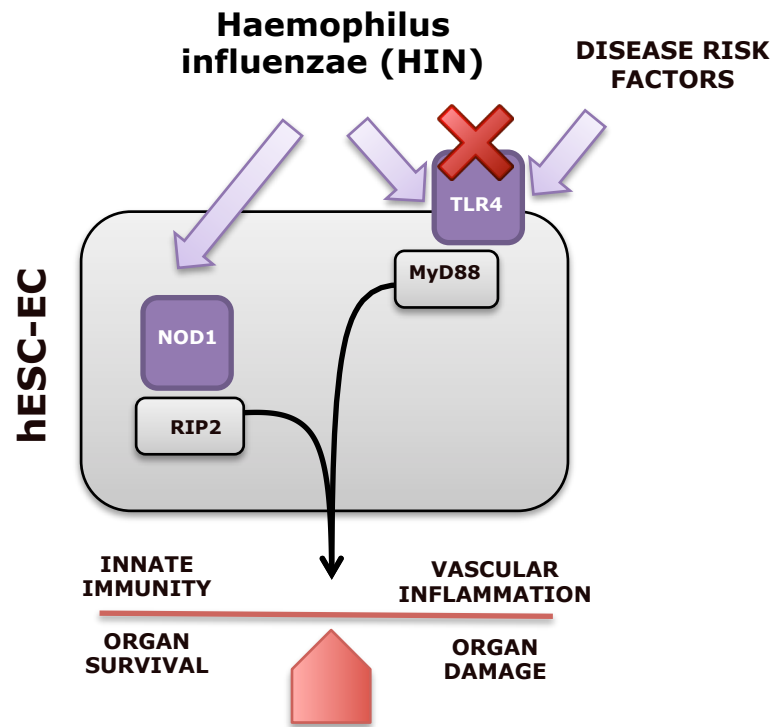
4.4 SUMMARY

In the previous chapter it was determined that hESC-EC, unlike endothelial cells from adult progenitors (BOEC) or induced pluripotent stem cells (iPSC-EC), or endothelial cells from vessels do not respond to the TLR4 agonist, LPS. hESC-EC do however respond to NOD1 agonist to release CXCL8.

In this Chapter I have shown that:

- In addition to CXCL8, hESC-EC do not respond to the TLR4 agonist LPS to release a range of additional pro-inflammatory cytokines, and this is explained through lower TLR4 expression compared to HUVEC.
- However, despite no TLR4 function, hESC-EC are able to sense the Gram-negative bacteria *Haemophilus influenzae*.
- Using a molecular and pharmacological approach, NOD1 and its associated adapter protein RIP2 accommodate and drive responses in hESC-EC to C12-iE-DAP and Gram-negative bacteria.

As TLR4 is now implicated in atherosclerosis (Edfeldt et al., 2002) these observations enabled me to speculate that the lack of TLR4 on hESC-EC might afford these cells an advantage over other stem cell derived endothelial cells when used in engineered organs or as cell therapies. This is discussed in more detail in Chapter 7 and in the context of potential benefits and limitations of hESC-EC. The full implications of observations in this chapter and how they relate to others in my thesis are discussed in detail in Chapter 7.



Summary Figure 3. Human embryonic stem cell derived endothelial cells (hESC-EC) do not express TLR4 or respond to TLR4 agonists (LPS) to release any cytokines associated with inflammation. hESC-EC do express NOD1, and utilise this receptor to sense Gram negative bacteria such as *Haemophilus influenzae* (HIN) which results in cytokine release essential for innate immunity and organ survival. As many cytokines associated with the activation of TLR4 in disease drive vascular inflammation, these cells might be protected for disease risk factor activated inflammation.

4.5 LIMITATIONS

The data presented in this chapter are limited in a number of ways, which are outlined and discussed below:

Whilst the *Haemophilus influenzae* was a good representative Gram-negative bacteria, the inclusion of other Gram-negative bacteria might have been useful and provided support to the conclusions drawn in this chapter. Examples of other, perhaps more relevant (to cardiovascular disease) would be *Chlamydia* and *Listeria* species which have been implicated in cardiovascular disease. Bacteria that have been associated with cardiovascular disease and reviewed by Libby et al. (Libby et al., 1997) and discussed in more detail in Chapter 7.

Given that hESC-EC did not have TLR4 function I chose to investigate these cells in particular over other stem cell derived endothelial cells. However, it may also have been of interest to look at responses of other stem cell derived endothelial cells, as well as other hESC-EC lines, to live bacteria but this was beyond the premise of this chapter.

Other methods to assess the signalling pathway involved in responses of hESC-EC to *Haemophilus influenzae* are also available. These include short hairpin-RNAs and additional siRNAs to all the various proteins involved in innate immune signalling, as well as omics approaches to look at genes, proteins and lipids. Whilst this would have been of interest and added some support to the conclusions drawn, this was beyond the scope and purpose of the protocols in this chapter.

Furthermore, whilst I was able to show that incubation of hESC-EC with NOD1 targeting siRNA resulted in (i) a decrease in NOD1 mRNA expression and (ii) a decrease in the ability of the cells to release cytokines in response to a NOD1 selective agonist; I did not measure directly transfection efficiency and this is a limitation of this experiment. This limitation would be addressed by co-transfecting with a fluorescently labelled siRNA (for example, with green fluorescent protein; GFP) which can be visualised by microscopy and this can be used to calculate transfection efficiency as a function of fluorescent cells versus total cells. Whilst this would have been important to do, in this chapter I have combined a molecular approach with a pharmacological approach to conclude that NOD1 receptors drive responses to bacteria and NOD1 agonist in hESC-EC. Whilst I was able to show effects of the NOD1 targeting siRNA versus non-targeting siRNA using the same transfection reagents, it would have been useful to determine the effect of the transfection reagent alone on the cells to provide a mock transfection control.

Expression of TLR4 in hESC-EC and HUVEC was based on data from a multi-gene array which could have been confirmed using specific primers for TLR4 and a semi-quantitative-RT-PCR approach. Conclusions based on this data however were based on head to head experiments for hESC-EC versus HUVEC and so the effect of this limitation is somewhat reduced by this design. In this Chapter I have also speculated that through significantly lower expression of TLR4 mRNA, and lack of function, that hESC-EC might be protected from atherosclerosis. To assess this fully, an animal model of post-transplant atherosclerosis in which stem cell derived organs have been transplanted, and where the atherosclerosis has been shown to be a result of TLR4 activation, would be needed, and this is currently not available. Unfortunately, it was beyond the scope of my PhD to establish such a model.

4.6 FIGURES

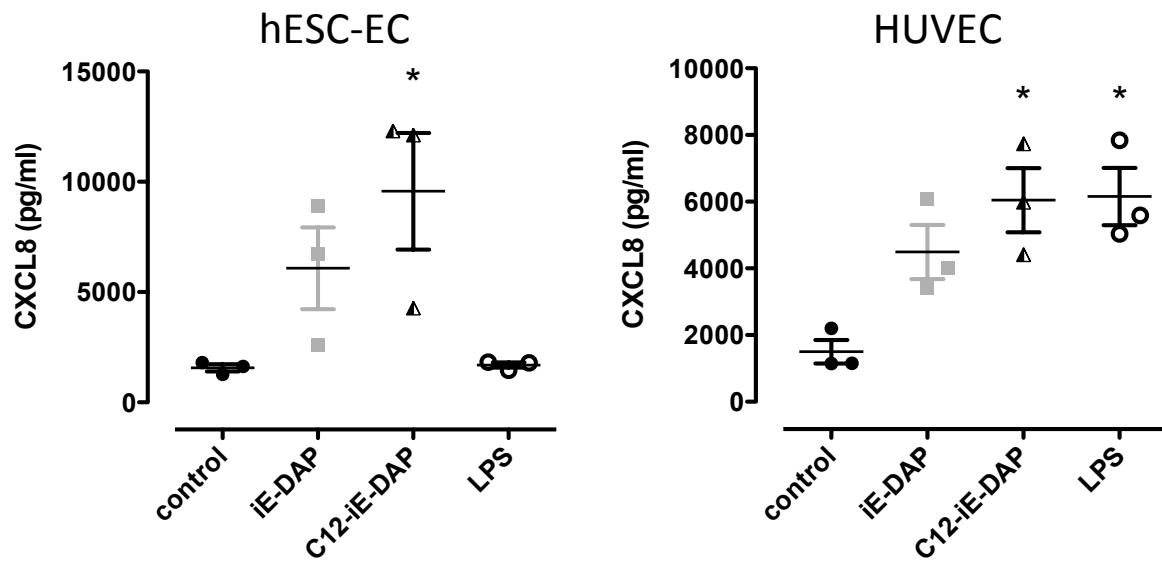


Figure 4.1 CXCL8 release from human embryonic stem cell derived endothelial cells (hESC-EC) (left) and human umbilical vein endothelial cells (HUVEC) (right) in response to TLR4 and NOD1 agonist. Cells were treated +/- iE-DAP (1 μ g/ml), C12-iE-DAP (10 μ g/ml) or LPS (1 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=3). Statistical significance was determined by one-way ANOVA followed Dunnett's multiple comparison test (*p<0.05).

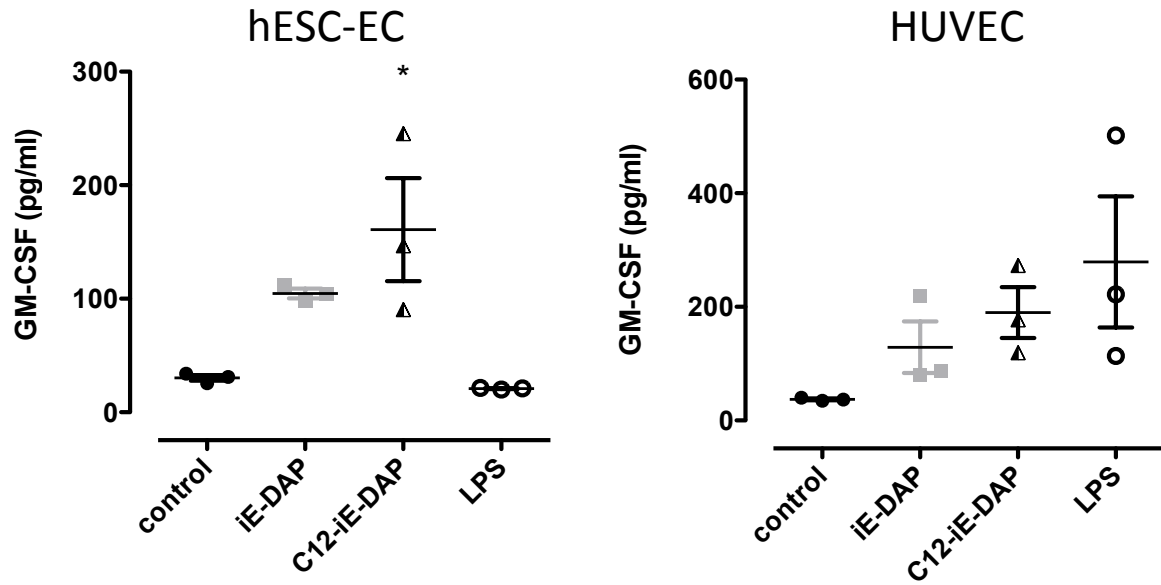


Figure 4.2 GM-CSF release from human embryonic stem cell derived endothelial cells (hESC-EC) (left) and human umbilical vein endothelial cells (HUVEC) (right) in response to TLR4 and NOD1 agonist. Cells were treated +/- iE-DAP (1 μ g/ml), C12-iE-DAP (10 μ g/ml) or LPS (1 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=3). Statistical significance was determined by one-way ANOVA followed Dunnett's multiple comparison test (*p<0.05).

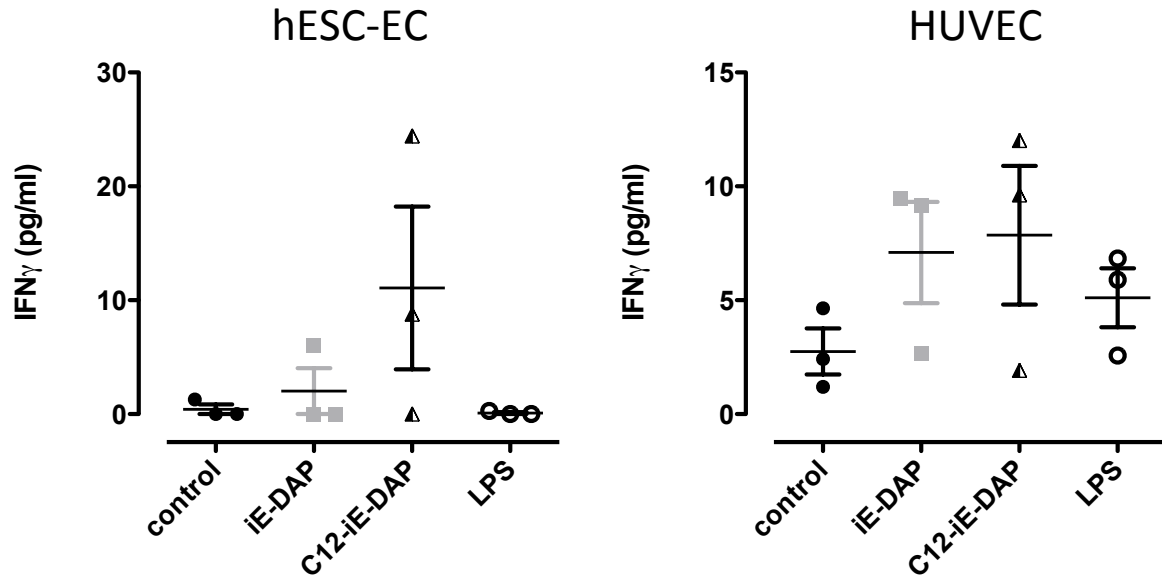


Figure 4.3 IFN γ release from human embryonic stem cell derived endothelial cells (hESC-EC) (left) and human umbilical vein endothelial cells (HUVEC) (right) in response to TLR4 and NOD1 agonist. Cells were treated +/- iE-DAP (1 μ g/ml), C12-iE-DAP (10 μ g/ml) or LPS (1 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=3). Statistical significance was determined by one-way ANOVA followed Dunnett's multiple comparison test (*p<0.05).

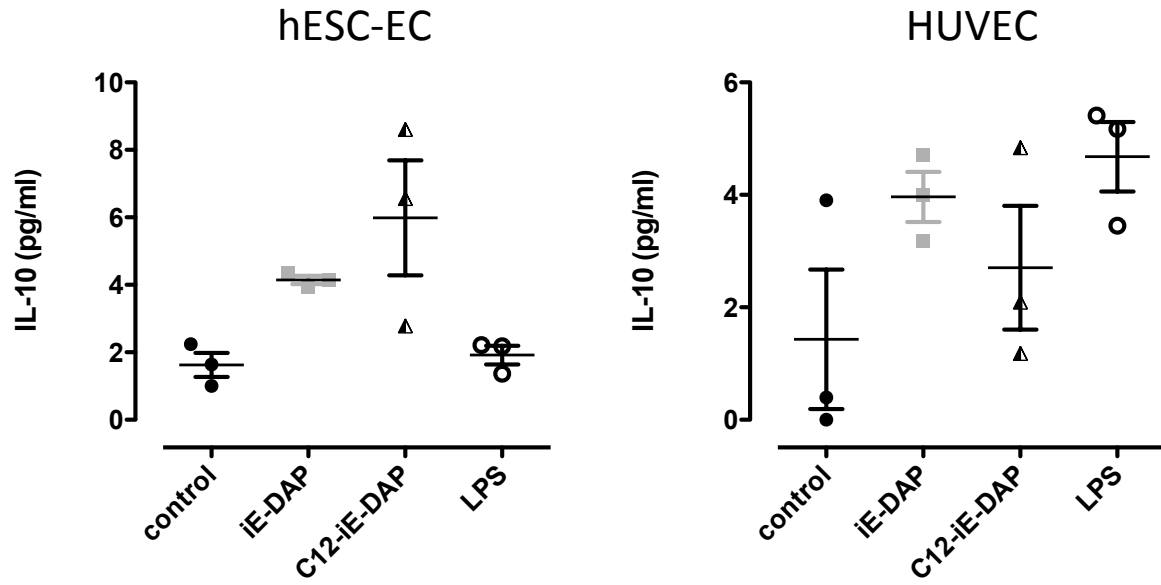


Figure 4.4 IL-10 release from human embryonic stem cell derived endothelial cells (hESC-EC) (left) and human umbilical vein endothelial cells (HUVEC) (right) in response to TLR4 and NOD1 agonist. Cells were treated +/- iE-DAP (1 μ g/ml), C12-iE-DAP (10 μ g/ml) or LPS (1 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=3). Statistical significance was determined by one-way ANOVA followed Dunnett's multiple comparison test (*p<0.05).

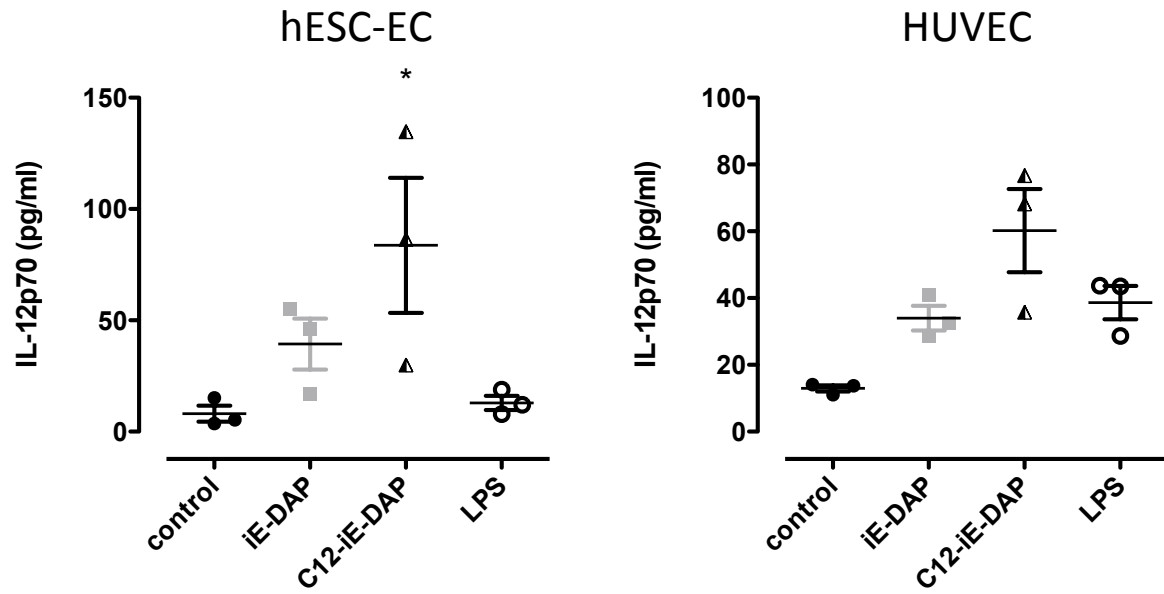


Figure 4.5 IL-12p70 release from human embryonic stem cell derived endothelial cells (hESC-EC) (left) and human umbilical vein endothelial cells (HUVEC) (right) in response to TLR4 and NOD1 agonist. Cells were treated +/- iE-DAP (1 μ g/ml), C12-iE-DAP (10 μ g/ml) or LPS (1 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=3). Statistical significance was determined by one-way ANOVA followed Dunnett's multiple comparison test (*p<0.05).

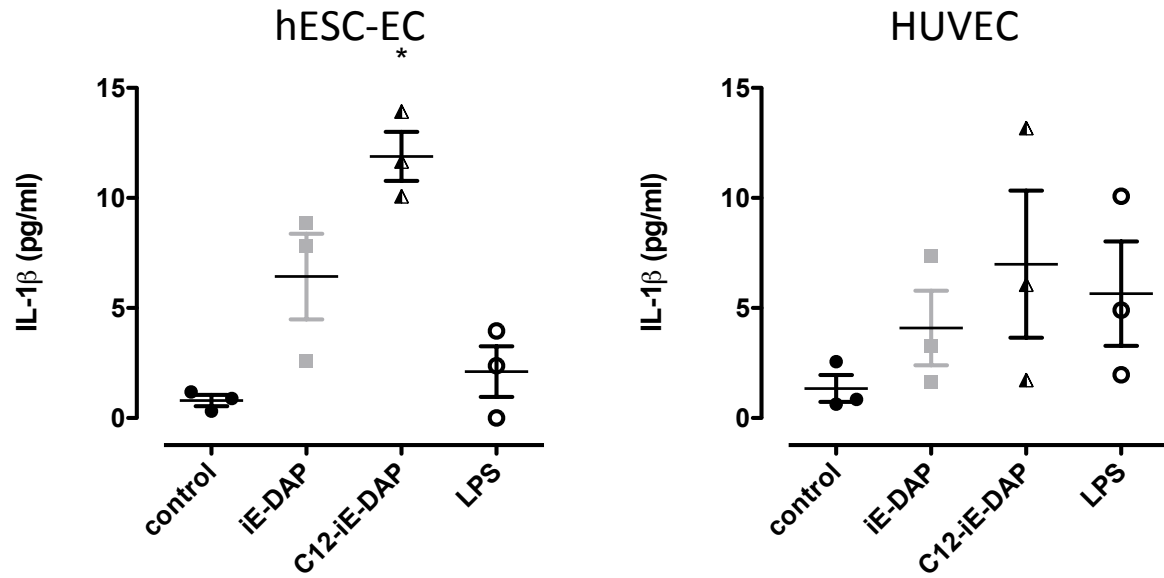


Figure 4.6 IL-1 β release from human embryonic stem cell derived endothelial cells (hESC-EC) (left) and human umbilical vein endothelial cells (HUVEC) (right) in response to TLR4 and NOD1 agonist. Cells were treated +/- iE-DAP (1 μ g/ml), C12-iE-DAP (10 μ g/ml) or LPS (1 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=3). Statistical significance was determined by one-way ANOVA followed Dunnett's multiple comparison test (*p<0.05).

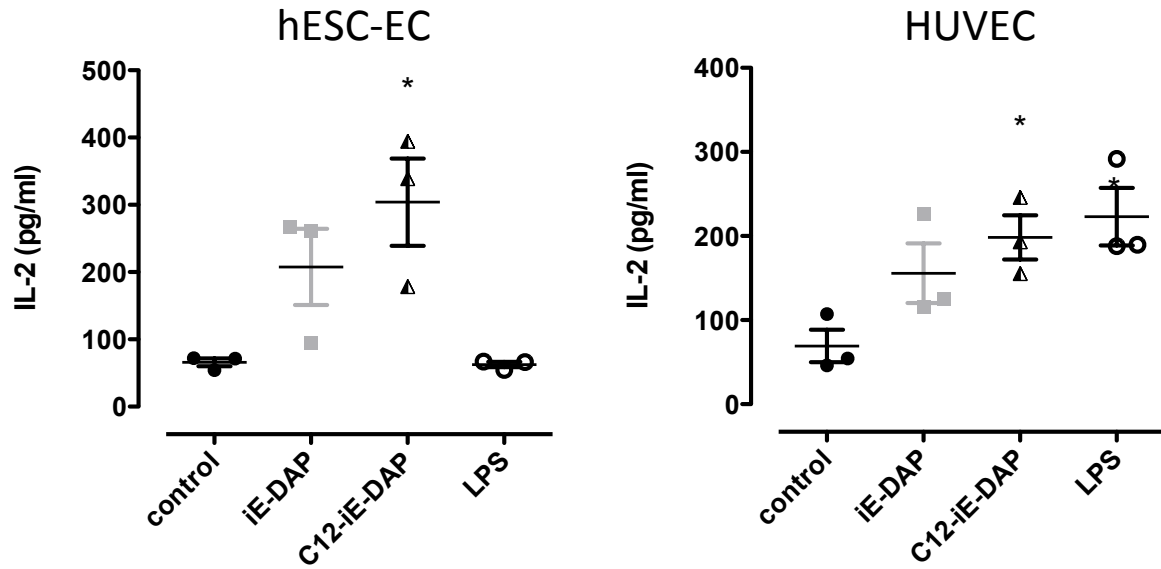


Figure 4.7 IL-2 release from human embryonic stem cell derived endothelial cells (hESC-EC) (left) and human umbilical vein endothelial cells (HUVEC) (right) in response to TLR4 and NOD1 agonist. Cells were treated +/- iE-DAP (1 μ g/ml), C12-iE-DAP (10 μ g/ml) or LPS (1 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=3). Statistical significance was determined by one-way ANOVA followed Dunnett's multiple comparison test (*p<0.05).

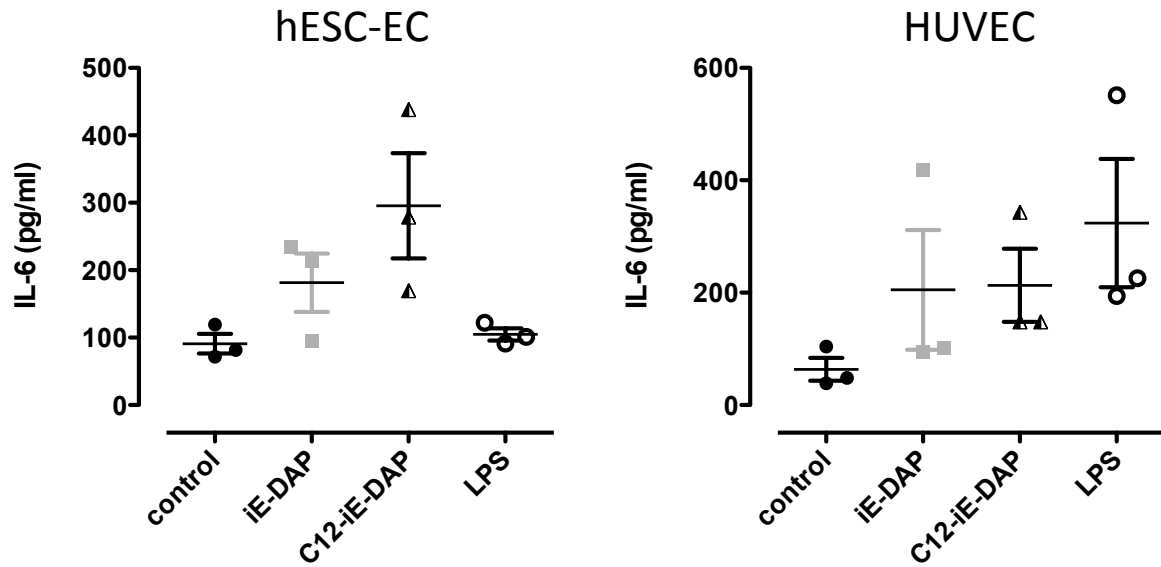


Figure 4.8 IL-6 release from human embryonic stem cell derived endothelial cells (hESC-EC) (left) and human umbilical vein endothelial cells (HUVEC) (right) in response to TLR4 and NOD1 agonist. Cells were treated +/- iE-DAP (1 μ g/ml), C12-iE-DAP (10 μ g/ml) or LPS (1 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=3). Statistical significance was determined by one-way ANOVA followed Dunnett's multiple comparison test (*p<0.05).

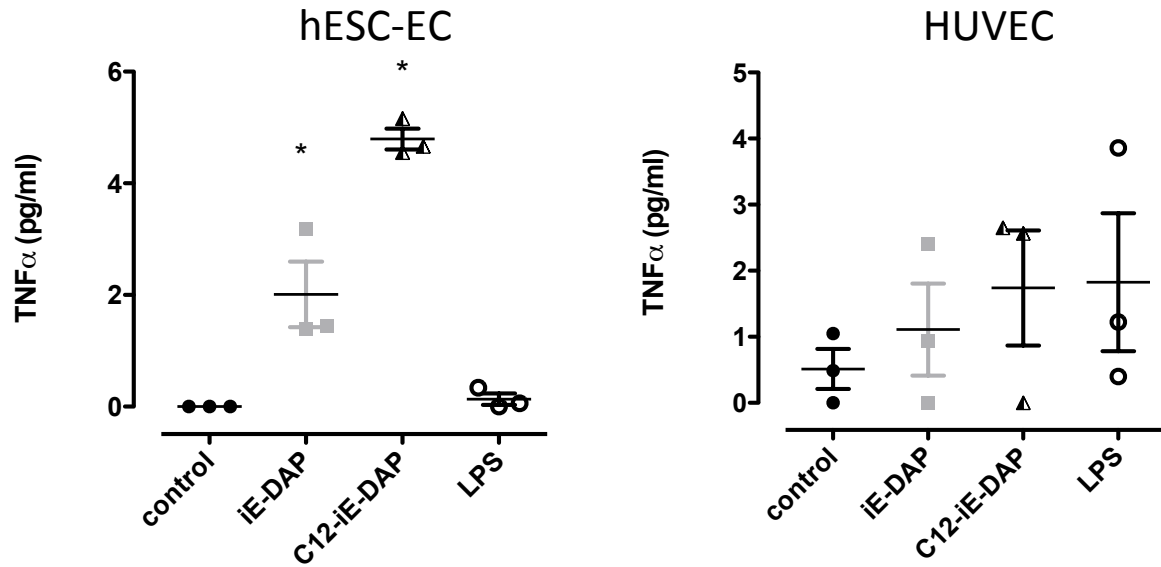


Figure 4.9 TNF α release from human embryonic stem cell derived endothelial cells (hESC-EC) (left) and human umbilical vein endothelial cells (HUVEC) (right) in response to TLR4 and NOD1 agonist. Cells were treated +/- iE-DAP (1 μ g/ml), C12-iE-DAP (10 μ g/ml) or LPS (1 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=3). Statistical significance was determined by one-way ANOVA followed Dunnett's multiple comparison test (*p<0.05).

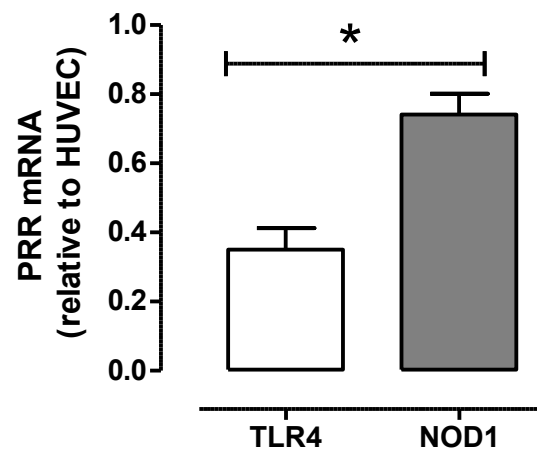


Figure 4.10 TLR4 and NOD1 expression in human embryonic stem cell derived endothelial cells (hESC-EC). TLR4 and NOD1 expression was determined relative to expression in HUVEC *in vitro*. Data are mean $\pm$ SEM (n=3-4) and are normalised to GAPDH for each cell type. Statistical significance was determined by one-sample t-test (*p<0.05) for NOD1 vs. TLR4 expression.

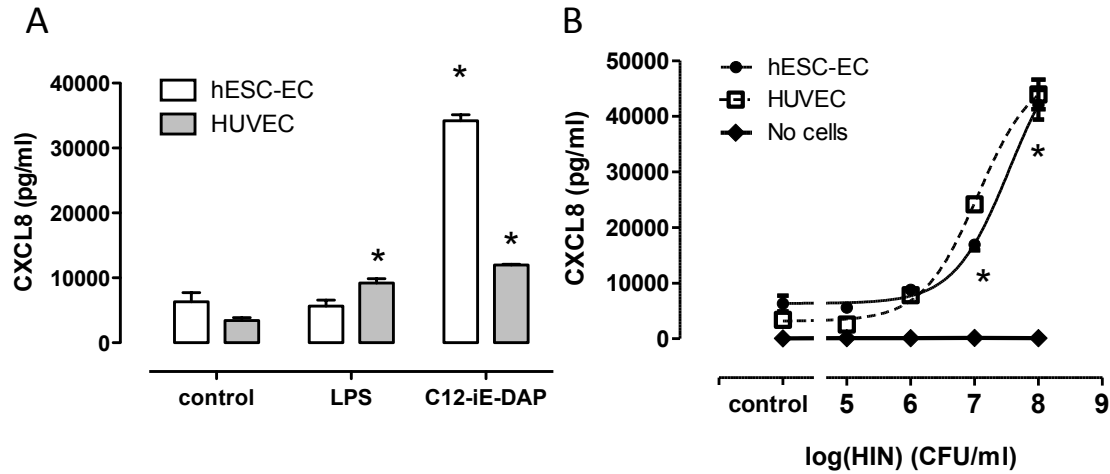


Figure 4.11 Responses of human embryonic stem cell derived endothelial cells (hESC-EC) and human umbilical vein endothelial cells (HUVEC) to Gram negative bacteria. **(A)** CXCL8 release from cells (open bars, hESC-EC; filled bars, HUVEC) were treated for 24h with LPS (1 μ g/ml) or C12-iE-DAP (10 μ g/ml). **(B)** CXCL8 release from cells (hESC-EC, solid line; HUVEC, dashed line) treated for 24h with *H.influenzae* (HIN) (10⁵-10⁸ CFU/ml). Experiments were also run with HIN in the absence of cells (no cells; filled diamonds). Data are mean $\pm$ SEM; n=3 representative of 6 hESC-EC isolations. Statistical significance for responses to drugs or bacteria was determined by one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$).

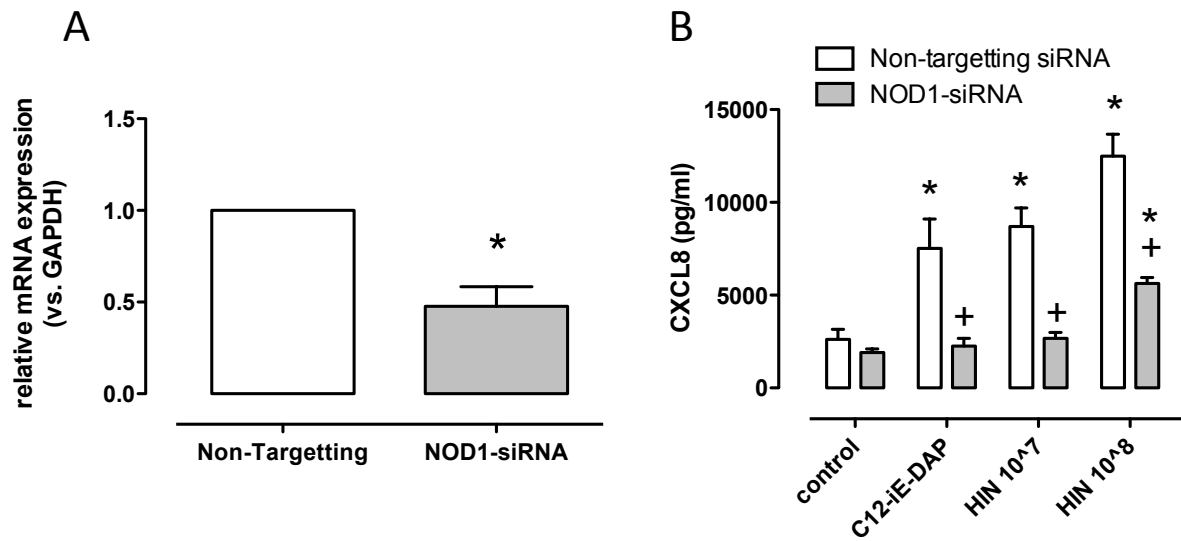


Figure 4.12 Effect of NOD1 siRNA knockdown on responses of human embryonic stem cell derived endothelial cells (hESC-EC) to HIN and C12-iE-DAP. **(A)** Relative expression (vs. GAPDH) of NOD1 following 48h incubation with NOD1 siRNA normalized to non-targetting siRNA (n=6). **(B)** CXCL8 release from hESC-EC following 48h pre-incubation with non-targetting siRNA (open bars) or NOD1-siRNA (filled bars) and 24h treatment +/- C12-iE-DAP ($10\mu\text{g/ml}$) or *Haemophilus influenzae* (HIN) (10^7 - 10^8 CFU/ml) (n=7-8). For panel A, statistical significance was determined by one-sample t-test. For panel B, statistical significance within siRNA groups was determined by one-way ANOVA followed by Dunnett's multiple comparison test (* $p < 0.05$), and between groups by two-way ANOVA followed by Bonferroni's post-test (+ $p < 0.05$)

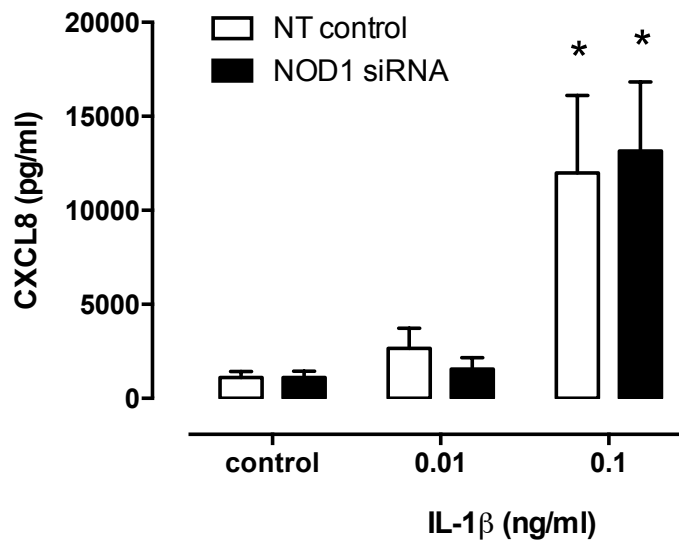


Figure 4.13 Effects of NOD1 siRNA targeting on IL-1 β induced CXCL8 release from human embryonic stem cell derived endothelial cells (hESC-EC). CXCL8 release from hESC-EC following 48h pre-incubation with non-targeting siRNA (open bars) or NOD1-siRNA (filled bars) and 24h treatment with/without IL-1 β (0.01-0.1ng/ml). Data are mean $\pm$ SEM (n=6-8). Statistical testing was carried out by one-way ANOVA followed by Dunnett's multiple comparison test for the effect of IL- β (* p <0.05) and by two-way ANOVA followed by Bonferroni's post test (p >0.05).

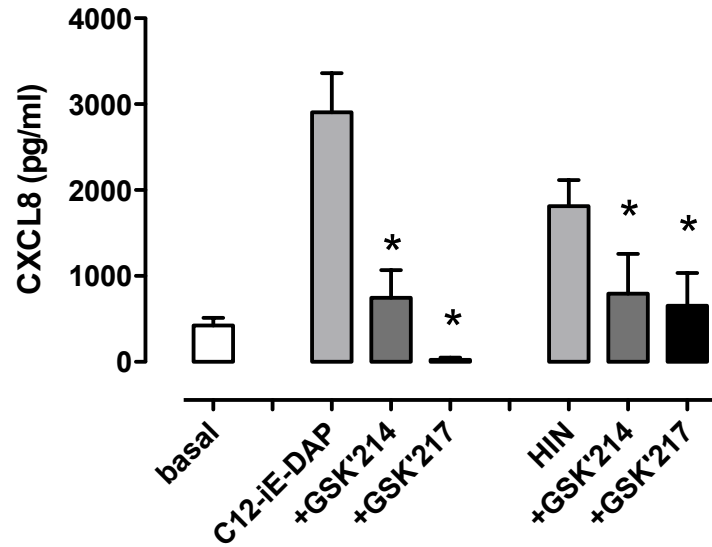


Figure 4.14 Effect of inhibition of NOD1 and RIP2 on responses of human embryonic stem cell derived endothelial cells (hESC-EC) to NOD1 agonist and *Haemophilus influenzae* (HIN; 10^7 CFU/ml). CXCL8 release from hESC-EC pre-treated for 30min with GSK'214 (300nM; RIP2 inhibitor) or GSK'217 (300nM; NOD1 inhibitor) or vehicle (0.1% DMSO), followed by 24h treatment with HIN (10^8 CFU/ml) or C12-iE-DAP (10 μ g/ml) (n=4). It should be noted that GSK drugs increased CXCL8 release under basal, for each experiment this was subtracted from the C12-iE-DAP and HIN treatment groups. Statistical significance for the effects of inhibitor of C12-iE-DAP or HIN induced CXCL8 was determined by one-way ANOVA followed by Dunnett's multiple comparison test (*p<0.05).

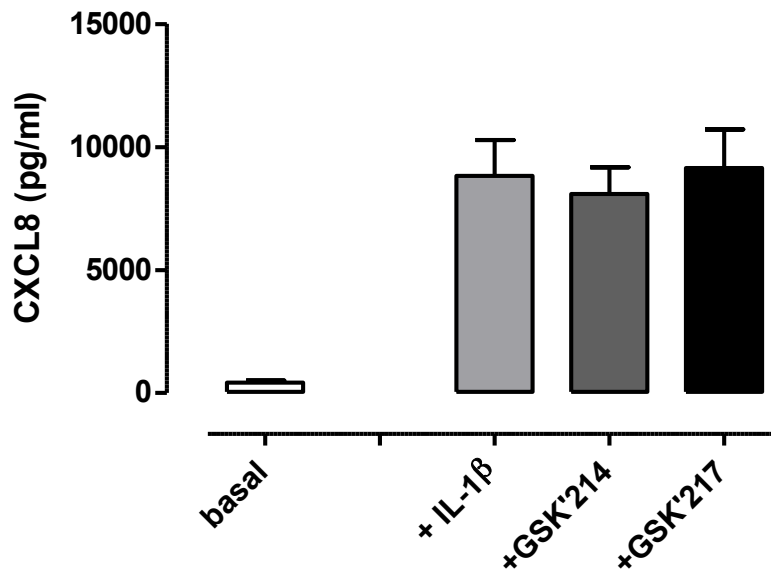


Figure 4.15 Effects of NOD1 and RIP2 inhibition on IL-1 β induced CXCL8 release from human embryonic stem cell derived endothelial cells (hESC-EC). CXCL8 release from hESC-EC following 30 minute pre-treatment with GSK'214 (RIP2 inhibitor; 300nM) or GSK'217 (NOD1 inhibitor; 300nM) followed by 24h treatment with/without IL-1 β (0.1ng/ml). Data are mean $\pm$ SEM (n=4). Data were handled and corrected for inhibitor drug induced CXCL8 background as in Figure 4.14. Statistical testing for the effects of inhibitor on IL-1 β induced CXCL8 release were determined by one-way ANOVA followed by Dunnett's multiple comparison test (p>0.05)

Absorbance (570-610nm)	media	NT siRNA	NOD1 siRNA
control	0.308 ± 0.007	0.313 ± 0.009	0.311 ± 0.007
C12-iE-DAP	0.306 ± 0.001	0.304 ± 0.005	0.306 ± 0.005
HIN	0.331 ± 0.008	0.326 ± 0.015	0.345 ± 0.016
IL-1 β	0.319 ± 0.009	0.314 ± 0.008	0.308 ± 0.009

Table 4.1 Effect of NOD1 siRNA and *Haemophilus influenzae* on cell viability of human embryonic stem cell derived endothelial cells (hESC-EC). AlamarBlue assay for hESC-EC pre-treated for 48h with NOD1 siRNA followed by 24h treatment with C12-iE-DAP (10 μ g/ml), IL-1 β (0.01ng/ml) or HIN (10⁷-10⁸CFU/ml). Data are mean $\pm$ SEM (n=3 representative of 3 experiments)

	$-\log(\text{IC}_{50})$	$E_{\max}$ (% inhibition)
SB203580: C12-iE-DAP IL-1 β	5.5 $\pm$ 0.22 4.17 $\pm$ 0.77	59.4 $\pm$ 2.3 * 35.15 $\pm$ 6.4*
SC-514 C12-iE-DAP IL-1 β	5.1 $\pm$ 0.24 4.8 $\pm$ 0.43	52.7 $\pm$ 15.0 42.2 $\pm$ 7.3*
BIRB0796 C12-iE-DAP IL-1 β	- -	35.7 $\pm$ 6.6* 24.7 $\pm$ 6.3*

Table 4.2 Effect of inhibition of RIP2, p38 and IKK on NOD1 agonist and IL-1 β induced responses in human embryonic stem cell derived endothelial cells (hESC-EC). hESC-EC were pre-treated for 30 minutes with SB203580 (RIP2 and p38 inhibitor; 0.01-10 μ M) or SC-514 (IKK inhibitor; 0.1-30 μ M) or BIRB0796 (p38 selective inhibitor; 0.1-30 μ M) followed by 24h treatment with C12-iE-DAP (10 μ g/ml) or IL-1 β (1ng/ml). Data are $-\log\text{IC}_{50} \pm \text{SEM}$ and $E_{\max}$ is expressed as % inhibition of agonist induced CXCL8 release (n=3-4). Statistical significance was determined by one-sample t-test to a theoretical value of 0 (*p<0.05).

A

NF-κB nuclear intensity	control	+C12-iE-DAP
control	79.71 ± 2.71	133.79 ± 5.00
GSK'214	62.30 ± 6.19	78.80 ± 6.20
GSK'217	48.93 ± 1.42	67.40 ± 6.08

B

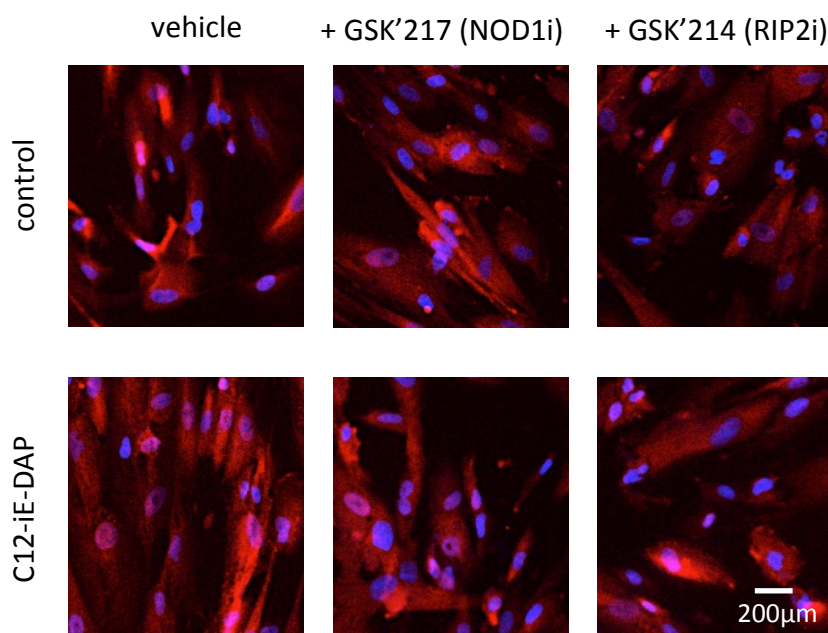


Table 4.3 and Figure 4.16 Effect of inhibition of NOD1 and RIP2 on responses of human embryonic stem cell derived endothelial cells (hESC-EC) to NOD1 in terms of NF-κB nuclear translocation. **(A)** Change in nuclear NF-κB/cell (average of 100 cells for nucleus-cytosolic intensity difference) for hESC-EC pre-treated for 30min with RIP2 inhibitor (GSK'214; 300nM) or NOD1 inhibitor (GSK'217; 300nM) or vehicle (0.1% DMSO) followed by 24h treatment C12-iE-DAP (10μg/ml) for 1h. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test ($p > 0.05$). **(B)** Representative images of NF-κB staining (red) and 10μg/ml DAPI (nuclei) of hESC-EC in A. Data are mean ± SEM (n=3 from one experiment)

**CHAPTER 5: USE OF BLOOD OUTGROWTH ENDOTHELIAL
CELLS (BOEC) IN A PHARMACOLOGICAL BIOASSAY TO
DETECT CYTOKINE STORM REACTIONS TO BIOLOGICS**

5.1 RATIONALE

The overall aim of my thesis is to study endothelial cells derived from stem cell populations in order to better understand how they can be utilised as therapeutically. One way in which stem cells are currently being used is in the screening of drug toxicity. In this chapter I have specifically addressed the idea of using endothelial cells derived from stem cells to develop and validate a novel assay using autologous cells in co-culture.

In 2006 a clinical study held at Northwick Park to test the anti-CD28 superagonist, TGN1412, resulted in all 6 healthy male volunteers suffering a profound cytokine storm response. This was despite the drug passing all preclinical safety testing, including tests of human isolated T-lymphocytes (Stebbing et al., 2007). In this way, TGN1412 defined an urgent unmet need for improved bioassays to detect cytokine storm inducing drugs (Hünig, 2012; Stebbings et al., 2012). We now know that TGN1412 does not activate T-lymphocytes or peripheral blood mononuclear cells (PBMCs) to release cytokines when antibodies are added in aqueous phase, unless there is an endothelial cell interface present (Findlay et al., 2011b; Stebbings et al., 2007). This can be mimicked when TGN1412 is immobilised by non-specific binding to a solid phase platform such as plastic (Findlay et al., 2010; Findlay et al., 2011b). Current endothelial cell:PBMC co-culture assays have employed endothelial cells derived from human umbilical vein or cell lines (Findlay et al., 2011b). However, this type of assay relies on cells from two separate donors, which inevitably results in tissue mis-match responses, including those driven by heterologous major histocompatibility complex (MHC) reactions (Huang et al., 1994). Clearly, the best type of assay would be one where endothelial cells and PBMCs are isolated from the same donor.

Up until now, same donor endothelial cell: PBMC co-culture has not been possible due to difficulties in isolating and growing endothelial cells from vessels of living donors. As stem cells offer the opportunity to grow cells from living donors, I considered blood outgrowth endothelial cells (BOEC) as a viable source of cells from which PBMCs could be obtained from the same donor for future co-culture bioassay protocols. In this chapter I have therefore shown how autologous BOEC and PBMCs can be cultured together and how they respond, compared to HUVEC:PBMC co-cultures to inflammatory agents. Specifically these have included pathogen associated molecular patterns (PAMPs), cytokines and 'proof of concept' biologic antibodies, including a TGN1412-like anti-CD28 superagonist (ANC28).

The specific questions addressed in this chapter are:

1. How do co-cultures of BOEC and PBMCs derived from the same donor respond to PAMPs and cytokines compared to HUVEC and PBMCs in co-culture?
2. How do co-cultures of BOEC and PBMCs derived from the same donor respond to therapeutic antibodies used clinically compared to a TGN1412-like anti-CD28 superagonist (ANC28) to release CXCL8, as well as range of pro-inflammatory cytokines and chemokines?
3. How well do BOEC:PBMC co-culture assays distinguish cytokine storm causing biologics from control antibodies when run through an unbiased principal components analysis?

5.2 SUMMARY OF METHODS

A full description of the methods used in this chapter is given in Chapter 2 and are summarised below.

5.2.1 BOEC culture

BOEC were isolated as described in chapter 2. Briefly, PBMCs were isolated from blood and expanded on tissue culture plates for up to 22 days in Lonza-EGM2 with 10% foetal bovine serum (FBS) on 5.2µg/cm² collagen coated plates. Typically between 5 and 22 days in culture, endothelial cell colonies emerged and were expanded for use in experiments. Cells were maintained in Lonza-EGM2 media with 10% FBS.

5.2.2 Autologous BOEC:PBMC cytokine storm assay

For autologous co-culture, BOEC were plated on 1% gelatinised 96-well plates (Nunc, Denmark) and allowed to adhere overnight. The same donor from whom the BOEC were derived was then recalled and PBMCs isolated using a Histopaque gradient separation, as detailed in the general methods. Same donor PBMCs were then added to wells with and without BOECs. Cells were added at the following seeding densities: BOEC; 7000 cells/well, PBMC at 100000 cells/well in 96-well plates. For assessment of responses to PAMPs, cells were treated with agonists of Toll like receptors (TLR)1-9 or nucleotide oligomerisation domain receptor-1 (NOD1) (see figure legends and Table 2.3 in general methods) for 24 hours. Cells were also treated IL-1β (0.1-1ng/ml) (R & D Systems, UK) for 24 hours. Cytokine storm inducing drugs: the TGN1412-like anti-CD28 superagonists (ANC28. 1.5D10) (10µg/ml), which models TGN1412 (Waibler et al., 2008), and Campath (anti-CD52:

alemtuzumab) (10µg/ml) were then added. Avastin (anti-VEGFR2: bevacizumab) (10µg/ml), Herceptin (anti-EGFR2: trastuzumab) and Arzerra (anti-CD20: ofatumumab) (10µg/ml), which are therapeutic antibodies that do not cause cytokine storm reactions, were included as negative controls. Drugs were a gift from Huntingdon Life Sciences (Cambridgeshire, UK). Plates were then incubated for 24 hours. Supernatants were collected and stored at -20°C for further analysis for CXCL8 by ELISA and at -80°C for analysis of multiple cytokines using MSD technology. Some experiments were carried out at the National Institute of Biological Standards and Control (NIBSC). Unless otherwise stated in figure legends, all experiments were carried out in media containing 10% FBS.

5.2.3 Measurement of CXCL8

CXCL8 was measured by ELISA (R and D Systems) according to manufacturer's instructions. Optical density was determined using a microplate reader (Dyne, Madellan Biosciences) with absorbance readings at 450-570nm.

5.2.4 Measurement of pro-inflammatory cytokines using a Meso Scale discovery (MSD) platform

Levels of 9 pro-inflammatory cytokines and 9 chemokines were measured using separate '*human pro-inflammatory 9-Plex MULTISPOT 96-well -10 spot*' plates purchased from MSD (Gaithersburg, Maryland). Samples were diluted 1:10 in assay buffer and assays carried out according to manufacturer's instructions. MSD plates were read using an MSC Sector Imager 2400 and analysed using MSD Discovery® Workbench software. Cytokines analysed were TNFα, IL-2, CXCL8, IL-12p70, IL-1β, GM-CSF, IFNγ, IL-6, IL-10. Chemokines analysed were eotaxin, MCP-1, MIP-1β, eotaxin-3, MCP-4, TARC, IP-10 and MDC.

5.2.5 Measurement of cell viability

Cell viability was determined using commercially available AlamarBlue® assay (Invitrogen, UK). Unless otherwise stated none of the treatments greatly affected cell viability (Table 5.1).

5.2.6 Assessment of cell proliferation

Cell proliferation in response to TGN1412-like anti-CD28 superagonist (ANC28) was determined using a ³H-thymidine incorporation assay. Proliferation experiments were carried out at the National Institute of Biological Standards of Control (NIBSC), using an in-house assay (Stebbing et al., 2007).

5.2.7 Principal component analysis

Principal component analysis based on co-variances between 17 cytokines and chemokines across all conditions was carried out using commercially available software.

5.3 RESULTS

5.3.1 Characterization of classical inflammatory responses in same-donor BOEC:PBMC or blood bioassays

As discussed in the foregoing chapters, endothelial cells respond to PAMPs and cytokines. Others have shown that endothelial cells interact with PBMCs to enhance these responses in some *in vitro* assays (Ward et al., 2009). At the level of TLR4 for example, it is known that endothelial cells from HUVEC interact with PBMCs *in vitro* to increase CXCL8 release in response to LPS (Ward et al., 2009). It was important therefore to consider how BOEC responded to PAMPs in the absence and presence of same donor PBMCs.

Same donor BOEC:PBMC co-cultures, and mono-cultures of either cell type, were treated with LPS (TLR4 agonist), Pam₃CSK4 (TLR2 agonist), C12-iE-DAP (NOD1 agonist), and the cytokines, IL-1 β and TNF α . PBMCs and BOEC monocultures released low levels of CXCL8 under control culture conditions (Figure 5.1A). Co-culture of same donor BOEC and PBMCs had no discernible effect above additive on levels of CXCL8 release when compared to levels released by either cell type alone under control conditions. All inflammatory agonists tested tended to increase CXCL8 release by BOEC, with statistically significant responses seen with LPS and IL-1 β (Figure 5.1A). On the whole CXCL8 release stimulated from PBMCs was less than seen for BOEC (Figure 5.1A). When cells were stimulated in co-culture CXCL8 release was essentially equivalent to the release from BOEC alone or an additive amount from BOEC and PBMC cultures, except for experiments with LPS where there was a tendency for a greater than additive effect (Figure 5.1A). With the exception of LPS, broadly similar results

were seen when HUVEC:PBMC co-cultures were treated with the same panel of PAMPs and cytokines (Figure 5.1B).

5.3.2 Responses of the autologous/same-donor BOEC: PBMC bioassay to cytokine storm inducing biologics: *CXCL8* and proliferation

For same donor endothelial cell: PBMC bioassays to be useful it should show cytokine release to therapeutic antibodies known to cause cytokine storm in man, but not to those used in man with no discernible cytokine storm associated side effects. I therefore tested a small panel of therapeutic antibodies with different cytokine storm inducing characteristics (see Table 2.5). Notably, this included two positive control antibodies, a TGN1412-like anti-CD28 superagonist known to bind a region of CD28 on PBMCs that activates an identical signaling pathway to TGN1412 (Waibler et al., 2008) and a CD52 antibody alemtuzumab (Campath). Three negative control antibodies (Herceptin, Avastin and Arzerra), which are used therapeutically, but don't cause cytokine storm responses were also included. When CXCL8 was measured as a read out, PBMC or BOEC cultures alone did not respond appreciably to any of the antibodies tested (Figure 5.2A). Similarly co-cultures of PBMCs and BOEC incubated with Herceptin, Avastin or Arzerra did not release increased levels of CXCL8. Co-cultures of same donor PBMCs and BOEC released increased levels of CXCL8 when stimulated with the TGN1412-like anti-CD28 superagonist (ANC28) or with Campath where ANC28>Campath. This correlates with the expected severity of cytokine storm for these agents (Eastwood et al., 2010; Suntharalingam et al., 2006). The effects of the TGN1412-like anti-CD28 superagonist, ANC28, were concentration dependent with maximal effects seen at 10µg/ml (Figure 5.2B). Furthermore, and in line with what is expected for TGN1412 in *in vitro* assays, the TGN1412-like anti-CD28 superagonist, ANC28 resulted in a proliferation

response in same donor BOEC:PBMC co-cultures (Figure 5.3). Unlike TGN1412 however, in these assays ANC28 resulted in proliferation of PBMC monocultures, and this is in line with what is known for ANC28 versus TGN1412 activity (Figure 5.3). Notably, when the Alamarblue assay was used to determine cell number/proliferation, no response to ANC28 was detected (Table 5.1). Whilst not conclusive, one possible explanation for this is based in differences between the assays to measure 'proliferation'. As ^3H -thymidine must enter the cell where it is incorporated into DNA and RNA of active cells, this can occur prior to cell division, which may not have occurred at 24 hours. The Alamarblue assay on the other hand relies on cell respiration to oxidise components of the reagent which typically corresponds with cell number. Thus, these cells may have been activated to proliferate as suggested by the ^3H -thymidine incorporation but not yet undergone cell division at 24 hours as suggested by the Alamarblue data. In published literature showing proliferative responses of human lymphocytes to TGN1412 (Stebbing et al., 2007) these have typically been carried out following 3 day incubations with drugs.

5.3.3 Responses of the autologous/same-donor BOEC: PBMC bioassay to cytokine storm inducing biologics: *full range of 'cytokine storm' cytokines*

Although CXCL8 is key cytokine associated with cytokine storm responses (Suntharalingam et al., 2006), vascular inflammation and sepsis (Gatheral et al., 2012); cytokine storm responses are defined by the concerted release of a broad range of cytokines (Findlay et al., 2011a; Harrison, 2010; Suntharalingam et al., 2006). Specifically, TGN1412 *in vivo* induced increases in CXCL8, IL-2, IL-6, IFN γ and TNF α (Suntharalingam et al., 2006). Therefore, it was important to analyse this autologous assay further, and measure a full range of cytokines. In addition to CXCL8; GM-CSF, IL-6, IL-10, IFN γ and IL-2 were significantly increased whilst

TNF α and IL-12p70 tended to increase in response to the TGN1412-like superagonist, ANC28 in BOEC:PBMC co-cultures (Figure 5.4 to 5.9). The pro-inflammatory chemokines: eotaxin, MCP-1 and TARC were also increased by the TGN1412-like superagonist, ANC28, in BOEC:PBMC co-cultures but not monocultures of either cell type (Figure 5.7 to 5.9). Importantly and in line with data in Figure 5.2A, with the exception of eotaxin, less cytokine/chemokine release was detected in response to Campath than ANC28. The only cytokine/chemokine measured that was released by monocultures of PBMCs stimulated with ANC28 was MIP-1 β (Figure 5.7) Interesting, unlike other cytokines measured Campath induced more MIP-1 β than ANC28 (Figure 5.7)

5.3.4 Principal Components Analysis of responses of BOEC, PBMC and BOEC:PBMC co-culture cytokine/chemokine responses to biologics

Principal components analysis allows for complex data with multiple groups and variables to be transformed in to a coordinate system that reveals patterns and sources of variation in an unbiased way. I considered this to be important to carry out on this data to allow for unsupervised analysis of the 17 cytokines/chemokines measured in response to treatment of same donor BOEC:PBMC co-cultures, and respective monocultures, to the biologics tested. Taking this kind of approach when testing new drugs in individual patient groups using autologous BOEC:PBMCs co-culture assays could conceivably reveal new and unique patterns of cytokine release that are associated with certain phenotypes and/or drugs (particularly biological drugs such as therapeutic antibodies where multiple readouts are required). Principal components analysis confirmed the TGN1412-like anti-CD28 superagonist, ANC28, treated BOEC:PBMC co-culture group as a major contributor to

variation (increases or decreases) in cytokine/chemokine levels (Figure 5.10). There was a clear dissociation of this condition from similarly treated mono-cultures or co-cultures with any other antibody tested (Figure 5.10). The loadings plot indicated positive correlations between all cytokines and chemokines measured (Figure 5.11).

5.3.5 Effect of therapeutic antibodies on heterologous BOEC:PBMC and HUVEC:PBMC co-culture assays

In separate experiments I also compared responses of non-same (heterologous) donor mixes of BOEC:PBMC to therapeutic antibodies. Heterologous donor BOEC:PBMC co-culture assays appeared to give more variable results but detected ANC28 as well as other, albeit, milder cytokine storm inducing drugs such as Campath (Figure 5.12). For experiments where BOEC were replaced by HUVEC (Figure 5.13), results typical of HUVEC:PBMC assays (Bailey et al., 2012; Findlay et al., 2011b) were seen where the TGN1412-like superagonist ANC28 and Campath resulted in comparable CXCL8 release.

5.4 SUMMARY

In the previous chapters I have shown that, like endothelial cell from vessels, BOEC have cobblestone morphology, align under shear stress, respond to PAMPs and release prostacyclin and endothelin-1. As these cells are easily accessible and have undergone minimal manipulation *in vitro*, this made them viable candidates for application in a pharmacological bioassay.

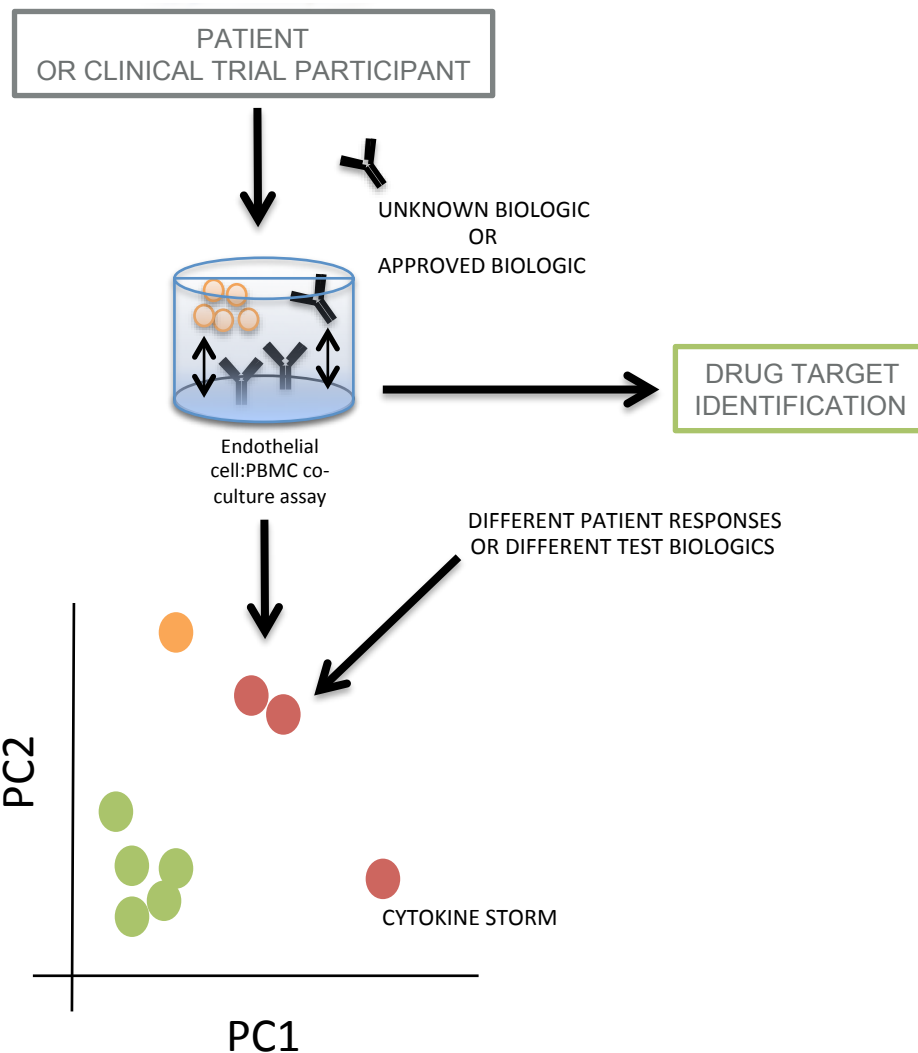
In this chapter I have shown that:

- Same donor BOEC:PBMC co-cultures respond to PAMPs and cytokines and to release CXCL8.
- Same donor BOEC:PBMC respond to 'proof of concept' therapeutic antibodies to release CXCL8 with rank order TGN-like anti-CD28 superagonist (ANC28)>Campath and did not respond to negative control antibodies (Herceptin, Avastin and Arzerra).
- Same donor BOEC:PBMC co-culture also results in proliferative responses and released additional key cytokines and chemokines in response to ANC28.
- Principal components analysis indicated that for same donor BOEC:PBMC co-cultures, the TGN1412-like anti-CD28 superagonist, ANC28, treated group was separate from Campath treated cells and negative control antibodies. ANC28 drove positive correlations in 17 cytokines and chemokines.
- Mixed donor BOEC:PBMC co-cultures, like HUVEC:PBMC co-cultures also responded to TGN1412-like anti-CD28 superagonist but with more variable responses to non-cytokine storm causing biologics.

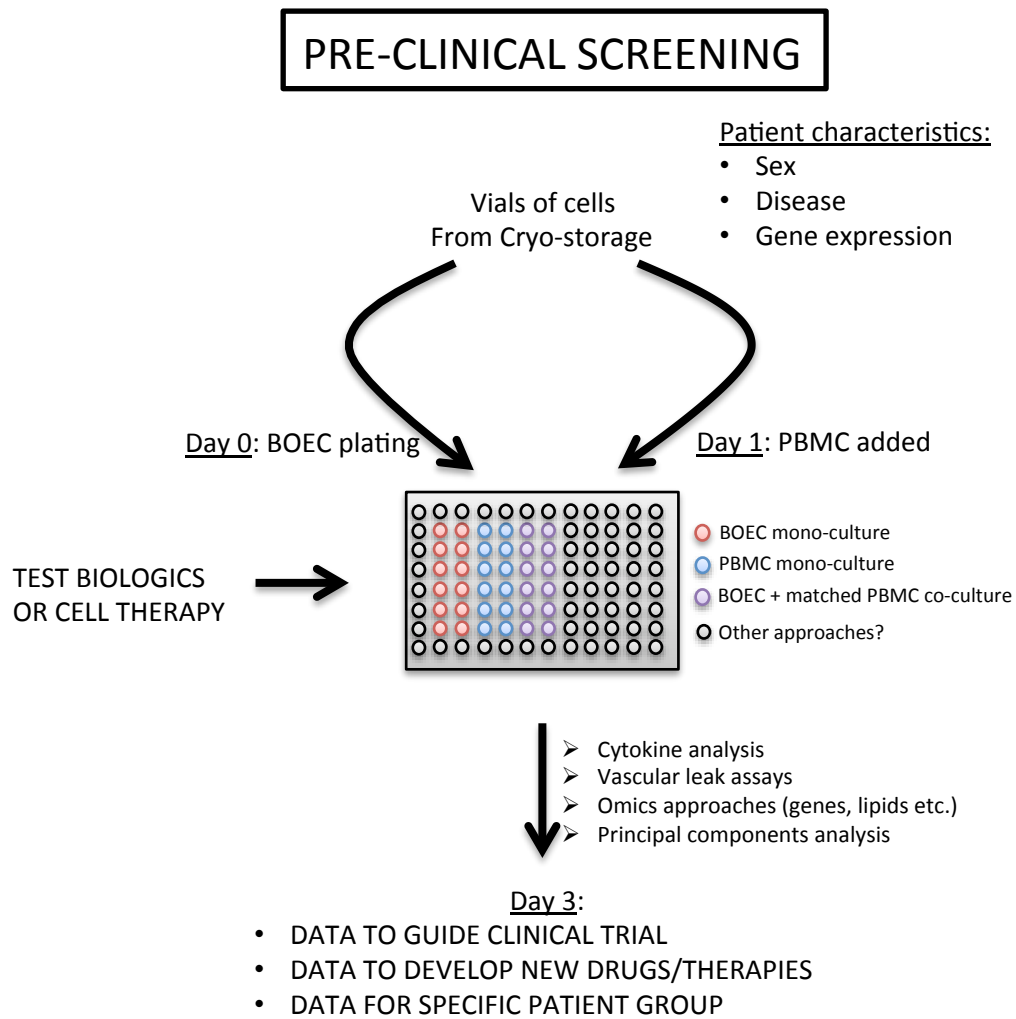
Cytokine storm responses represent a significant clinical problem in the field of biologic drug use and development. Any new assays that are developed to test for cytokine response side effects must first be shown to perform 'at least' as well as current gold standard assays. In this chapter I have characterised responses of same donor BOEC:PBMC co-cultures as being able to respond to key cytokine storm causing drugs in a manner 'at least' as well as HUVEC:PBMC assays. The advantage of HUVEC:PBMC assay over other assays is accepted, as these assays recapitulate the cell interactions occurring in the vasculature that drive cytokine storm reactions (Findlay et al., 2011b). Same donor BOEC:PBMC assays take this same advantage further, as they do not rely on endothelial cells and PBMCs from different donors and so are not limited by mixed donor cell interactions.

Clearly, using endothelial cells and PBMCs from the same donor would recapitulate even further the true interactions between endothelial cells and PBMCs in the vasculature. Up until now, same donor endothelial cell:PBMC co-culture, has not been possible due to difficulties and technical limitations in how endothelial cells are sourced from tissue, typically removed during surgery or reliant on HUVEC which represent foetal cells.

Autologous BOEC:PBMC assays have clear implications in personalised medicine and the study of immune signalling in the vasculature, both as an assay to screen therapies and as a tool to study immune signalling. As this assay is the first report of using stem cell derived cells to create an autologous endothelial cell:PBMC co-culture assay this invention was filed for a patent on 23rd March 2013 on which I am an author, and named as 'contributor to the work of invention'



Summary Figure 4. Summary for chapter 5 showing applications of same donor BOEC: PBMC co-culture assays in personalised medicine. BOEC: PBMC assays could also be used to screen for 'drugable' targets in patients with cancer and rare diseases. Shown on the left BOEC: PBMC assays could also be used to screen for immune responses of patients to cell therapy which will be important to avoid rejection of allogeneic therapy if patient cells are unsuitable due to disease. Principal component analysis (PC) of patterns of cytokine release in certain patients will also be important to study, visualise and relate back to the clinic.



Summary Figure 5. Summary for chapter 5 showing applications of same donor BOEC: PBMC co-culture assays as an off-the-shelf assay to determine cytokine storm risk for a drug or therapy in a clinical trial or prior to use in patient groups.

5.5 LIMITATIONS

The data presented in this chapter are limited in a number of ways, which are outlined and discussed below:

During the course of my PhD I did not have access to authentic TGN1412 and relied on the substitute ANC28, which has been used in this way by others (Waibler et al., 2008) and is commercially available. However, ANC28 is not identical to TGN1412 in that it is thought to bind a different region of the domain of CD28 that confers superagonist activity (personal communication with Richard Stebbings, NIBSC). It is important that the assay described here be repeated with authentic TGN1412. To this end, I have formed collaborations with NIBSC to pursue optimisation of the assay with TGN1412 and a related in-house anti-CD28 superagonist. Subsequent to this, data has arisen from the collaboration which shows that same donor BOEC:PBMC co-cultures respond to original TGN1412 recovered from the Northwick Park clinical trial to release IL-6, IL-2, TNF α and CXCL8 and that this assay is superior to HUVEC:PBMC co-cultures.

Throughout this Chapter, I have suggested that same donor BOEC:PBMC co-culture assays represent a new standard assays that can be used to test therapies for individual patients. It is noteworthy however, that the time to grow BOEC from a patient is currently up to 20 days and patients are required to make a second visit for the isolation of PBMCs. This currently limits the use of this assay in clinical practice and as an off-the-shelf assay. It would be important in the future to investigate how to scale this assay up for industry use. For example, it is important to know how BOEC and PBMCs respond after being frozen in stocks and how they response after multiple passages. However, many treatment regimens for

critically ill patients can take many weeks to develop and so this lag time might be acceptable for patients with rare disease and requiring testing in a high-end assay.

However, in pilot studies, and as part of other projects in the group, I have shown that BOEC can be cryo-preserved in liquid nitrogen and recovered with their phenotype and viability intact. In the future therefore, BOEC from different patient groups could be biobanked and upon requesting of testing of a therapeutic agent, BOEC would be recovered from liquid nitrogen and PBMCs added either freshly isolated or also from cryo-storage. This in turn, would make the assay a 2-3 day process. The feasibility of this is under investigation.

Finally, given the disparity in proliferation data from two different readouts (^3H -thymidine incorporation and Alamarblue) the optimum condition to detect a proliferative response in these assays will be important to establish in future studies.

It is important to note that whilst BOEC are highly proliferative, yield of these cells for large scale assays might be a limiting factor. Therefore, developing protocols to improve yield of BOEC will be important going forward. In my PhD, and for purpose of this assay, I have chosen to apply BOEC as a same donor endothelial cell interface. Other sources of same donor endothelial cells also exist in the form of iPSC-EC which could be applied to same donor assays and stored in large numbers of use in assays. This however was beyond the scope of my PhD where I have established protocols within the group to grow BOEC and apply them to key assays. The extent to which both BOEC and iPSC-EC from patients retain a relevant phenotype in *in vitro* assays will be a critical consideration in the personalised medicine assay field.

5.6 FIGURES

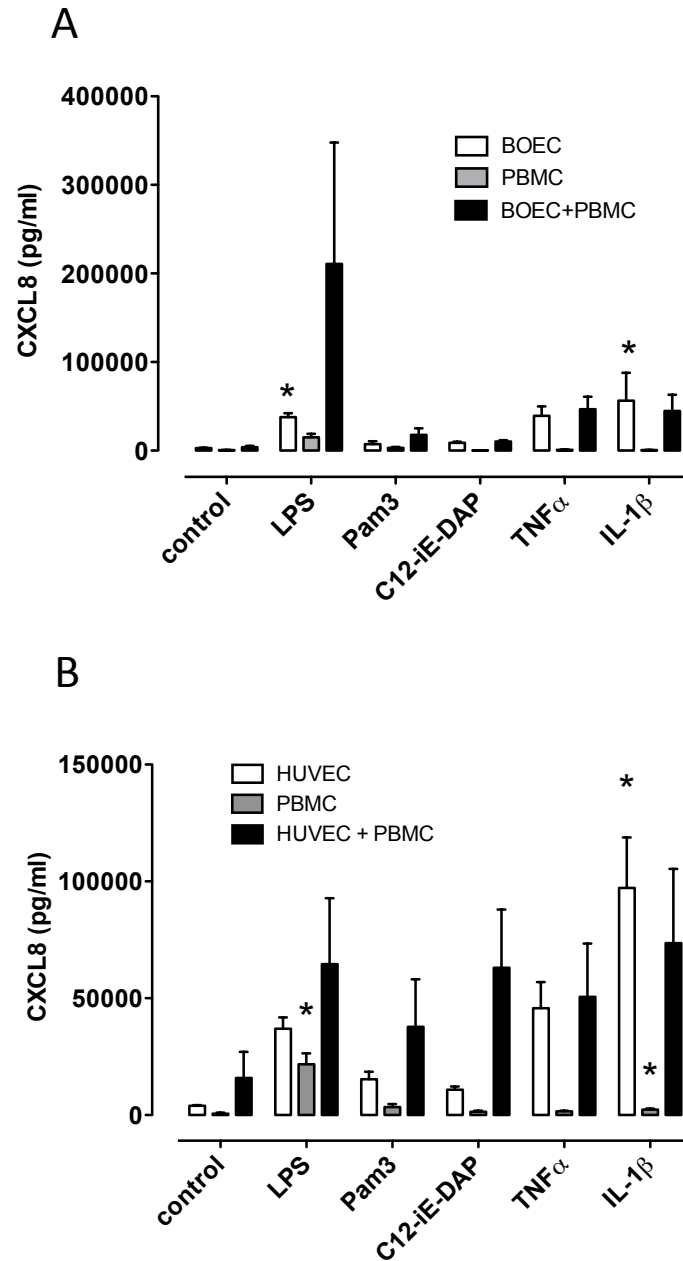


Figure 5.1 Effect of co-culture of blood outgrowth endothelial cells (BOECs) with autologous peripheral blood mononuclear cells (PBMCs) on responses to pathogen associated molecular patterns (PAMPs). **(A)** BOECs and **(B)** HUVEC with/without PBMCs were treated with LPS (1 μ g/ml), which activates TLR4, Pam₃CSK4 (Pam3) (1 μ g/ml), which activates TLR2, C12-iE-DAP (10 μ g/ml), which activates NOD1 and TNF α (10ng/ml) and IL-1 β (1ng/ml) which activate similar inflammatory pathways via cytokine receptors and independent of TLRs. Data are mean $\pm$ SEM (panel A; n=5, panel B; n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test (*p<0.05). NB. Different scales in panel A and B.

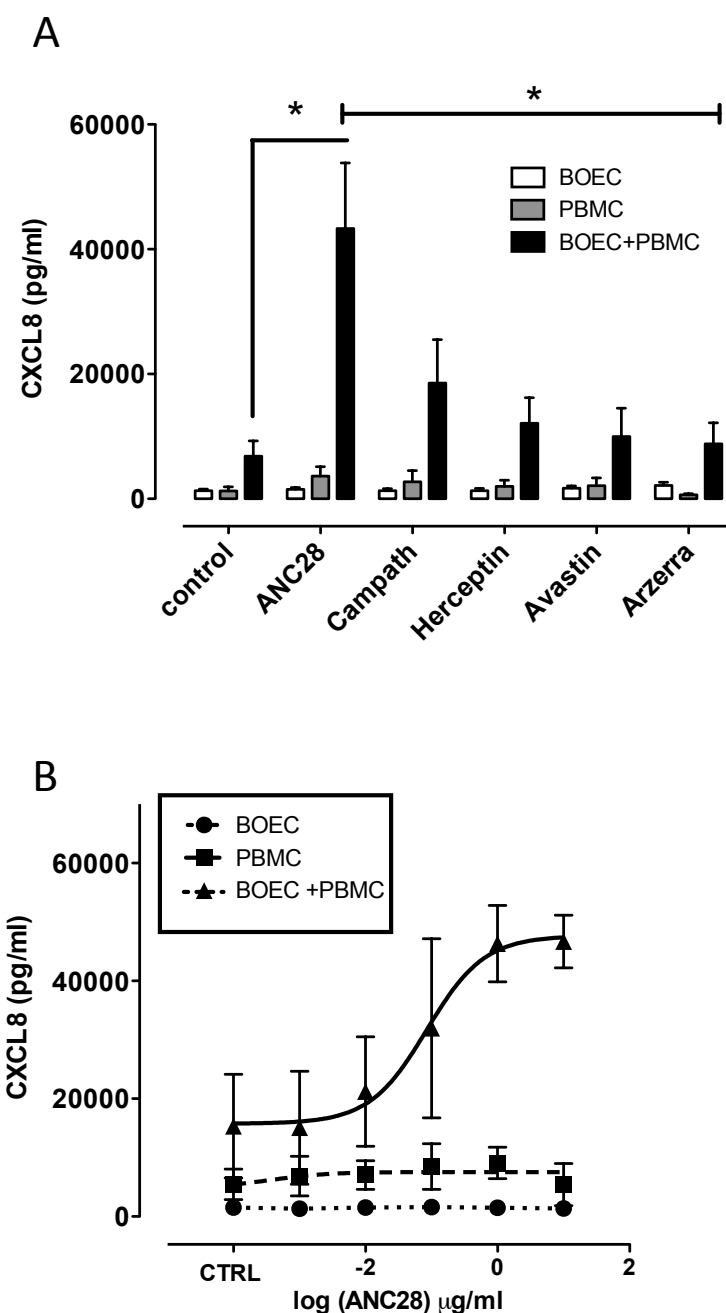


Figure 5.2 Response of same donor blood outgrowth endothelial cell (BOEC) and peripheral blood mononuclear cell (PBMC) co-culture assays to biologics. **(A)** Effect of therapeutic antibodies on CXCL8 release by mono cultures and co-cultures of same donor BOEC and PBMCs. Cells were treated with the TGN-like anti-CD28 superagonist; ANC28 (1/5D10; 10 μ g/ml), Campath (10 μ g/ml), Herceptin (10 μ g/ml) Avastin (10 μ g/ml) or Arzerra (10 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=8). Statistical significance was determined by one-way ANOVA (*p<0.05) followed by Bonferroni post-test. **(B)** Concentration response curve for BOEC and PBMC monocultures of co-cultures treated with ANC28 (0.001-10 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=3). Statistical testing was carried out by one-way ANOVA followed by Bonferroni's post-test.

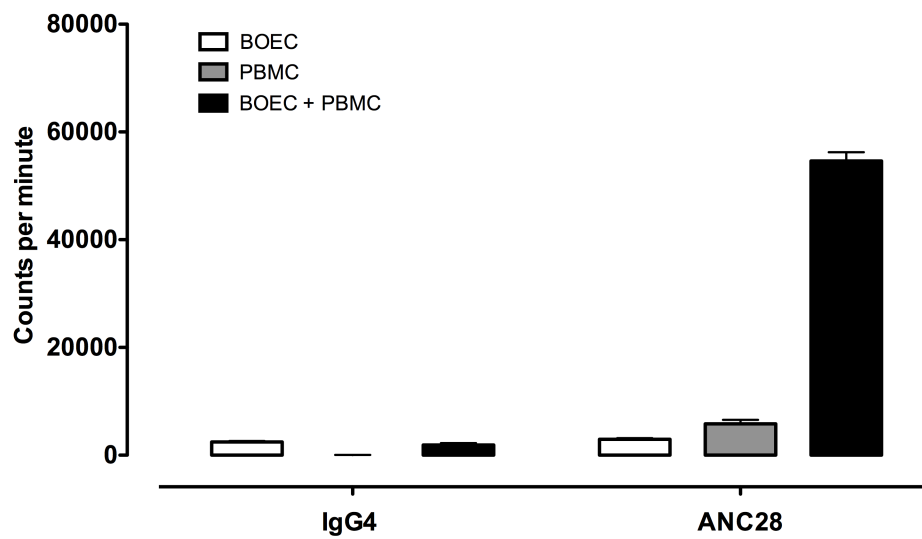


Figure 5.3 Proliferative response of same donor blood outgrowth endothelial cell (BOEC) and peripheral blood mononuclear cell (PBMC) co-culture assays to TGN1412-like anti-CD28 superagonist (ANC28). Cells were treated with the TGN-like anti-CD28 superagonist; ANC28 (1/5D10; 10 μ g/ml) or IgG4 isotype control, for 24h. Data are mean $\pm$ SEM (n=3 from one pilot experiment). Statistical significance was determined by paired t-test (*p<0.05)

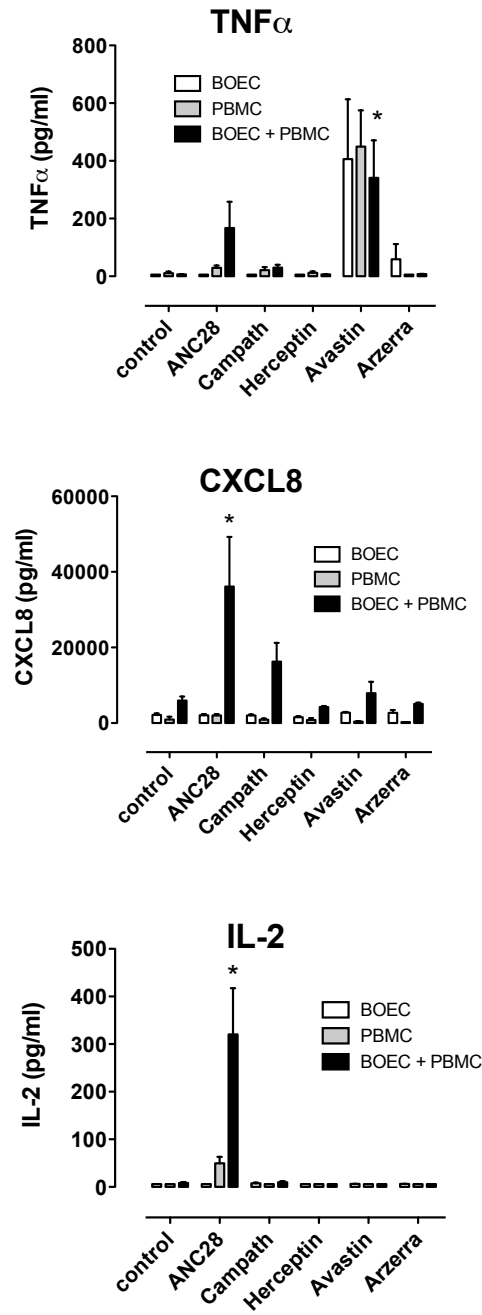


Figure 5.4 TNF α , CXCL8 and IL-2 release from blood outgrowth endothelial cell (BOEC) and peripheral blood mononuclear cell (PBMC) mono-cultures and same donor BOEC:PBMC co-cultures. Data are as per Figure 5.2A except measurements were carried out using an MSD 10-spot 9-plex Pro-Inflammatory Assay. Data are mean $\pm$ SEM (n=5). Statistical testing was carried out by one-way ANOVA followed by Dunnett's multiple comparison test (*p<0.05).

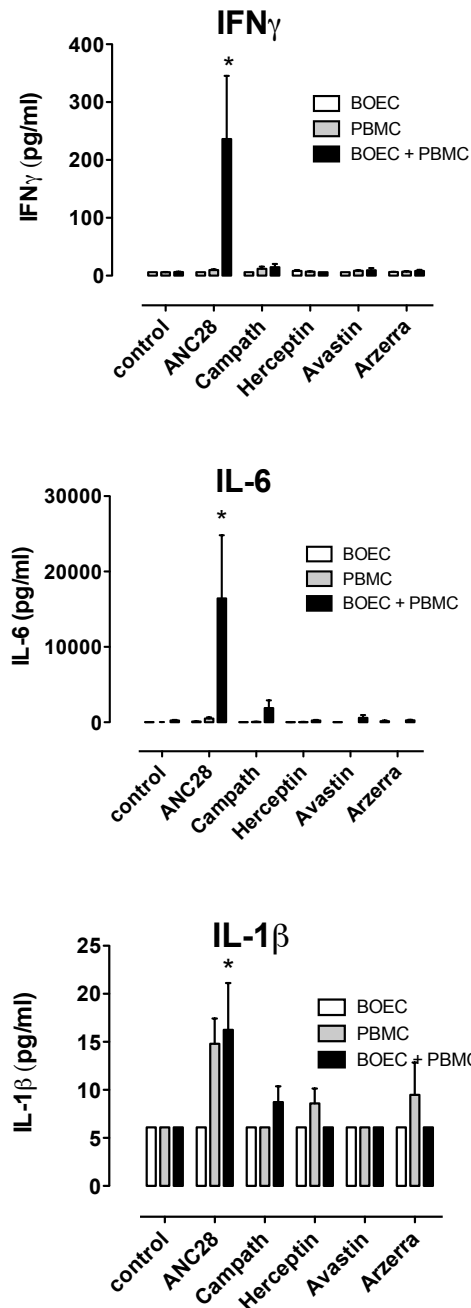


Figure 5.5 IFN γ , IL-6 and IL-1 β release from blood outgrowth endothelial cell (BOEC) and peripheral blood mononuclear cell (PBMC) mono-cultures and same donor BOEC:PBMC co-cultures. Data are as per Figure 5.2A except measurements were carried out using an MSD 10-spot 9-plex Pro-Inflammatory Assay. Data are mean $\pm$ SEM (n=5). Statistical testing was carried out by one-way ANOVA followed by Dunnett's multiple comparison test (*p<0.05). Where cytokines were not detected the minimum detectable level was added to the data (6.1pg/ml)

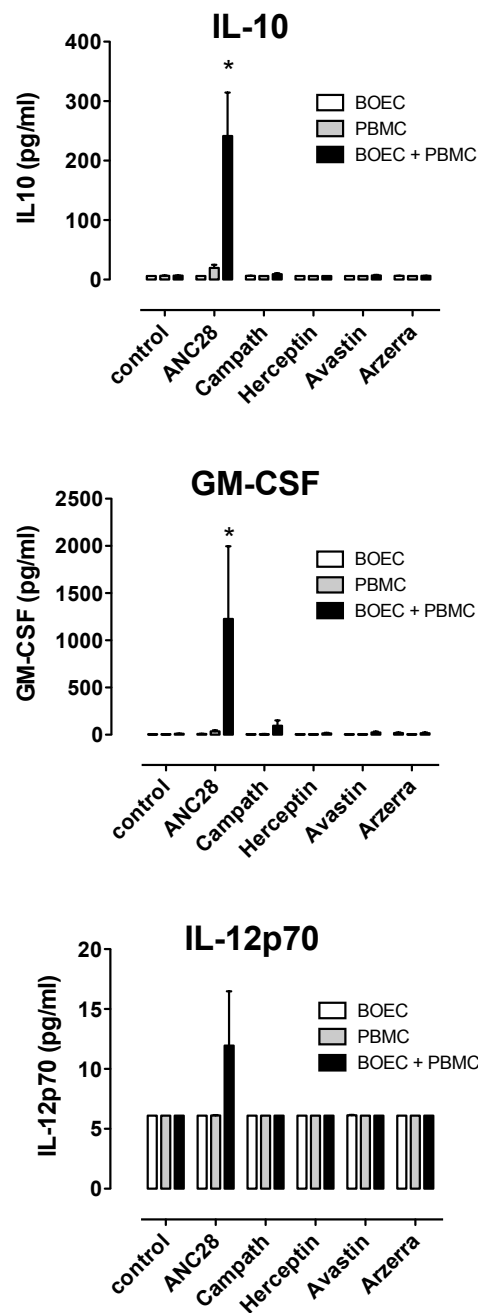


Figure 5.6 IL-10, GM-CSF and IL-12p70 release from blood outgrowth endothelial cell (BOEC) and peripheral blood mononuclear cell (PBMC) mono-cultures and same donor BOEC:PBMC co-cultures. Data are as per Figure 5.2A except measurements were carried out using an MSD 10-spot 9-plex Pro-Inflammatory Assay. Data are mean $\pm$ SEM (n=). Statistical testing was carried out by one-way ANOVA followed by Dunnett's multiple comparison test (* $p < 0.05$). Where cytokines were not detected the minimum detectable level was added to the data (6.1pg/ml)

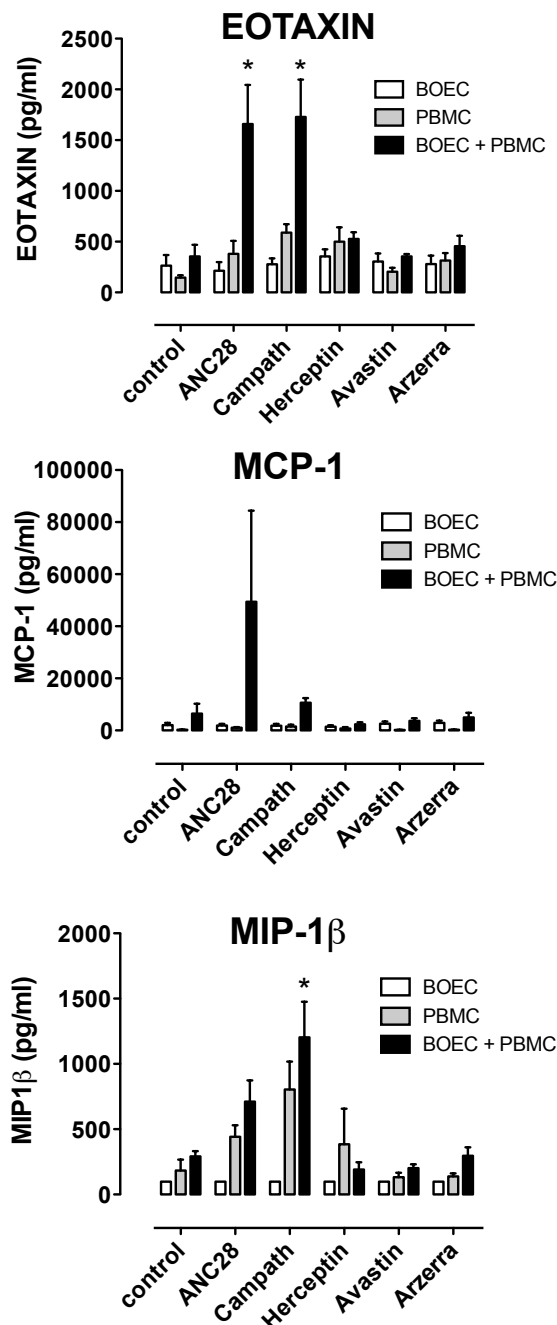


Figure 5.7 Eotaxin, MCP-1 and MIP-1 β release from blood outgrowth endothelial cell (BOEC) and peripheral blood mononuclear cell (PBMC) mono-cultures and same donor BOEC:PBMC co-cultures. Data are as per Figure 5.2A except measurements were carried out using an MSD 10-spot 9-plex Pro-Inflammatory Assay. Data are mean $\pm$ SEM (n=5). Statistical testing was carried out by one-way ANOVA followed by Dunnett's multiple comparison test (*p<0.05).

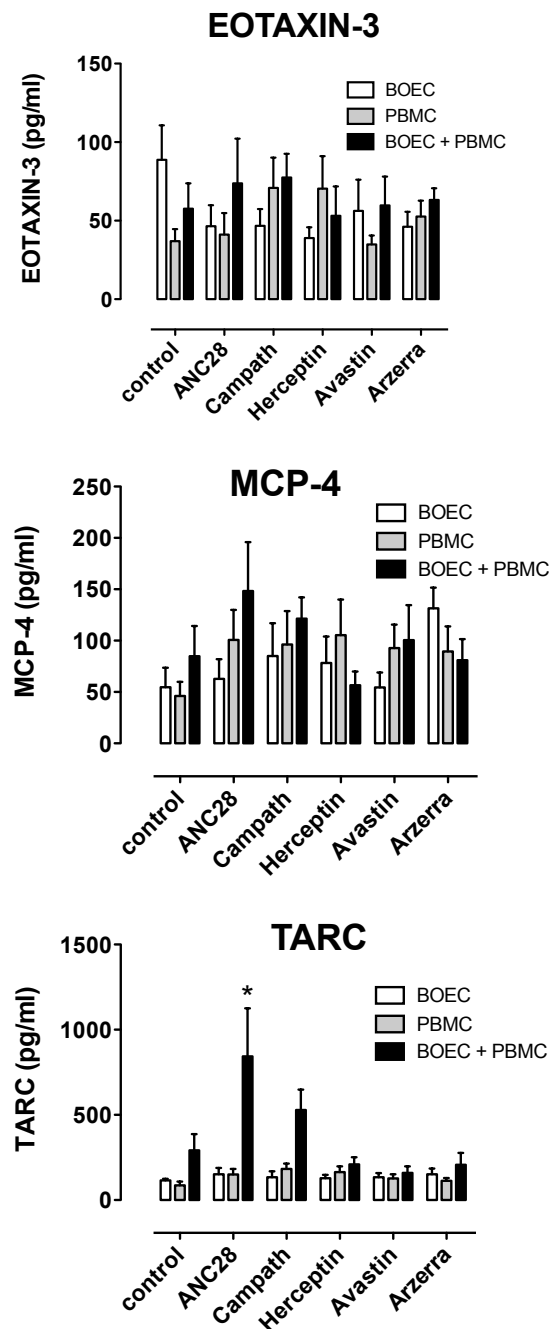


Figure 5.8 Eotaxin-3, MCP4 and TARC release from blood outgrowth endothelial cell (BOEC) and peripheral blood mononuclear cell (PBMC) mono-cultures and same donor BOEC:PBMC co-cultures. Data are as per Figure 5.2A except measurements were carried out using an MSD 10-spot 9-plex Pro-Inflammatory Assay. Data are mean $\pm$ SEM (n=5). Statistical testing was carried out by one-way ANOVA followed by Dunnett's multiple comparison test (*p<0.05).

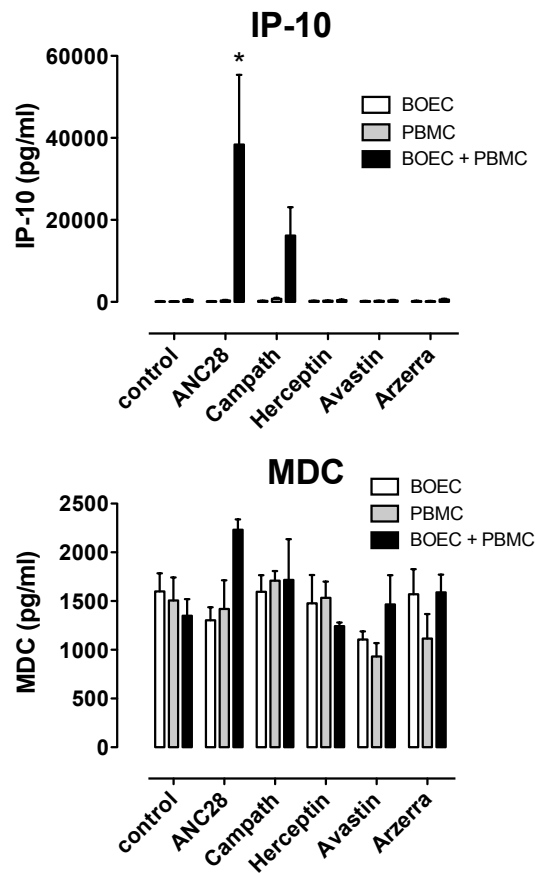


Figure 5.9 Eotaxin-3, MCP4 and TARC release from blood outgrowth endothelial cell (BOEC) and peripheral blood mononuclear cell (PBMC) mono-cultures and same donor BOEC:PBMC co-cultures. Data are as per Figure 5.2A except measurements were carried out using an MSD 10-spot 9-plex Pro-Inflammatory Assay. Data are mean $\pm$ SEM (n=5). Statistical testing was carried out by one-way ANOVA followed by Dunnett's multiple comparison test (* $p < 0.05$).

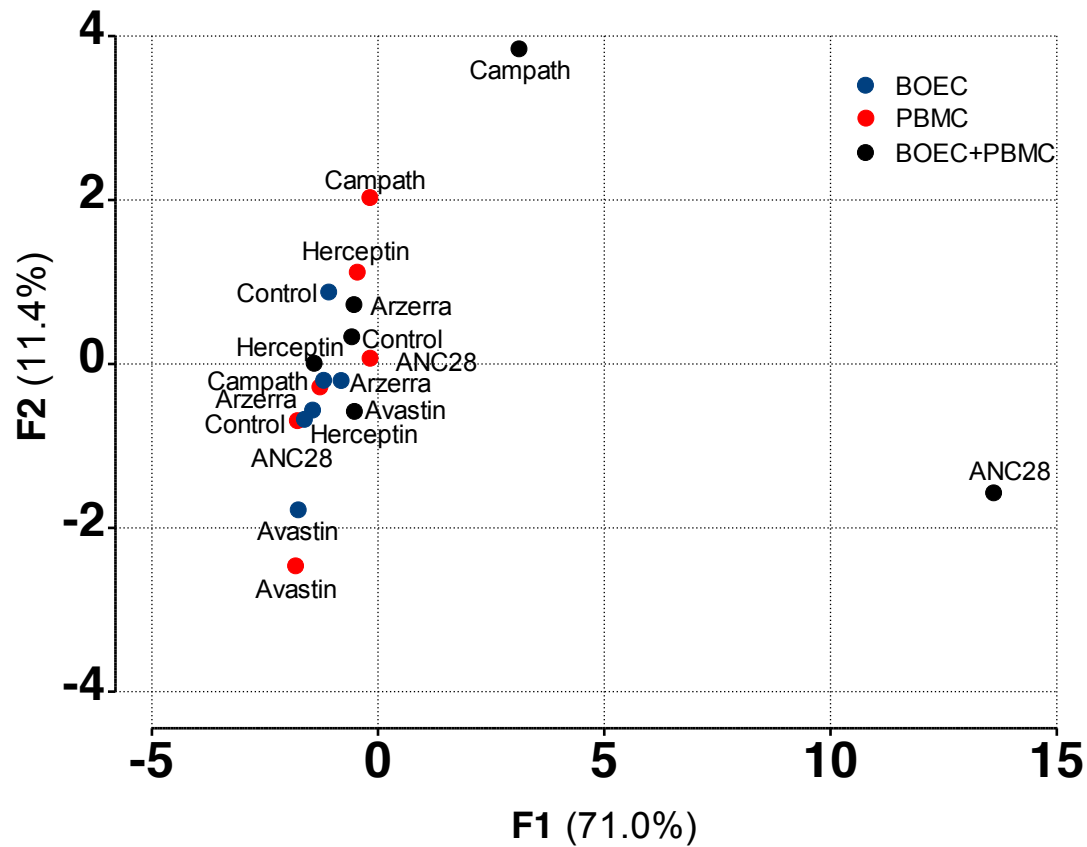


Figure 5.10 Scores plot for principal component analysis of blood outgrowth endothelial cell (BOEC) and peripheral blood mononuclear cell (PBMC) monocultures and same donor BOEC:PBMC co-cultures treated with the TGN-like anti-CD28 superagonist; ANC28 (1/5D10; 10µg/ml), Herceptin (10µg/ml), Campath (10µg/ml), Herceptin (10µg/ml), Avastin (10µg/ml) or Arzerra (10µg/ml) for 24h and the following cytokines and chemokines were measured and included in the analysis; GM-CSF, IFN γ , IL-2, IL-6, CXCL8, TNF α , IL-1 β , IL-10, IL-12p70, Eotaxin, Eotaxin 3, IP10, MCP-1, MCP-4, MDC, MIP-1 β , TARC. Values were entered for each variable for all treatment conditions which were the average of n=5 donors.

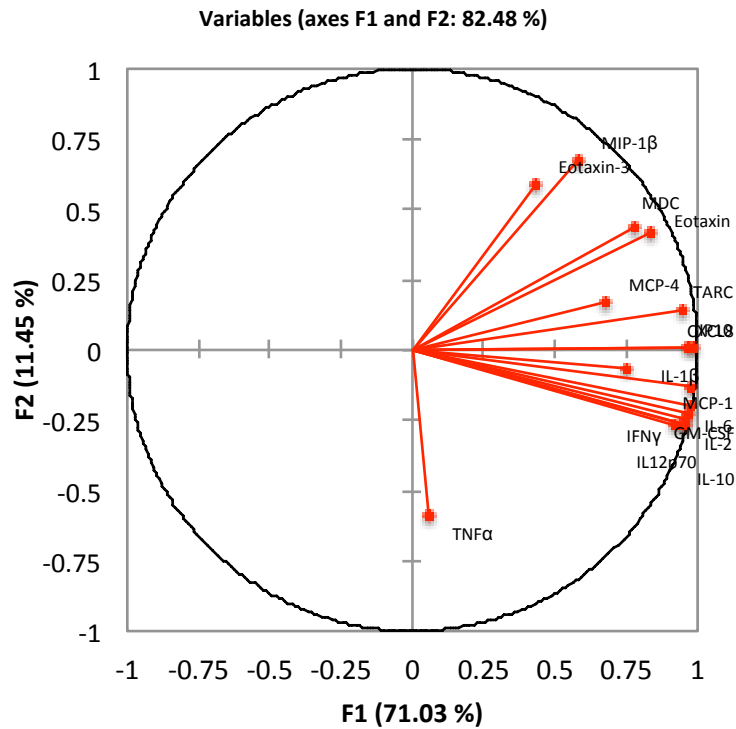


Figure 5.11 Loadings plot for principal component analysis of blood outgrowth endothelial cell (BOEC) and peripheral blood mononuclear cell (PBMC) monocultures and same donor BOEC:PBMC co-cultures treated with the TGN-like anti-CD28 superagonist; ANC28 (1/5D10; 10µg/ml), Herceptin (10µg/ml), Campath (10µg/ml), Herceptin (10µg/ml), Avastin (10µg/ml) or Arzerra (10µg/ml) for 24h and the following cytokines and chemokines were measured and included in the analysis; GM-CSF, IFN γ , IL-2, IL-6, CXCL8, TNF α , IL-1 β , IL-10, IL-12p70, Eotaxin, Eotaxin 3, IP10, MCP-1, MCP-4, MDC, MIP-1 β , TARC. Values were entered for each variable for all treatment conditions which were the average of n=5 donors.

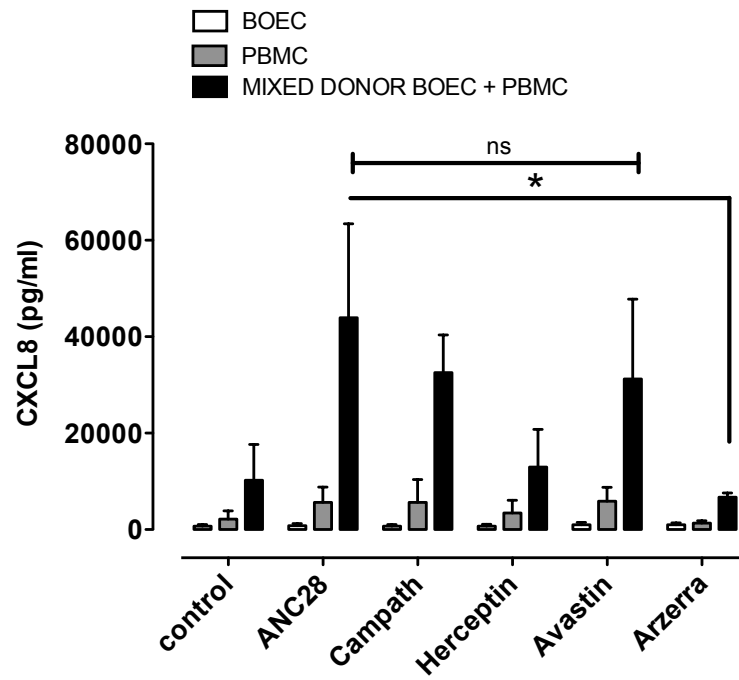


Figure 5.12 Response of mixed donor blood outgrowth endothelial cell (BOEC) and peripheral blood mononuclear cell (PBMC) co-culture assays to biologics. Cells were treated with the TGN-like anti-CD28 superagonist; ANC28 (1/5D10; 10 μ g/ml), Campath (10 μ g/ml), Herceptin (10 μ g/ml) Avastin (10 μ g/ml) or Arzerra (10 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=3). Statistical significance was determined by one-way ANOVA (*p<0.05) followed by Bonferroni post-test.

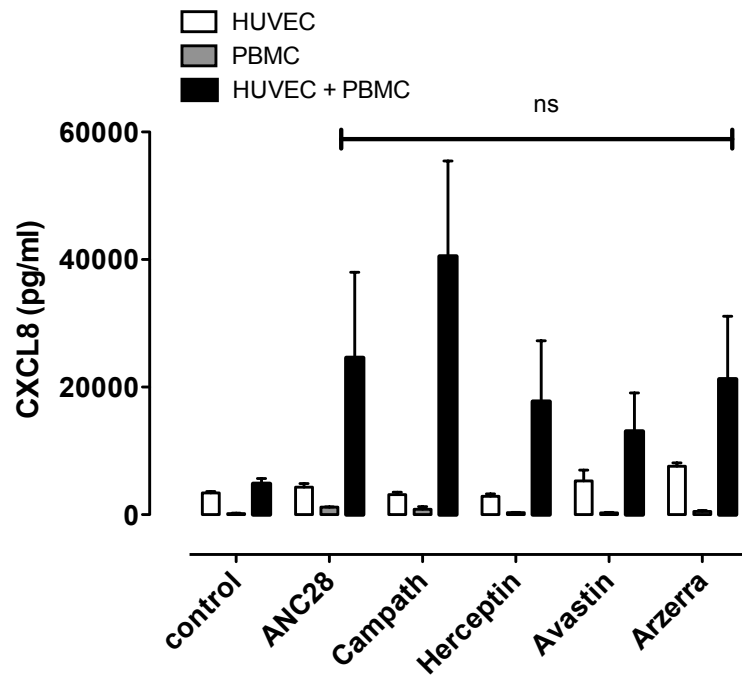


Figure 5.13 Response of human umbilical vein endothelial cell (HUVEC) and peripheral blood mononuclear cell (PBMC) co-culture assays to biologics. Cells were treated with the TGN-like anti-CD28 superagonist; ANC28 (1/5D10; 10 μ g/ml), Campath (10 μ g/ml), Herceptin (10 μ g/ml) Avastin (10 μ g/ml) or Arzerra (10 μ g/ml) for 24h. Data are mean $\pm$ SEM (n=3). Statistical significance was determined by one-way ANOVA (*p<0.05) followed by Bonferroni post-test.

Absorbance (570-610nm)	BOEC	PBMC	BOEC + PBMC
MEDIA	0.434 ± 0.069	0.378 ± 0.080	0.420 ± 0.047
ANC28	0.433 ± 0.066	0.423 ± 0.109	0.402 ± 0.036
CAMPATH	0.423 ± 0.066	0.352 ± 0.067	0.384 ± 0.040
HERCEPTIN	0.420 ± 0.063	0.348 ± 0.059	0.385 ± 0.040
AVASTIN	0.423 ± 0.062	0.342 ± 0.054	0.392 ± 0.037
ARZERRA	0.423 ± 0.059	0.339 ± 0.054	0.394 ± 0.044

Table 5.1 AlamarBlue® data for effect of therapeutic antibodies on cell activity of mono cultures and co-cultures of autologous blood outgrowth endothelial cells (BOEC) and peripheral blood mononuclear cells (PBMCs). Cells were treated with the TGN-like anti-CD28 superagonist; ANC28 (1/5D10; 10µg/ml), Campath (10µg/ml), Herceptin (10µg/ml) Avastin (10µg/ml) or Arzerra (10µg/ml) for 24h. Data are mean ± SEM (n=6). alamar blue® measurements were taken at wavelength of 570-620nm at 6h post addition of 10% AlamarBlue® reagent in DMEM with 10% FBS.

CXCL8 (pg/ml) BOEC+PBMC	Donor A	Donor C	Donor F	Donor Q	Donor Z	Donor S	Donor AG	Donor AK
MEDIA	2694.92	4203.864	5242.842	3250.47	6518.04	23856.94	10261.76	797.53
ANC.28	37936.5	12230.51	35161.56	108258.0	15609.40	38014.00	52866.86	49218.52
CAMPATH	23703.28	6588.711	11634.86	2342.246	18190.27	52583.4	46919.76	5132.27
HERCEPTIN	3800.45	3432.637	4490.92	4528.572	2562.80	31786.66	28641.6	3847.91
AVASTIN	3712.35	7333.211	5677.08	3320.974	3164.62	41707.1	3288.744	13888.85
ARZERRA	3168.66	5521.58	5504.42	4774.344	9247.12	32982.08	8008.88	4334.41

Table 5.2 Effect of therapeutic antibodies on CXCL8 release by same donor BOEC:PBMC co-cultures from 8 healthy donors. Cells were treated with the TGN-like anti-CD28 antibody; ANC28 (1/5D10; 10µg/ml), Herceptin (10µg/ml), Campath (10µg/ml), Herceptin (10µg/ml), Avastin (10µg/ml) or Arzerra (10µg/ml) for 24h. Individual data points are expressed.

**CHAPTER 6: APPLICATION OF BLOOD OUTGROWTH
ENDOTHELIAL CELLS (BOEC) TO (I) VALIDATE THE
PHENOTYPE OF A PATIENT WITH A GENETIC MUTATION
AND (II) FURTHER STUDY OF PHOSPHOLIPASE-A₂ ISOFORM
FUNCTION IN PROSTACYCLIN RELEASE FROM ENDOTHELIAL
CELLS**

6.1 RATIONALE

One of the important applications of blood outgrowth endothelial cells (BOEC) derived from donor stem cells is the ability to use them to phenotype vascular function in patients. In this chapter I have used BOEC to do this in a patient who has a frame shift mutation in the PLA2G4A gene, which results in loss of function of cytosolic phospholipase-A_{2α} (cPLA_{2α}). This results in severe clinical symptoms including gastrointestinal inflammation and renal failure. These clinical manifestations are consistent with a person taking long-term high dose non-steroidal anti-inflammatory drugs (NSAIDs). Indeed, my group has shown full clinical and genetic characteristics of this patient and relatives and that platelets from this patient do not release cyclooxygenase (COX) products when stimulated *in vitro* (Brooke et al., 2012).

PLA₂ encompasses a family of enzymes that cleave the *sn*-2 ester bonds of phospholipids to liberate free fatty acids (Funk, 2001); including arachidonic acid. As discussed previously, arachidonic acid serves as a substrate for COX. Numerous isoforms of PLA₂ exist and these fit broadly in to three families. These are secreted PLA₂ (sPLA₂), cytosolic PLA₂ (cPLA₂), and calcium-independent PLA₂ (iPLA₂). It is thought to be the Ca²⁺-dependent cPLA₂ isoform that is required for arachidonic acid release associated with prostaglandin synthesis (Adler et al., 2008; Brooke et al., 2012). As loss of prostacyclin in the gastric and renal circulations is associated with the type of symptoms experienced by the patient lacking cPLA_{2α}, this is consistent with the idea that cPLA₂ supports prostacyclin synthesis *in vivo*. However, others have reported that in blood vessels iPLA₂ is linked to arachidonic acid release and prostacyclin production by endothelial cells (Sharma et al., 2011) and vascular smooth muscle (Liu et al., 2012).

In this Chapter, I sought to grow BOEC from the patient with a mutation in the cPLA_{2α} gene, PLA2G4A and to phenotype these cells for ability to release prostacyclin as well as a full range of eicosanoids.

The specific questions addressed in this chapter are:

1. Can BOEC be successfully grown out of the blood from the patient with the PLA2G4A mutation, and how do their differentiation kinetics compare to cells from healthy controls?
2. How does the phenotype, in terms of morphology and response to shear stress, of BOEC from the patient with the PLA2G4A polymorphism compare with cells from healthy donors?
3. How does loss of cPLA_{2α} affect the ability of BOEC to release prostacyclin and other eicosanoids?
4. What is the relative role of cPLA₂ vs iPLA₂ in the release of prostacyclin from BOEC from healthy donors?
5. What is the role of cPLA₂ vs iPLA₂ in prostacyclin release from intact blood vessels

6.2 SUMMARY OF METHODS

A full description of the methods used in this chapter is given in Chapter 2 and are summarised below.

6.2.1 Patient characteristics

Blood was collected from a patient defined by our group as having homozygous mutation in the cPLA_{2α} gene, PLA2G4A. The clinical characteristics and medical history of the affected female used in my PhD are shown in Table 6.1

6.2.2 BOEC culture from healthy donors and a patient with PLA2G4A mutation

BOEC were isolated from healthy volunteers and a patient with a homozygous mutation in the cPLA_{2α} encoding gene, PLA2G4A. This patient was identified previously by our group (Brooke et al., 2012). Blood was collected, and cells were grown, at Barts and The London Hospital. Cells derived from the patient, as with BOEC used previously, were characterised for alignment under shear stress, and expression of CD31, and VE-cadherin.

6.2.3 Assessment of the ability of BOEC from a cPLA_{2α}-deficient patient to align under shear stress

Endothelial cell alignment under shear stress was determined using a model previously defined by our group and developed by my colleague Claire Potter (Potter et al., 2011) and in chapters 2 and 3 of this thesis. Briefly, cells were cultured on standard 1% gelatinised 6-well plates and placed on an orbital shaker. The movement of the shaker results in a wave of media that oscillates around the well resulting in a complex pattern of shear applied with

directional (laminar) shear towards the edge of the well, and non-directional (oscillatory) shear at the centre. Alignment of cells was visualised by light microscopy and by fluorescence imaging of cells stained with appropriate markers as described above.

6.2.4 BOEC treatment protocols

BOEC from healthy volunteers or the cPLA_{2α}-deficient patient were incubated with or without IL-1β (1ng/ml) for 24 hours to activate COX. They were then stimulated with thrombin (1U/ml; phosphate buffered saline (PBS)) or Ca²⁺ ionophore (A23187; 30μM; dimethyl sulphoxide (DMSO) 0.1% final) to activate endogenous iPLA₂ and cPLA₂ enzymes, respectively, or with arachidonic acid (50μM; ethanol 0.05% final), which stimulates prostacyclin formation without the requirement of PLA₂ and so bypasses this part of the pathway to prostacyclin release. In some experiments, cells were pre-treated for 30 minutes with or without the cPLA₂ inhibitor pyrrophenone (0.1-10μM) or vehicle (0.1% DMSO).

6.2.5 Mouse aortic ring experiments

Isolation of aortic rings in this chapter was carried out by my colleague Dr. Nick Kirkby. Aortic rings were isolated from CO₂-euthanised mice (male, BALB/c, 10 weeks; n=6), and incubated for 1 hour at 37°C. Mouse aortic rings were then incubated for 30 minutes with the cPLA₂ inhibitor, pyrrophenone (PYR; 0.1-10μM), or the iPLA₂ inhibitor bromoenol lactone (BEL; 0.1-10μM) or vehicle (0.1% dimethyl sulphoxide (DMSO)) as above. Mouse aortic rings do not respond robustly to thrombin, so for these experiments, prostacyclin release was stimulated for 30 minutes with acetylcholine (ACh; 1μM; saline), which also acts

via a G_q-protein coupled receptor signalling pathway to activate PLA₂ pathways in a similar fashion to thrombin. Supernatants were collected and stored at -80°C for analysis.

6.2.6 Measurement of prostacyclin release

In all assays, prostacyclin release was measured in the supernatant after 24 hours or 30 minutes as its breakdown product, 6-ketoPGF_{1α} by ELISA. Optical density was determined using a microplate reader (Dyne, Madellan Biosciences) with absorbance readings at 450nm

6.2.7 Lipidomics by LC/MS/MS

To consider a more complete profile of eicosanoids released by BOEC, supernatants were also analysed using a liquid chromatography – tandem mass spectrometry (LC/MS/MS) ‘lipidomics’ approach. This allows for unbiased analysis of a wide range of biologically active lipid mediators within a small sample volume (Kirkby et al., 2013; Masoodi and Nicolaou, 2006). For this, supernatants from A23187 or vehicle treated BOEC were sent to the National Institute of Environmental and Health Research, USA, under collaboration with Dr. Matthew Edin and Prof. Daryl Zeldin. Prostanoids were extracted and analysed as previously described (Edin et al., 2011; Newman et al., 2002).

Briefly, samples were spiked with PGE₂-d₄, 10(11)-epoxyheptadecanoic acid and 10(11)-dihydroxynonadecanoic acid internal standards then mixed with 0.1 volumes for 1% acetic acid (volume/volume) in 50% methanol. Samples were then pre-extracted using Oasis HLB C18 3ml columns, and then further separated by high pressure liquid chromatography with Phenomenex Luna C18(2) columns. Extracts were analysed using negative ion electrospray

ionization tandem mass spectrometry in triplicate using an MDS Sciex API 3000 instrument with Applied Biosystems TurbolonSpray source. Lipid mediator abundance was quantified from peak areas of characteristic fragments and normalised to the extraction efficiency of internal standards.

6.3. RESULTS

6.3.1 Characterisation of BOEC from a cPLA_{2α} deficient patient

As in previous chapters, it was important to characterise BOEC grown from the cPLA_{2α}-deficient patient. Colonies of BOEC grown from the cPLA_{2α}-deficient patient, like healthy BOEC, had cobblestone morphology (Figure 6.1A). However, BOEC from the cPLA_{2α}-deficient patient emerged sooner and in greater number than for healthy donors (Figure 6.1B). Similarly to BOEC from healthy donors, cells grown from the patient with a homozygous deletion of cPLA_{2α} expressed CD31, and VE-cadherin, and aligned under shear stress (Figure 6.2)

6.3.2 Prostacyclin release from cPLA_{2α} deficient BOEC

As expected, BOEC from healthy patients released prostacyclin in response to thrombin, A23187 and arachidonic acid. cPLA_{2α}-deficient BOEC, whilst able to release prostacyclin in response to arachidonic acid, released lower levels of prostacyclin in response to A23187 and thrombin (Figure 6.3).

6.3.3 Lipidomics analysis of eicosanoid release from BOEC from a cPLA_{2α} deficient patient

To further explore the effect of cPLA_{2α} deficiency on a range of eicosanoid pathways in endothelial cells, in addition to prostacyclin, a lipidomics approach was employed. This approach, which employs a combination of tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) (Edin et al., 2011; Newman et al., 2002) allowed for a more complete analysis of the lipids that might be regulated by cPLA_{2α} in BOEC. Across all the

eicosanoids measured, in response to A23187, healthy BOEC released increased levels of AA-derived COX products (prostacyclin, $\text{PGF}_{2\alpha}$, PGE_2 , PGD_2), AA-derived P450/LOX products (15-HETE, 12-HETE, 11-HETE, 8-HETE and 5-HETE), LA-derived P450 products (13-HODE, 9-HODE, 12,13-DHOME and 9,10,13-THOME), and the DHA P450 product, 19,20-EpDPE (Figure 6.4). Release of all these eicosanoids, except $\text{PGF}_{2\alpha}$ and 8-HETE, were reduced in BOEC lacking $\text{cPLA}_{2\alpha}$.

6.3.4 Effect of pharmacological inhibition of cPLA_2 in BOEC from healthy donors

As shown, thrombin, A23187, and AA induced robust prostacyclin release from BOEC from healthy donors (Figure 6.3). Furthermore, pre-treatment of BOEC with the cPLA_2 selective inhibitor, pyrrophenone, resulted in a concentration-dependent inhibition of prostacyclin release in response to both A23187 (Figure 6.6) and thrombin (Figure 6.7). Inhibition of cPLA_2 had no effect on arachidonic acid induced prostacyclin release (Figure 6.8).

6.3.5 Prostacyclin release from mouse aortic ring preparations

In line with observations in BOEC, inhibition of cPLA_2 with pyrrophenone, but not iPLA_2 with bromoenol lactone, resulted in a concentration dependent decrease in prostacyclin release from mouse thoracic aortic rings (Figure 6.9).

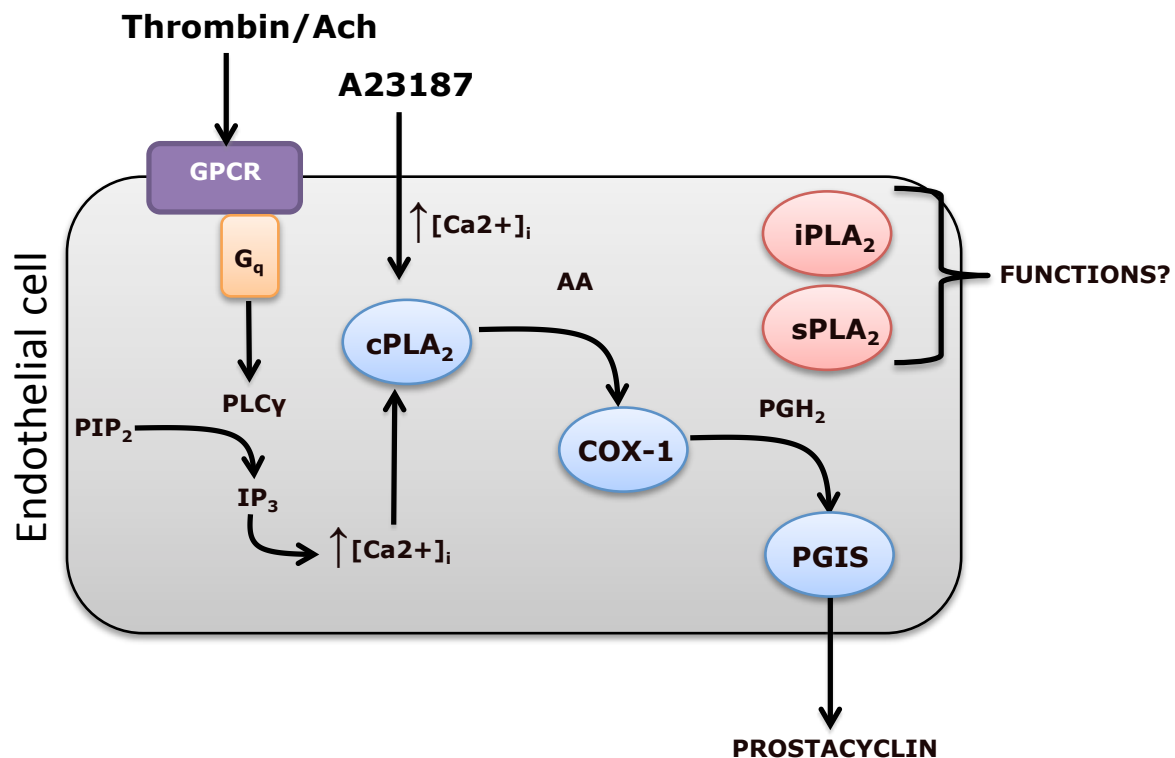
6.4 SUMMARY

In this chapter I have shown that:

- BOEC from a cPLA_{2α}-deficient patient, like BOEC from healthy donors, expressed CD31 and VE-cadherin and aligned under shear stress.
- BOEC lacking cPLA_{2α} released lower levels of prostacyclin, as well as other eicosanoids, in response to Ca²⁺ ionophore (A23187) and thrombin.
- Pharmacological inhibition of cPLA₂ reduced Ca²⁺ ionophore (A23187) and thrombin induced prostacyclin release from healthy BOEC.
- Pharmacological inhibition of cPLA₂ but not iPLA₂ reduced Ach induced prostacyclin release from mouse thoracic aorta.

This chapter serves as an important proof of concept that BOEC can be used to phenotype a patient with a genetic disorder and retain differences in key pathways associated with disease. In terms of endothelial cell function, loss of a key enzyme in prostacyclin synthesis, like cPLA₂ provides a gold standard research tool to investigate the function of this pathway in endothelial cell biology. Understanding the pathways that drive prostacyclin synthesis in endothelial cells is essential. We have previously shown that COX-1, and not COX-2 drives prostacyclin release from vessels (Kirkby et al., 2012; Kirkby et al., 2013). Using BOEC from a cPLA_{2α} deficient patient and healthy donors I have shown that cPLA₂ contributes significantly to prostacyclin production in endothelial cells. Clearly with this data it is reasonable to speculate that similar, less obvious, disease phenotypes impact on endothelial cells and it is possible to capture and study these using BOEC.

As a second outcome I was able to use these cells to test directly the role of PLA₂ in prostacyclin release by vascular cells. This is important to address and conflicting reports exist in the literature. The findings of this chapter are summarised in Summary Figure 6



Summary Figure 6. Endothelial cells express G-protein coupled receptors (GPCRs) which respond to physiological levels of agonist such as thrombin or acetylcholine (Ach). These receptors activate G_q which activates phospholipase-C γ (PLC γ) which cleaves membrane bound Phosphatidylinositol 4,5-bisphosphate (PIP₂) to liberate inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ subsequently results in an increase in intracellular Ca²⁺ concentration which activates cytosolic Ca²⁺-dependent PLA₂ (cPLA₂). cPLA₂ then cleaves the *sn*-2 ester bond of membrane bound phospholipids to liberate the cyclooxygenase (COX) substrate arachidonic acid (AA) which through the concerted actions of COX-1 and prostacyclin synthase (PGIS) in endothelial cells is converted to prostacyclin.

6.5 LIMITATIONS

The data presented in this chapter are limited in a number of ways, which are outlined and discussed below:

As data presented in this chapter were gathered in the latter stages of my PhD time constraint became apparent. It would have been valuable to run repeat experiments as well as further experiments including the iPLA₂ inhibitor, bromoenol lactone, in BOEC protocols. This would address whether thrombin is in fact signalling via iPLA₂ in BOEC similar to Ach in vessels.

I also acknowledge that only BOEC from the cPLA₂ deficient patient was carried out without comparison with family relatives including a sibling with the same mutation and a parent with a heterozygote mutation.

Whilst this approach, using pharmacological inhibition of cPLA₂ was valuable it would have also been of interest to use gene knock down approaches where cPLA₂, iPLA₂ and sPLA₂ could have been targeted. Other inhibitors also include commercially available inhibitors of G_q and G_s proteins which are thought to activate cPLA₂. This would provide some elucidation of the mechanism by which physiological agonists activate cPLA₂ and subsequent prostacyclin release.

Whilst it would have been valuable, in this chapter, it was not possible, due to time constraints, to measure the PLA₂ and COX enzyme expression both at the gene and protein level.

Indeed, the expression of different PLA₂ isoforms, and pathways regulating their expression and function is now under investigation by our group. Furthermore, as the BOEC derived from the cPLA_{2α} mutant patient in this chapter clearly represent a valuable tool to study PLA₂ signalling in the vasculature, these cells are also under further investigation by the group.

6.6 FIGURES

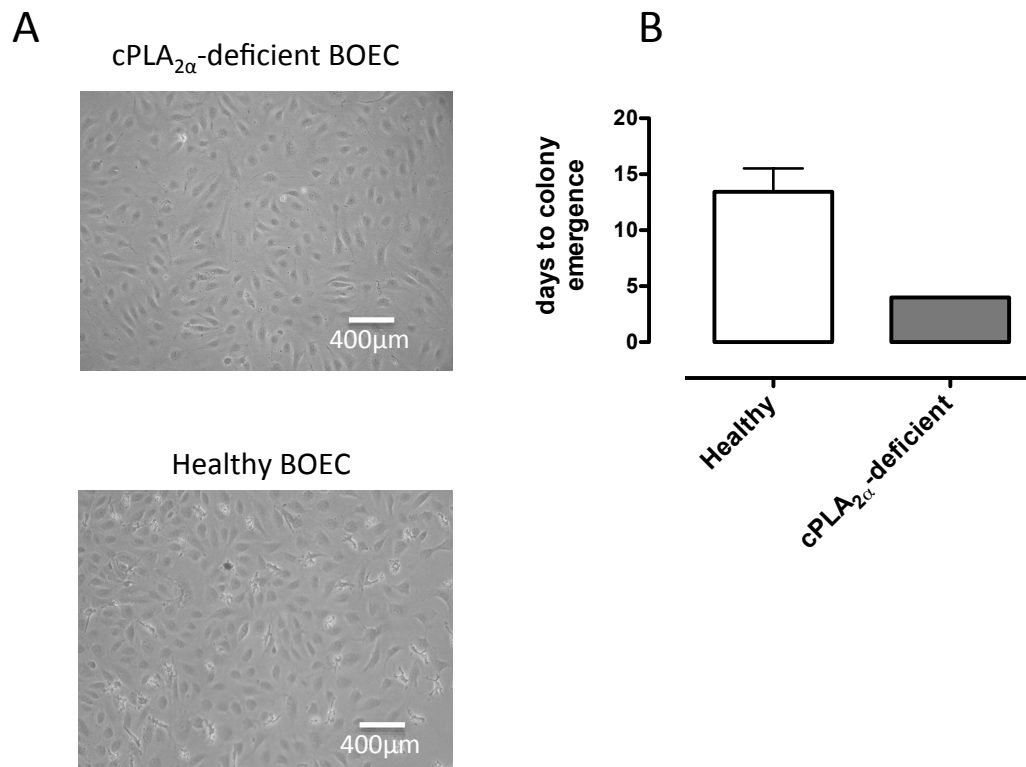


Figure 6.1 Characterisation of blood outgrowth endothelial cells (BOEC) from a patient with a homozygous deletion of cPLA_{2α}. **(A)** Images of BOEC from a cPLA_{2α}-deficient patient (top) and healthy donors. **(B)** Number of colonies emerging from successful isolations (healthy; n=4, cPLA_{2α} deficient patient n=1). Images in panel A were captured using a light microscope with a Nikon VR ISO3200 camera attachment. Contrast and brightness enhancements were applied equally to all images. All images are at 10 x magnifications with scale bars indicated.

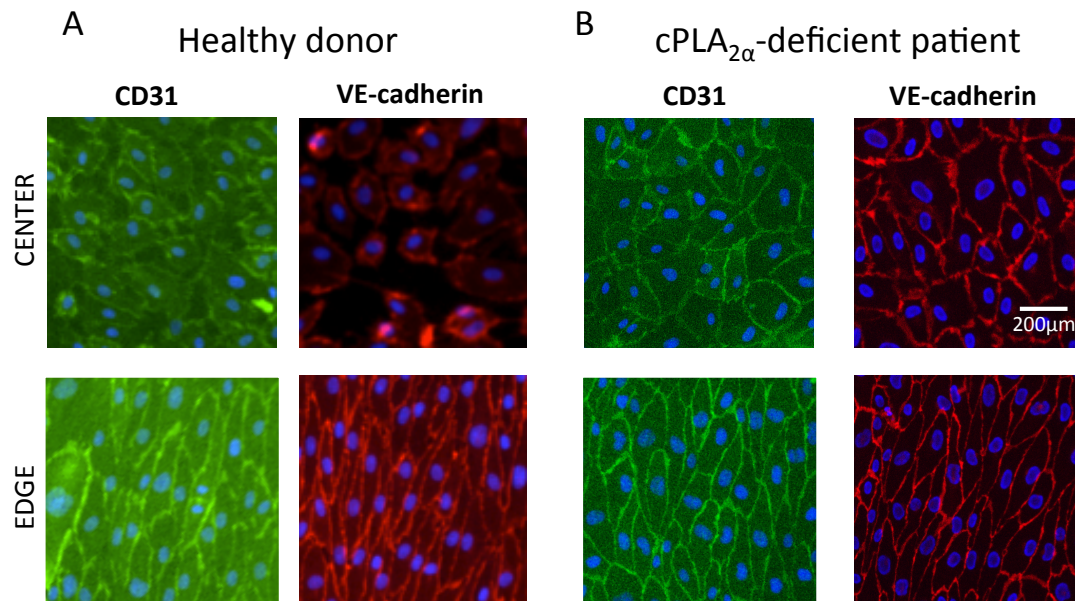


Figure 6.2 Characterisation of blood outgrowth endothelial cells (BOEC) from a patient with a homozygous deletion of cPLA_{2α}. BOEC from **(A)** healthy donors and **(B)** a cPLA_{2α}-deficient patients were cultured for 4 days shear stress conditions. Images were taken at the edge of the well of cells, where shear stress is unidirectional and cells align and at the center of the well where shear stress has no preferred direction and cells appear cobblestone in morphology. Cells were stained for CD31 (green), and VE-cadherin (red) and imaged using a Cellomics® HCS Arrayscanner. Images were captured at 10x magnification.

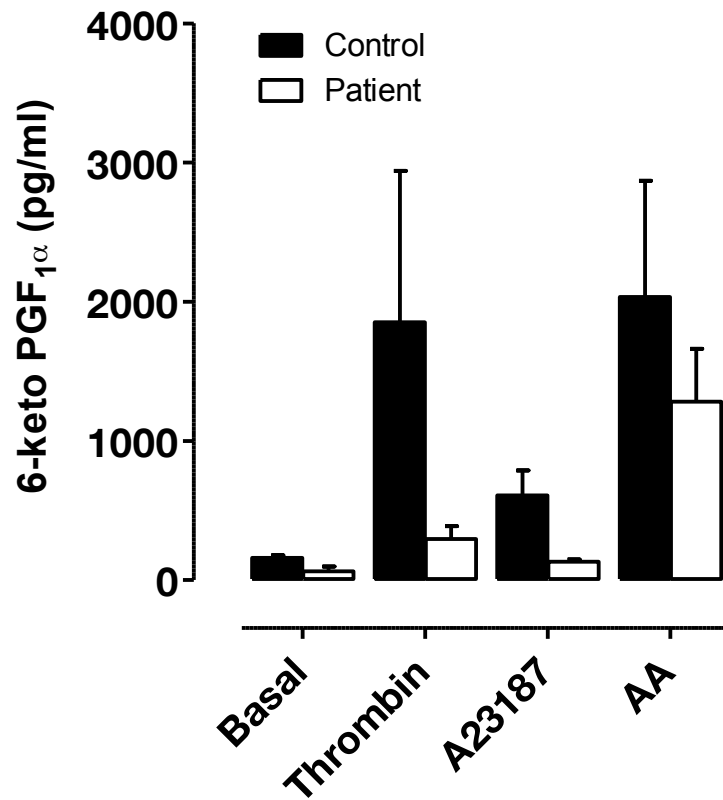


Figure 6.3 Effect of homozygous deletion of cPLA_{2α} on prostacyclin release from BOEC. BOEC from healthy patients (n=2-4) and a cPLA_{2α}-deficient patient (n=1) were pre-treated for 24h with IL-1β (1ng/ml) followed by 30 min treatment +/- thrombin (1 unit/ml), A23187 (50μM) or arachidonic acid (AA; 30μM) Data are mean ± SEM. Statistical significance between healthy and patient cells was not carried out as only one patient is available.

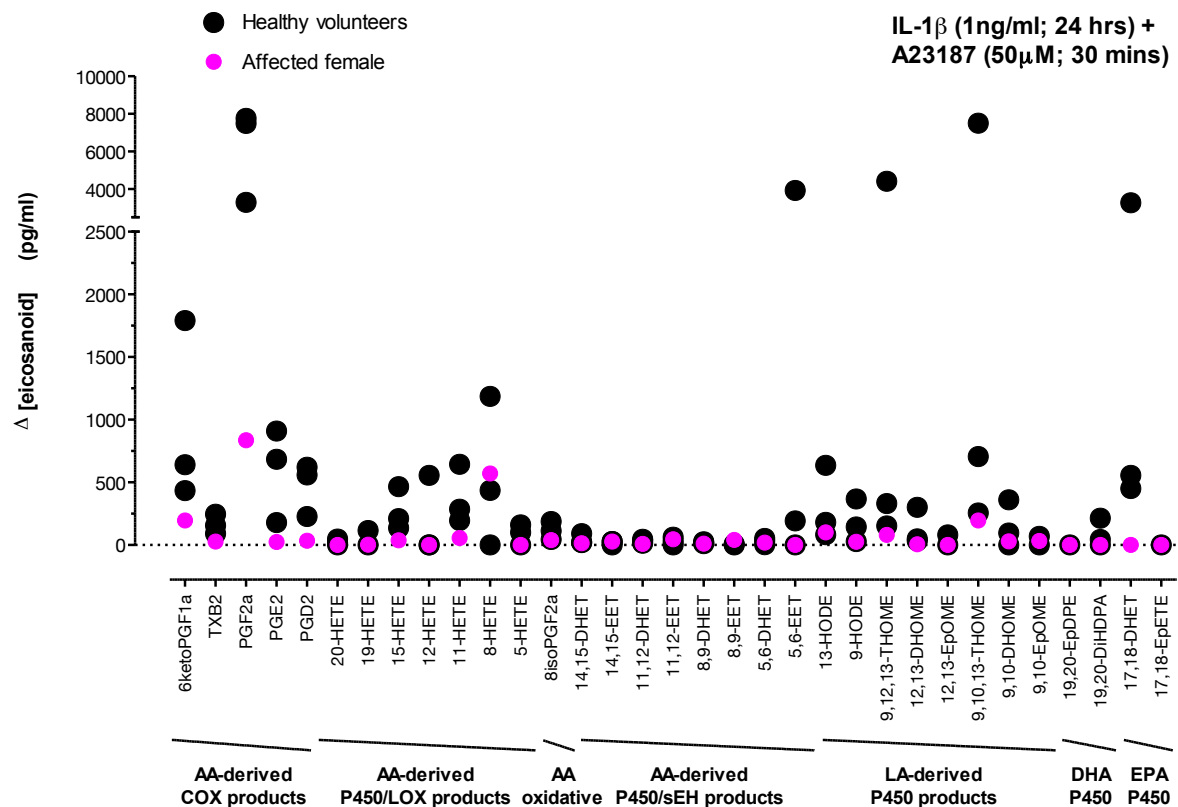


Figure 6.4 Lipid release profile measured by LC-MS/MS from blood outgrowth endothelial cells (BOEC) from healthy (black; n=3) or a cPLA $_{2\alpha}$ -deficient patient (pink; n=1) pre-treated for 24h with IL-1 β (1ng/ml) followed by 30min treatment with A23187 (50 μ M).

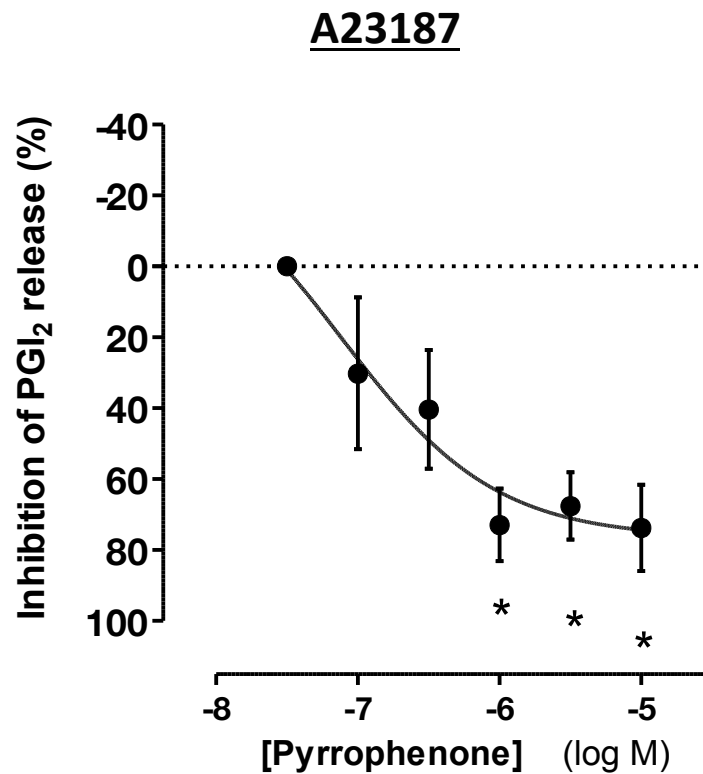


Figure 6.6 Effect of inhibition of cPLA₂ on A23187 induced prostacyclin release from blood outgrowth endothelial cells (BOEC). Concentration response curve for the effect of the cPLA₂ inhibitor, pyrrophenone (10 μ M- 0.1 μ M), on 6-ketoPGF_{1 α} production from BOEC following 24h pre-treatment with IL-1 β (1ng/ml) then 30min treatment +/- A23187 (50 μ M). Data are mean $\pm$ SEM (n=4). Statistical significance was determined by one-sample t-test vs. a theoretical value of 0.

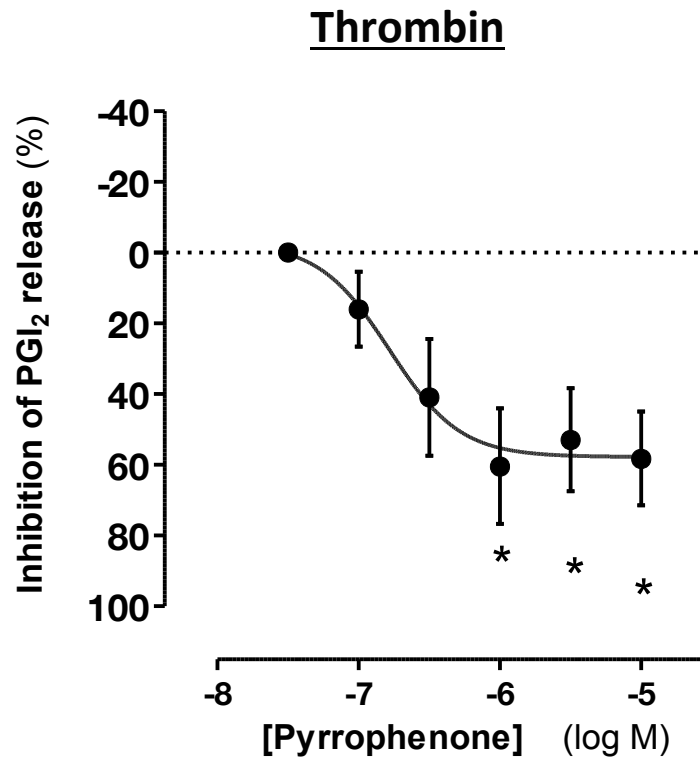


Figure 6.7 Effect of inhibition of cPLA₂ on thrombin induced prostacyclin release from blood outgrowth endothelial cells (BOEC). Concentration response curve for the effect of the cPLA₂ inhibitor, pyrrophenone (10 μ M- 0.1 μ M), on 6-ketoPGF_{1 α} production from BOEC following 24h pre-treatment with IL-1 β (1ng/ml) then 30min treatment +/- Thrombin (1unit/ml). Data are mean $\pm$ SEM (n=4). Statistical significance was determined by one-sample t-test vs. a theoretical value of 0.

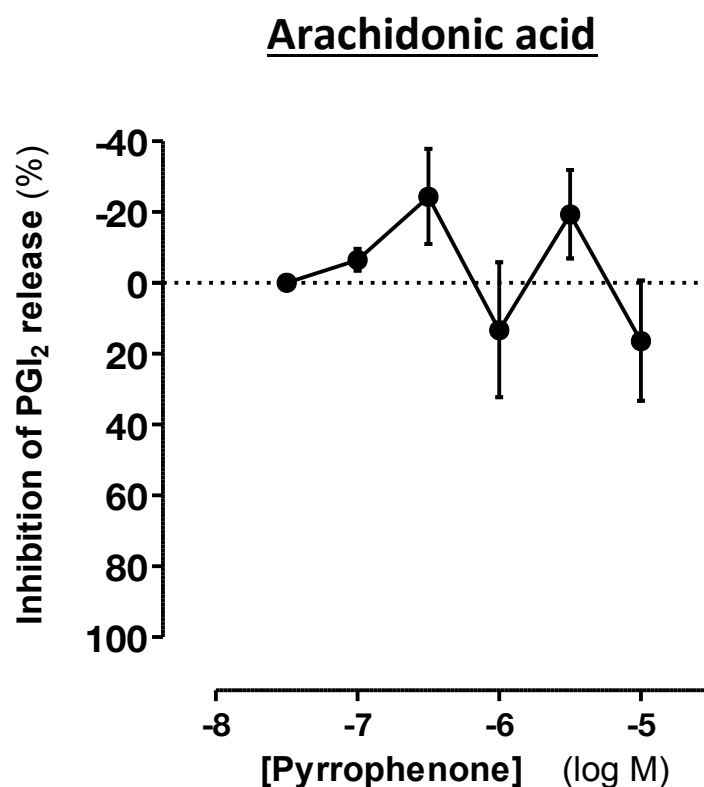


Figure 6.8 Effect of inhibition of cPLA₂ on AA induced prostacyclin release from blood outgrowth endothelial cells (BOEC). Concentration response curve for the effect of the cPLA₂ inhibitor, pyrrophenone (10μM- 0.1μM), on 6-ketoPGF_{1α} production from BOEC following 24h pre-treatment with IL-1β (1ng/ml) then 30min treatment +/- AA (30μM). Data are mean ± SEM (n=3). Statistical significance was determined by one-sample t-test vs. a theoretical value of 0.

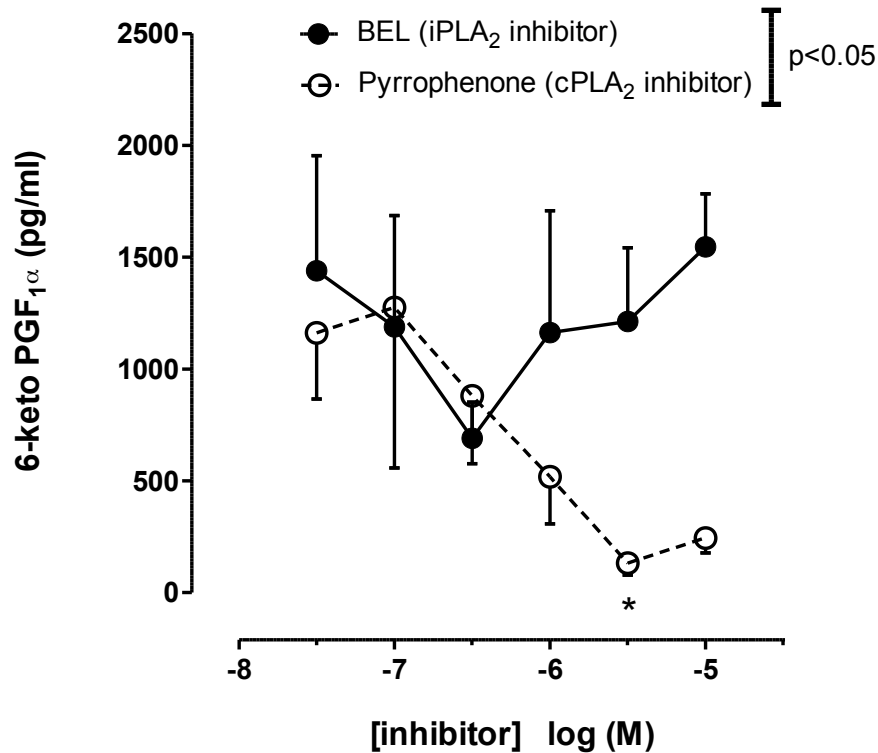


Figure 6.9 Effect of inhibition of cPLA₂ and iPLA₂ on Ach induced prostacyclin release from mouse aorta. Concentration response curves for the effect of the cPLA₂ inhibitor, pyrrophenone (10μM- 0.1μM) or the iPLA₂ inhibitor, bromoenol lactone (BEL) (10μM- 0.1μM), on prostacyclin production (measured as 6-ketoPGF_{1α}) from mouse aortic ring preparations pre-treated with inhibitors for 30 minutes followed by 30min treatment with Ach (1μM). Data are mean ± SEM (n=6 per group). Statistical significance for the effect of the individual inhibitors was determined using a Kruskal-Wallis test for non-normal data (*p<0.05). Statistical significance between inhibitors was determined by two-way ANOVA.

- Sex: Female born 1966.
- Presented at 2 years of age with peptic ulceration.
- Gastriejunostomy in 1980 for further peptic ulceration and duodenal stenosis.
- Diagnosed with pernicious anaemia.
- Surgery in 2000 for ileal perforation with *Salmonella enteritidis* infection.
- Renal biopsy in 2000 and following two episodes of *Campylobacter enteritis* lead to diagnosis of xanthogranulomatous pyelonephritis; patient continues with chronic renal failure.
- In 2001, patient experienced another ileal perforation and ileoileal fistula complicated by *Candida* septicaemia, resistant staphylococcal chest infection, acute respiratory distress syndrome and acute renal failure requiring haemofiltration.
- In 2009, patient underwent transverse colon resection for volvulus.
- In 2010, patient presented malnourished with hypoalbuminaemia and oedema, and started total parenteral nutrition.
- In 2010, patient developed biliary reflux with severe oesophagitis and strictures and required repeated oesophageal dilatation in 2010–2011, but developed bronchiectasis secondary to recurrent aspiration pneumonia.
- Further complications include endometriosis (1990), gall stones (2006), left ventricular concentric hypertrophy with a small cavity, and a fibrotic, unstable bladder with carbapenem stones, requiring ureteric stents (both 2009) and infertility.
- Based on this presentation, she was diagnosed in 2012 with cryptogenic multifocal ulcerating stenosing enteritis (CMUSE).

Table 6.1 Summary of history and characteristics of patient with homozygous mutation of cPLA_{2α}. Information was collected from Brooke et al. (Brooke et al., 2012)

CHAPTER 7: GENERAL DISCUSSION

Stem cell research is changing the way we think about and treat human disease. Stem cells have applications that pervade all areas of modern medicine, and in many cases, will be critical for success in the ongoing quest to treat human diseases. Stem cells, and cells derived from stem cells can be used to:

- Engineer tissues and organs for transplant, thus overcoming the 'organ waiting list' problem. In fact, many stem cell approaches will allow for personalised organs to be engineered in the laboratory.
- To develop regenerative cell therapies, whereby cells can be injected to reverse cell dysfunction.
- Study disease *in vitro* as cells can be grown from individual patients and used to phenotype cells. This 'disease in a dish' concept sits at the core of translational and personalised medicine whereby *in vitro* studies can be related directly to the clinic, and indeed to individual patients or groups.

In line with all of these, and in particular the concept of 'disease in a dish', stem cells can also be used to develop translational *in vitro* assays to assess the safety and efficacy of other therapies in modern medicine, such as biologics, gene therapies, and even autologous cell therapies for individual patients. Studying stem cells has already resulted in paradigm shifts in how we think about disease. Extensive examples exist in the literature illustrating the application of different stem cells. Many of these will be discussed later in the context of stem cell therapy; stem cell derived endothelial cells and pharmacological assay development.

In my PhD, I have explored some of these applications and performed characterization studies which will be essential for their use in the areas outlined above.

As discussed in detail in Chapter 1, three key families of stem cells exist each with benefits and limitations. These include human embryonic stem cells (hESC), adult progenitor/stem cells and induced pluripotent stem cells (iPSC). hESC have largely been considered as a cornerstone of stem cell research. These cells have applications in organ engineering, and have been used to study developmental aspects of human biology. In this regard, hESC have also been critical to our knowledge of stem cell phenotype and 'pluripotency' which is the property assigned to cells that are able to differentiate in to any cell in the body. Using this new knowledge of stem cells it is now possible to grow a plethora of cells and tissues in the lab. In my PhD I have acquired or isolated these cells and differentiated them in to endothelial cells. This gives rise to stem cell derived endothelial cells, which are truly revolutionising cardiovascular disease research. In fact, given the importance of endothelial cells in the vasculature of any organ, stem cell derived-endothelial cells will be critical in any and all organ regeneration programmes.

As discussed, endothelial cells are critical mediators of cardiovascular health whilst dysfunctional endothelial cells result in disease. It is now thought that endothelial cell health in the body can also be regulated by adult stem cells (Hill et al., 2003; Timmermans et al., 2009) and in particular endothelial progenitor cells. A caveat to success in using stem cell derived endothelial cells in the future has been the need to characterise them as 'authentic' endothelial cells.

Indeed, any stem cell derived endothelial cell used in therapy or in assays must be able to perform as a healthy endothelial cell and display cardinal hallmarks of endothelial cell phenotype. Looking at some of these pathways can also reveal differences in phenotype of endothelial cells between patient groups. This improves our understanding of cardiovascular disease as well as serves as a safety screen to ensure cell functions are preserved for cell therapy.

In my PhD, I set out to study stem cell endothelial cells in terms of some cardinal endothelial cell functions. These were ability to align under complex shear stress, respond to pathogen associated molecular patterns (PAMPs) and release endothelial cell hormones prostacyclin and endothelin-1 (ET-1). I have therefore split my discussion in to characterisation followed by applications of stem cell derived endothelial cells, namely blood outgrowth endothelial cells (BOEC), that I have carried out in my PhD.

PART I: CHARACTERISATION OF STEM CELL DERIVED ENDOTHELIAL CELLS

Alignment of endothelial cells is the result of the exposure of endothelial cells on vessels to shear stress exerted by the flow of blood against the endothelial cell surface. These forces have profound effects on the morphology and function of endothelial cells. This was studied extensively in the PhD thesis of my colleague Dr. Claire Potter. In my PhD, I have applied this knowledge to ensure that stem cell derived endothelial cells display the same pattern of responses to shear stress, and thus validate them as endothelial cells. Modeling of shear stress *in vitro* can be achieved using a system previously developed by our group (Potter et al., 2011). In this model, endothelial cells are grown on 6-well culture flasks and placed on an orbital shaker. This creates a wave of media, which flows over the endothelial cell monolayer. Cells grown at the centre of the well experience oscillatory/non-directional shear stress whilst cells at the edge experience laminar/uni-directional shear stress. Cells grown under non-directional shear stress have cobblestone morphology whilst those under uni-directional shear stress are elongated and aligned (see Chapter 2: General Methods). Indeed, this replicates the types of shear stress that occur *in vivo*, which are typified in the aortic arch (Suo et al., 2007). In the lesser curvature of the arch, shear stress is non-directional, cells are cobblestone in appearance and prone to atherosclerotic lesions. In the outer curvature, endothelial cells are aligned and elongated and atheroprotected (Suo et al., 2007).

This kind of characterisation is highly relevant to the use of stem cell derived endothelial cells. Particularly in vessel and organ engineering since in the short term organs will need to be coated with endothelial cells that can align under shear stress and assume an atheroprotected phenotype. Notably, stem cell-derived cells have already been used in

vessel engineering. These include autologous bone marrow derived mononuclear cells being seeded on to biodegradable scaffolds and implanted in to patients requiring cavopulmonary connection grafts (Matsumura et al., 2006; Matsumura et al., 2003). At a median follow up of 16.7 months, no thrombosis or restenosis was reported. Whether the efficacy of these grafts is attributable to the ability of bone marrow derived cells to form endothelial cells, which subsequently align, or whether flanking vasculature coats the graft *in vivo* is unknown. In other studies, it has been shown that bone marrow derived cells can form endothelial cells and smooth muscles, and so it appears likely that there is some role of endothelial progenitor cells early after surgery. Patients at high risk of atherosclerosis however may require 'enhanced' endothelial cell functions and these may include grafts with pre-aligned stem cell derived endothelial cells. In the case of whole organ engineering, and with the fact that post-transplant atherosclerosis is a major problem, it is reasonable to speculate that endothelial cell alignment might be critical to success. Indeed, the importance of vascularisation of engineered tissues has been recognized (Chen et al., 2009), and is currently a limitation in the organ engineering field. Equally, in patients with profound endothelial cell dysfunction, including dysfunction in bone marrow-endothelial progenitor cell pathways, the ability of cells to align under shear stress might be compromised. In many ways therefore, assessment of the ability of stem cell derived endothelial cells to align under shear stress is important.

Human embryonic stem cell derived endothelial cells (hESC-EC) can now be derived using established protocols. These cells, as in other fields, have been used to study the development of the vascular system, and guided are understanding of adult progenitor stem cells. In this regard, hESC-EC are useful models of vasculogenesis and have applications in

therapy and *in vitro* assays for assessment of drugs effecting endothelial cells. These cells have been characterised as expressing CD31, VE-cadherin and being able to form vascular networks *in vivo*. One advantage of these cells has been the ability to grow these cells in large numbers as hESC have unlimited self-renewal capacity. In my PhD, I have shown that CD31+ hESC-EC, unlike endothelial cells from vessels; do not align under shear stress. However, these cells appear to already express an elongated phenotype under normal culture conditions. This elongated phenotype is typically associated with endothelial cells exposed to laminar/uni-directional shear stress, which are protected from atherosclerosis. It is conceivable therefore that these cells might already express a protected phenotype, which would be useful in organ engineering for the reasons already outlined. To assess this fully, an animal model of post-transplant atherosclerosis in which stem cell derived organs have been transplanted, would be needed, and this is currently not available. More knowledge on the fate and function of injected stem cell derived endothelial cells is also required and this was beyond the scope of my PhD. Conversely, the inability of hESC-EC to align under shear stress might raise some questions regarding their true endothelial cell phenotype. Again further investigation of how manipulation of the differentiation of hESC in to hESC-EC might affect this, whilst interesting, was beyond the scope of my PhD.

Others have also investigated the importance of eNOS expression and function in stem cell derived endothelial cells (Ward et al., 2011) and expression by hESC-EC has been illustrated. Since NO release by endothelial cells *in vitro* is low and typically requires highly sensitive assays employing mass spectrometry for NO to be detected (Palmer et al., 1987), characterisation of this pathway and making comparisons with endothelial cells from vessels is therefore more difficult but will be important to investigate in future studies in this field.

In my PhD however, I chose to focus on the pathways and functions described as these, at the time, can be measured in *in vitro* assays.

In terms of innate immunity, and as we have previously shown (Földes et al., 2010), hESC-EC also expressed some differences versus endothelial cells from vessels. As discussed in Chapter 1, endothelial cells are critical sensors of pathogens. PAMPs on or in pathogens are recognised by pattern recognition receptors, and in endothelial cells TLR4 and NOD1 are the best studied. HESC-EC, unlike endothelial cells from vessels did not respond to the TLR4 agonist LPS to release inflammatory cytokines that mediate immune reactions, and expressed lower levels of TLR4 than HUVEC. This again, could call to question to maturity and ‘endothelial-ness’ of these cells, certainly in the particular cell line studied. However, and as suggested in the summary of Chapter 3 and 4, a lack of TLR4 might afford hESC-EC some further protection from atherosclerosis. As discussed, TLR4 expression and activation is now centrally implicated in atherosclerosis (Edfeldt et al., 2002; Paul-Clark et al., 2012). In line with this, kidney transplants of patients with an Asp299Gly mutation in the TLR4 gene which confers reduced TLR4 function have been found to be less prone to post-transplant atherosclerosis. Whether this is a result of circulating cells expressing Asp299Gly TLR4 entering the graft or endothelial cells with Asp299Gly colonizing the graft is unknown. Indeed, TLR4 on endothelial cells has been directly linked with atherosclerosis and inhibition of TLR in endothelial cells can explain some of the atheroprotective effect of statins (Yang et al., 2010). However, a lack of TLR4 in any cell type could result in immune-compromise, and in the case of hESC-EC when used therapeutically, post-transplant infection and graft failure could occur. I therefore looked at another pattern recognition receptor expressed by endothelial cells, NOD1. NOD1, like TLR4, orchestrates responses to Gram-negative bacteria

as well as some Gram-positive bacteria. My group has previously described the role of NOD1 in endothelial cells from vessels (Gatheral et al., 2012). HESC-EC, like endothelial cells from vessels expressed NOD1, and responded to NOD1 agonists to release inflammatory cytokines. This raised the question as to whether hESC-EC were immune-compromised or able to sense Gram-negative bacteria through NOD1. To investigate this, I developed protocols with the support of Dr. Aurica Telcian and Dr. Mike Edwards in the Dept. of Respiratory Medicine, Imperial College London. In these protocols, hESC-EC responded to the Gram-negative bacteria *Haemophilus influenzae* to release the ubiquitous inflammatory cytokine CXCL8. As with other types of Gram-negative bacteria, *Haemophilus influenzae* is reportedly sensed by cells via TLR4 and/or NOD1 PRR pathways (Hirano et al., 2009; Ratner et al., 2007). Taking a pharmacological approach, using selective inhibitors of NOD1 and RIP2, and a molecular approach, using NOD1 targeting siRNA, I was able to demonstrate that this response was driven by NOD1 activation. Interestingly, knockdown of 50% of NOD1 mRNA was sufficient to result in an almost complete loss of function of NOD1 in terms of response to the NOD1 agonist, C12-iE-DAP and *Haemophilus influenzae*. This is likely to be due to the dynamic relationship between the agonists and the NOD1 receptor in terms of affinity and efficacy. It is conceivable that when an agonist binds its receptor this might not translate in to measurable responses and so loss of 50% of the binding sites (by loss of expression) results in apparently greater loss of function. In any case, these data clearly demonstrated that in TLR4-deficient hESC-EC, NOD1 was able to support inflammatory responses *in vitro* to *Haemophilus influenzae*.

It was also determined that hESC-EC do not release the endothelial cell hormone ET-1. In our group, we have since go on to show that the low ET-1 release from hESC-EC was

reflected by relatively low levels of gene expression when compared to HUVEC (Reed et al., 2014). ET-1 is typically released by endothelial cells from vessels under basal culture conditions, which *in vivo* is inhibited by interactions with other cells including vascular smooth muscle and fibroblasts (Stewart et al., 1990). High levels of ET-1 however are associated with cardiovascular disease. Thus, lack of ET-1 release under basal conditions might give hESC-EC a further therapeutic advantage. Conversely, low levels of prostacyclin release seen in hESC-EC, which is protective, could render these cells a potential adherent surface for platelets and leukocytes and result in graft failure. All of these features tested will be critical to success of stem cell derived endothelial cell therapy, and may represent bioassays to ensure safety and efficacy of stem cell therapies as they become established.

Responses to shear stress, TLR4 function and vasoactive hormone release also represent pathways that can be manipulated to improve therapeutic utility. As discussed, the idea of engineering cells *in vitro* to improve therapeutic utility is not new (Reed et al., 2012; Ward et al., 2011). It is conceivable, that the most useful therapeutic stem cell is one that is resistant to atherosclerosis and able to release high levels of protective hormones including prostacyclin. Assays to test the phenotype of these cells for individual patients will be equally critical to success.

Endothelial cells grown from adult progenitors, so called blood outgrowth endothelial cells (BOEC), did respond to shear stress. The ability of BOEC to align under shear stress has been shown previously as this is important for vessel engineering (Ensley et al., 2012). Nonetheless, given that the protocols to derive BOEC, and the definition thereof, are inconsistent across publications, it was important that I demonstrate this in BOEC grown for

my PhD. It has also been recognised that basic science studies on the biochemical and biomechanical signaling involved in endothelial cell alignment will be critical to our understanding of vascular disease (Glynn and Hinds, 2013). This is a research direction being pursued in the group and as BOEC can be derived from patients, these cells, when used in this shear stress model are an ideal tool to study endothelial cell alignment signaling in health and disease. It is conceivable, that BOEC derived from some patients have differences in the ability to align under shear stress and this will be important to recognise when considering use of autologous cells in vessel and organ engineering.

BOEC also responded to the TLR4 agonist LPS and the NOD1 agonist, C12-iE-DAP suggesting a fully mature innate immune phenotype comparable to endothelial cells from vessels. BOEC also released ET-1 and prostacyclin at levels at least comparable to endothelial cells from vessels, with a tendency for greater release. iPSC-derived endothelial cells (iPSC-EC) also responded to PAMPs and released prostacyclin and ET-1, although I was unable to test the ability of these cells to respond to shear stress. Nonetheless, my PhD represents the first comparative study of responses of different stem cell derived endothelial cells to in terms of these key endothelial cell hallmarks.

Indeed, iPSC-derived cells, like BOEC, can also be used to study patient cells and model 'disease in a dish'. iPSC-derived cells however have been through genetic manipulation that arguably takes them another degree of separation from the *in vivo* situation. Unlike adult progenitors, iPSC are also derived from somatic cells and are artificial. Using adult progenitor cells might be preferable to study vessel biology as these are derived from a cell type that exists putatively in vascular physiology. One major limitation of adult progenitor

cell studies and particularly for BOEC however, is that these cells are low in number in the circulation and are difficult to expand from blood. iPSC-EC however can be expanded in large numbers because of the highly pluripotent nature of iPSC *in vitro*. This has been addressed in the literature in a pioneering study by Geti et al. (Geti et al., 2012) wherein iPSC were derived from BOEC. This overcomes some of the limitation of BOEC but might also ensure that iPSC-EC retain representation of the vasculature. The requirement for genetic manipulation however remains an issue, and as discussed, transgene free approaches to engineering iPSC are being investigated. With this knowledge in mind, I choose to pursue BOEC as a tool to study endothelial cells and apply them to a pharmacological assay in which an endothelial cell interface is critical, and to phenotype a patient with a mutation in cPLA_{2α} which is important for endothelial cell function.

PART II: APPLICATION OF BLOOD OUTGROWTH ENDOTHELIAL CELLS IN PHARMACOLOGICAL BIOASSAYS

Testing the potential of biologics to cause cytokine storm responses

The case for the requirement of improved human tissue bioassays to detect cytokine release syndrome was emphasised by the unexpected and life threatening cytokine release caused by the CD28 superagonist, TGN1412 (Suntharalingam et al., 2006). This occurred despite TGN1412 passing the required preclinical safety tests. Importantly, these tests not only included the use of standard laboratory animals and primates but also human T-cells *in vitro* (Stebbings et al., 2007) which were the target cell for this therapy. It was therefore not clear how TGN1412 could appear innocuous in animal models, and target human cells, but precipitate such rapid and profound side effect in humans. In this way, TGN1412 defined an urgent unmet clinical need for improved human cell-based bioassays to detect cytokine storm in response to biological therapies.

We now know that TGN1412 does not activate PBMCs to release cytokines when antibodies are added in aqueous phase, unless there is an endothelial cell interface present (Findlay et al., 2011b; Stebbings et al., 2007). The interaction with endothelial cells and PBMCs likely contributes receptor interactions, co-stimulatory signals and immobilisation of the antibody, which in turn activates cytokine release. This can be mimicked when TGN1412 is immobilised by non-specific binding to a solid phase platform such as plastic (Findlay et al., 2010; Findlay et al., 2011b). Whilst, immobilising biologics to plastic has its advantages and provides a simpler solution than combining PBMCs with endothelial cells, it is well recognised that this represents a non-physiological system (Bugelski et al., 2009; Findlay et

al., 2011a). In fact, the extent to which a test antibody or other biologic undergoes 'immobilisation' *in vivo* might be a physiological pathway and a determining factor in whether a cytokine storm occurs and this may occur differentially in different patients.

Pioneering studies by Stebbings *et al.* were the first to illustrate that endothelial cells and PBMCs in co-culture detect cytokine storm responses to TGN1412 (Stebbing et al., 2007). It is now known that like immobilised antibody assays, endothelial cell co-culture assays also sense other cytokine storm causing drugs including Campath and OKT3 to release IL-2, IL-8, IL-6, TNF α and IFN γ . As our group (Bailey et al., 2012) and others (Wolf et al., 2012) have recently described, an alternative approach is to test therapeutic antibodies for cytokine storm responses using undiluted human whole blood. Human whole blood responds readily to TGN1412-like antibodies in a way that does not require, and is not enhanced by, co-culture with endothelial cells (Bailey et al., 2012). However, whole blood assays have recently been criticised (Thorpe et al., 2013a) are less sensitive (Bailey et al., 2012; Thorpe et al., 2013a), do not discriminate between mild and severe cytokine storm inducing therapeutics (Bailey et al., 2012) and thus do not adequately recapitulate the full extent of cytokine storm responses.

Accordingly, a fundamental requirement for any cytokine release assay is that it can delineate mild and severe cytokine storm causing drugs from non-cytokine storm causing drugs (Thorpe et al., 2013a). The extent to which *in vitro* cytokine release should correlate with absolute values detected in serum has also been debated widely (Thorpe et al., 2013a, b; Wolf et al., 2013).

Whilst endothelial cell co-culture assays, as well as other assays, have been used to study some of the potential mechanisms associated with TGN1412 induced cytokine storm, these assays have, to date, not been applied directly to study patient responses and patterns of cytokine release in patients. However, current endothelial cell:PBMC assays where human umbilical vein endothelial cells (HUVEC) are grown in co-culture with the PBMCs of another donor, inevitably rely on mixing of heterologous cells, and so, in fact, cannot be applied to personalised medicine. Furthermore, heterologous mixtures of cells may be prone to inflammatory activation, which may interfere with cytokine storm detection. Indeed, recent data from our group showed that, in a whole blood: HUVEC mixed tissue bioassay, there was an inflammatory activation caused by mixing of HUVEC and blood in at least 10% of blood donors (Bailey et al., 2012). It is also known, for example, that lymphocytes from different donors differentially regulate inflammatory pathways in co-culture with endothelial cells from human aortic endothelial cells (Morris et al., 1994). In line with this, and more recently, key opinion leaders in field have also suggested that the use of heterologous co-culture assays might be a potential limitation in endothelial cell: PBMC co-culture assays used to test for cytokine storm responses (Finco et al., 2014).

Clearly and logically, the best form of endothelial cell: PBMC bioassay, where mixed donor immune responses could be avoided, would be one that uses cells from the same donor. Such an assay could then be used to study patient responses to biologics and yield new knowledge on cytokine release syndrome and how to use and develop biologics optimally. PBMCs are easy to obtain from blood donated by volunteers, but adult endothelial cells on blood vessels are inaccessible and can only be obtained from tissue removed in surgery or post mortem.

With the advent of stem cell technology and its application in translational and personalised medicine research, I sought to apply endothelial cells derived from adult progenitors (BOEC) to an endothelial cell: PBMC co-culture bioassay. As BOEC are derived from PBMCs, taking this approach uniquely allows both cell types to be derived from the same donor.

I chose to apply BOEC in this assay not least because they have a phenotype consistent with endothelial cells from vessels but, as discussed, can also be derived from patients. It is fully conceivable that differences in phenotype or target expression on BOEC could result in variation in susceptibility to cytokine storm as well as efficacy of the biologic being tested. In support of this, we have recently shown that BOEC from patients with pulmonary arterial hypertension have an increased inflammatory response to interferons (George et al., 2013). Others have shown that disease phenotype is retained in BOEC from patients with COPD (Paschalaki et al., 2013). I have also shown in this thesis (Chapter 6) that BOEC can be used to phenotype patients with genetic mutations affecting cardiovascular pathways. Using autologous/same donor BOEC:PBMC co-culture assays in preclinical testing has its obvious advantages however, the ultimate use of this approach will be in personalised medicine where drugs can be tested for safety and efficacy in individual patients with target disease.

For autologous BOEC:PBMC co-cultures to be useful, I first sought to ensure that autologous BOEC:PBMC co-cultures respond avidly to more classical inflammatory insults. As discussed at length, endothelial cells and PBMCs respond to pathogen stimuli and cytokines to orchestrate inflammatory reactions.

On the whole, CXCL8 release stimulated from PBMCs was less than seen for BOEC in response to PAMPs and this is expected for these cell types. When cells were stimulated in co-culture CXCL8 release was essentially equivalent to the release from BOEC alone, except for experiments with LPS where there was a tendency to a greater than additive effect. This is consistent with what is known for endothelial cell interactions with monocytes where cells in co-culture are known to bi-directionally regulate responses to TLR4 ligands(Ward et al., 2009).

As discussed, to validate this same donor BOEC:PBMC co-culture assay as a cytokine storm assay the following criteria were tested:

- Ability to sense TGN1412 to release IL-8, IL-6, IL-2, IFN γ and TNF α .
- To delineate severe (TGN1412) and mild (Campath) cytokine storm causing drugs from non-cytokine storm causing drugs (Herceptin, Avastin and Arzerra).

Given restrictions on the availability of original TGN1412 material, in my initial validation experiments for this assay I used a TGN1412-like anti-CD28 superagonist (ANC28) which can be used to model TGN1412 responses (Waibler et al., 2008).

When CXCL8 was measured as a read out PBMC or BOEC cultures alone did not respond appreciably to any of the antibodies tested when treated as mono-cultures. Similarly co-cultures of PBMCs and BOEC incubated with Herceptin, Avastin or Arzerra did not release increased levels of CXCL8. However, co-cultures of same donor PBMCs and BOEC released increased levels of CXCL8 in a concentration dependent manner when stimulated with the TGN1412-like anti-CD28 superagonist, ANC28, or with Campath where ANC28>Campath. This correlates with the expected severity of cytokine storm for these drugs (Eastwood et

al., 2010; Suntharalingam et al., 2006). Although CXCL8 is key cytokine associated with cytokine storm (Suntharalingam et al., 2006), vascular inflammation (Sprague and Khalil, 2009) and sepsis (Gatheral et al., 2012), accordingly, in order to fully characterise this assay I measured a full range of cytokines. Using an MSD multi-plex platform I was able to show that in addition to CXCL8, other obligatory members of the 'cytokine storm' response, including, GM-CSF, IL-6, IL-1 β , IL-10, IFN γ and IL-2 were significantly increased in response to the TGN1412-like anti-CD28 superagonist, ANC28. In addition to these standard cytokines, I also measured release of 8 chemokines by cells stimulated with ANC28. Of the chemokines measured ANC28 stimulated release of eotaxin, MIP-1 β , TARC and IP10 by co-cultures of BOEC and PBMCs. Interestingly, for both IL-1 β and MIP-1 β release, PBMCs did not require an endothelial cell interface. With the exception of eotaxin and MIP-1 β , less cytokine release was detected in response to Campath than seen with ANC28. It is also important to note that the absolute levels of cytokines induced by ANC28 in this autologous co-culture system were relatively high and much greater than seen in whole blood assays (Bailey et al., 2012).

Given the number of cytokines and chemokines measured in the different assay conditions, I considered it important to analyse this data using an unbiased approach to visualize the data. Furthermore, for TGN1412, as for new biological drugs, it is critical to assess any generic as well as drug specific pattern of cytokine release and to analyse this in an unbiased manner in order to confirm safety and/or efficacy principal components analysis allows for complex data with multiple groups and variables to be transformed in to a coordinate system that reveals clustering of groups of data. Principal components analysis confirmed that for 17 cytokine/chemokine readouts in the ANC28 treated BOEC:PBMC co-cultures group clustered

distinct from control antibodies and Campath, which clustered away from control antibodies. The loadings plot indicated positive correlations between all cytokines and chemokines measured as a source of variation and clustering.

In separate experiments it seemed logical to consider whether mixed donor BOEC: PBMC co-cultures gave results similar to HUVEC: PBMC co-cultures which are mixed donor assay by definition, and compare these with same donor co-cultures. Heterologous (or mixed donor) BOEC: PBMC co-culture assays appeared to give more variable results similar to HUVEC: PBMC co-cultures. Indeed, the effect of mixing endothelial cells and PBMCs from the different donors has been reported previously to influence immune signaling (Morris et al., 1994). The reason for variation in HUVEC: PBMC co-cultures and whether other cell types are required for clearer responses has been investigated (Findlay et al., 2011b). It has not been considered until recently (Finco et al., 2014) and as hypothesized in my PhD whether this is due to mixing of donors. The ultimate use of this approach will be in personalised medicine where drugs can be tested for safety and efficacy in individual patients with target disease.

Using BOEC to phenotype patients: cPLA₂ function in endothelial cells

The study of BOEC in patients with cardiovascular disease and cancer represents an exciting area of research that, as with other stem cell research programs, with inevitably change how we treat disease and develop personalised medicines. In the later stage of my PhD, I applied BOEC to phenotype a patient with a rare disease driven by a mutation in the gene encoding cPLA_{2α}. This served not only to support the rationale that including patient specific endothelial cells in bioassays allows one to capture patient specific phenotypes, but also

address important questions relating to cPLA_{2α} and arachidonic acid pathways in endothelial cells.

It is generally accepted that cPLA₂ is the dominant isoform responsible for prostaglandin production. However, it has been suggested that iPLA₂ is important for prostacyclin production by endothelial cells on vessels (Rastogi and McHowat, 2009; Sharma et al., 2011; Sharma et al., 2010) and prostaglandin production in cancer (Sanchez and Moreno, 2002). Given the reported role of iPLA₂ in cancer, PLA₂ inhibitor drugs have been advocated as potentially beneficial in cancer (Sharma et al., 2010). Indeed, it will be important that the role of cPLA₂ and iPLA₂ in cardiovascular health and cancer be unpicked to ensure therapeutic targeting of certain tissues and minimal side effects to the cardiovascular system. Using BOEC derived from a patient lacking cPLA₂ function, I was able to show that these cells, unlike healthy BOEC, did not respond to thrombin to release prostacyclin. This suggests that the pathway activated by thrombin results in activation of cPLA₂ which in turn drives prostacyclin production. I was able to validate these findings using an inhibitor of cPLA₂, pyrophosphate, in both healthy BOEC and mouse thoracic aortic tissue in culture. Taken together, these data suggested that cPLA₂ and not iPLA₂ is the dominant isoform responsible for the production of prostacyclin in endothelial cells. This provides support to the idea that inhibition of iPLA₂ might be beneficial in cancer, and importantly suggests that such a strategy might not result in cardiovascular side effects. Drugs that targets cPLA₂ however, may result in cardiovascular toxicity and this will require careful consideration. Further to this, these data provide a proof of concept that BOEC can be grown from patients and used to capture differences in phenotype versus healthy cells, or other patient cells.

Summary and future directions for stem cell derived endothelial cells

Stem cell derived endothelial cells have tremendous potential in cardiovascular research. As advocated throughout my thesis, the characterisation of any stem cell derived cell as an authentic phenotype will be essential to ensure safety of therapy, and usefulness in assays. Such characterisation can also reveal important differences in key pathways that might advance our understanding of disease. In my thesis, hESC-EC were, like endothelial cells from vessels, found to be innate immune replete. hESC-EC however express lower levels of TLR4 compared to endothelial cells from vessels and do not respond to the TLR4 agonist, LPS. Given the role of TLR4 in cardiovascular disease this might afford these cells some protection from inflammation when used therapeutically. Indeed, cells engineered to be 'TLR4-deficient' 'aligned' 'protective hormone releasing' endothelial cells may one day be a possibility and useful for therapy. Future research along these lines could also lead to the discovery of additional pathways that could be engineered *in vitro* to enhance the therapeutic application of stem cells.

Stem cell derived endothelial cells when studied *in vitro* will also improve our understanding of endothelial cells in health and disease and enable new drug targets to be identified. This is true for all avenues of stem cell research. In particular, I have applied BOEC to a pharmacological bioassay to detect cytokine storm responses to biological drugs. This bringing together of stem cell technology to guide biological therapy is a powerful example of the applications of stem cell derived endothelial cells *in vitro*. I have also demonstrated that BOEC can be used to phenotype patients with vascular disease, thus providing a proof of concept of how BOEC could be used to define new drugs targets and used in personalised

medicine. The application of stem cells in translational research and their clinical application will inevitably continue to change how we study and treat human disease.

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APPENDIX I: Extended information on n-numbers in each figure

Figure 3.7:

A:

hESC-EC; n=8 from 4 experiments in duplicate
BOEC; n=8 from 4 experiments in duplicate
iPS-EC; n=2 from 1 pilot experiment in duplicate
HUVEC; n=8 from 4 experiments in duplicate
HMVEC; n=6 from 2 experiments in duplicate

B:

hESC-EC; n=6 from 3 experiments in duplicate
HUVEC; n=6 from 3 experiments in duplicate
BOEC; n=1 from 1 pilot experiment in singlicate

Figure 3.9

hESC-EC; n=8 from 7 experiments; 1 in duplicate and 6 in singlicate
BOEC; n=8 from 6 experiments; 4 in duplicate and 2 in singlicate
iPSC-EC; n=2 from one pilot experiment
HUVEC; n=6 from 6 experiments in singlicate
HAEC; n=3 from 1 experiment in singlicate
HLF; n=2 from 1 experiment in duplicate

Figure 3.10

hESC-EC; n=5 from 4 experiments; 1 in duplicate and 3 in singlicate
BOEC; n=9 from 4 experiments; 4 in duplicate and 1 in singlicate
iPSC-EC; n=2 from one pilot experiment in duplicate
HMVEC; n=5 from 3 experiments; 2 in duplicate and 1 in singlicate

Table 3.1

hESC-EC; n=8-10 from 5 experiments in duplicate; PolyIC was run in singlicate in two experiments
BOEC; n=14 from 7 experiments in duplicate
HMVEC; n=4 from 2 experiments in duplicate
iPSC-EC; n=2 from one experiment in duplicate

Table 3.2

hESC-EC; n=6 from 3 experiments in duplicate
BOEC; n=6 from 3 experiments in duplicate
iPSC-EC; n=2 from one experiment in duplicate
HMVEC; from one experiment in duplicate

Table 3.3

hESC-EC; n=6 from 3 experiments in duplicate

HUVEC; n=6 from 3 experiments in duplicate
BOEC; n=6 from 3 experiments in duplicate
iPSC-EC; n=2 from 1 experiment in duplicate

Figure 4.1 – 4.9

hESC-EC; n= 3 from 3 experiments in duplicate. Due to expense of the MSD platform, duplicate wells were combined by mixing and added as singlicate samples to the assay
HUVEC; n= 3 from 3 experiments in duplicate. Due to expense of the MSD platform, duplicate wells were combined by mixing and added as singlicate samples to the assay

Figure 4.10

hESC-EC TLR4 and TLR4; n=3 from 3 experiments in singlicate
HUVEC TLR4 and TLR4; n=3 from 3 experiments in singlicate
hESC-EC TLR4 and NOD1; n=4 from 4 experiments in singlicate
HUVEC TLR4 and NOD1; n=3 from 3 experiments in singlicate

Figure 4.12

A: n=6 from 3 experiments in duplicate
B: n=7-8; for media and HIN; n= 8 from 2 experiments in triplicate and 1 in duplicate. For C12-iE-DAP n=7 from 2 experiments in duplicate and 1 in triplicate.

Figure 4.13

Media and 0.1ng/ml; n= 8 from 2 experiments in triplicate and 1 in duplicate. 0.01ng/ml; n=6 from 2 experiments in duplicate

Figure 4.14

N=4 from 2 experiments in duplicate

Figure 4.15

N=4 from 2 experiments in duplicate

Table 4.1

N=3 from experiment which had a CXCL8 profile consistent with that of pooled data. This suggests the effects of the agonists tested were not due to cell death

Table 4.2

SB203580 and BIRB0796; n=4 from 4 experiments in singlicate.
SC-514; n=3 from 3 experiments in singlicate

Table 4.3

N=3 from one pilot experiment in triplicate

Figure 5.1

A: n=5 from 5 experiments in singlicate
B: n=3 from 3 experiments in singlicate

Figure 5.2

A: n=8 from 8 experiments in singlicate

B: n=3 from 3 experiments in singlicate

Figure 5.3

N=3 from one pilot experiment in triplicate

Figure 5.4 – 5.11

N=5 from 5 experiments in singlicate

Figure 5.12 – 5.13

N=3 from 3 experiments in singlicate

Table 5.1

N=6 from 6 experiments in singlicate

Figure 6.3

Control (healthy cells); n=2-4 from 2-4 experiments in singlicate

Patient cells; n=1 from 1 pilot experiment in singlicate

Figure 6.4

Healthy; n=3 from 3 experiments in singlicate

Patient; n=1 from 1 pilot experiment in singlicate

Figure 6.6 – 6.7

N=4 from 4 experiments in singlicate

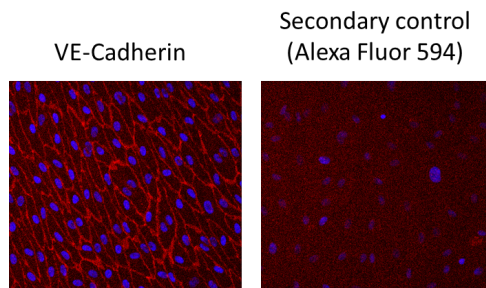
Figure 6.8

N=3 from 3 experiments in singlicate

Figure 6.9

N=6 from 6 experiments in 6 mouse vessels incubated in singlicate

APPENDIX II: Supplementary figure for secondary control staining for VE-cadherin



Supplementary Figure 1. Secondary control antibody (Alexa Fluor 594) staining for VE-cadherin. Left; blood outgrowth endothelial cells (BOEC) were incubated with primary antibodies for VE-cadherin followed by incubation with secondary control antibodies (Alexa Fluor 594; red). Right; BOEC were incubated with vehicle only without primary antibody (3% BSA/PBS) followed by incubation with secondary control antibody (Alexa Fluor 594). Images were captured using a Cellomics VTi Arrayscan at 10x magnification.