Characterisation of a novel endogenous anti-inflammatory activity from endothelial cells and its translational application in pathology

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A thesis submitted for the degree of Doctor of Philosophy

University College London

I, Laura Paneghetti confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Acknowledgements

I am grateful to Dr. Eric Ng for giving me the opportunity to work on this challenging but exciting project, for giving me the space to find my own way through the experiments and for pushing me to give my best and to always look at the positive side of things. I also thank prof. Dave Shima for his support and encouragement throughout my PhD.

I would like to thank Anne Goodwin for editing my thesis and for her useful and precious comments.

I thank my present and past colleagues in the TVR lab and the Institute of Ophthalmology, especially Asma, Meihua, Raúl and Margaret, for their immense patience and constant support, and for the chats and the good times.

A special “thank you” goes to my English relatives and Italian friends in London, who made my PhD experience less painful and more fun.

I am deeply thankful to my parents, my brother and Paul, who have always supported me and always will. I could not have done this without them.

Abstract

Endothelial injury often causes intimal hyperplasia, a disease characterised by local inflammation and critical narrowing or restenosis of the blood vessel. Endothelial cells (EC) grown on collagen particles are highly effective in inhibiting intimal hyperplasia in various animal models, and this effect appears to be, at least in part, the result of EC-derived soluble factors that suppress local vascular inflammation. To test this hypothesis, we produced EC on collagen particles-conditioned medium (ECPCM), which was expected to contain soluble anti-inflammatory factors. Indeed, EC treated *in vitro* with ECPCM together with pro-inflammatory cytokines including tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6) displayed reduced gene expression of the inflammation-related adhesion molecules E-selectin and VCAM-1. Investigation of the molecular mechanism of action for the anti-inflammatory activity excluded mRNA stability of E-selectin and VCAM-1, activation of signalling cascades via the NF-kB and Stat3 pathways, and nuclear localization of transcription factors. ECPCM did affect the TNFα-induced binding of p65, a subunit of the NF-kB transcription factor, to the E-selectin and VCAM-1 promoters. These results suggest that inhibition of gene transcription is responsible for the ECPCM-mediated suppression of inflammatory responses in EC. The therapeutic effects of ECPCM were supported by *in vivo* experiments performed on the mutant mouse strain JR5558, which develops spontaneous choroidal neovascularization (CNV) lesions associated with inflammatory cell recruitment and expression of inflammatory adhesion molecules. The CNV lesion area and recruitment of activated macrophages were both decreased in JR5558 mice given intraperitoneal injections of ECPCM. ECPCM might therefore have therapeutic potential in treating inflammatory vascular diseases.

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293PCM	HEK293 on particles-conditioned medium
AMD	age-related macular degeneration
AP1	activator protein 1
ATF	activating transcription factor
bFGF	basic fibroblast growth factor
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic AMP
ChIP	chromatin immunoprecipitation
CNV	choroidal neovascularization
ConA	concavalin A
COX	cyclooxygenase
DAPI	4',6-diamidino-2-phenylindole
DES	drug-eluting stents
DEX	dexamethasone
DMSO	dimethyl sulfoxide
EBM	endothelial basal medium
EC	endothelial cells
EC/matrix	EC engrafted in a collagen matrix sponge
ECM	extracellular matrix
ECPCM	EC on particles-conditioned medium
EGF	epidermal growth factor
EGM2	endothelial growth medium 2
ELISA	enzyme-linked immunosorbent assay
eNOS	constitutively active NO synthase
EPC	endothelial progenitor cell
FA	fluorescein angiography
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate

GPCR	G protein-coupled receptors
GR	glucocorticoid receptor
HAEC	human aortic endothelial cells
HEK293	human embryonic kidney 293 cells
HMG I	high mobility group protein I
HSPG	heparan sulfate proteoglycans
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobulin
IH	intimal hyperplasia
IKK	inhibitor of NF-kB kinase
IL	interleukin
IL-6R	IL-6 receptor α
iNOS	inducible NO synthase
IP	intra-peritoneal
IRF-1	interferon regulatory factor-1
IVT	intra-vitreous
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
miRNA	micro RNA
MMP	matrix metalloproteinases
NF-kB	nuclear factor-kB
NO	nitric oxide
P	postnatal day
PBS	phosphate-buffered saline
PCM	particle-conditioned medium
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PF4	platelet factor 4
PG	prostaglandin

PI3K	phosphatidylinositol 3-kinase
PMN	polymorphonuclear leukocytes
PTA	percutaneous transluminal angioplasty
RIP1	receptor-interacting protein 1
ROS	reactive oxygen species
RPE	retinal pigmented epithelium
RvD	resolvin D
RvE	resolvin E
SAP	serum amyloid P
SMC	smooth muscle cells
SMCPCM	SMC on particles-conditioned medium
ssRNA	single strand RNA
Stat3	signal transducer and activator of transcription 3
TBS	Tris-buffered saline
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of MMPs
TLR2	Toll-like receptor 2
TNFR	TNF receptor
TNFα	tumor necrosis factor-α
TRADD	TNFR-associated death domain protein
TRAF2	TNFR-associated factor 2
TX	thromboxane
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cells
vWF	von Willebrand factor
WPB	Weibel-Palade body

Chapter 1: Introduction

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Intimal hyperplasia is a state of abnormal vascular remodelling that represents a major issue in vascular surgery and biology. It is a complication that follows many vascular interventions and eventually can cause the failure of transplanted organs, venous and prosthetic bypass grafts, angioplasty and surgical repair (Nugent HM 1999; Newby AC 2000; Collins MJ 2012). This PhD project was inspired by the discovery of a novel and effective endothelial cell (EC)-based therapy for intimal hyperplasia, in which the collagen matrix Vascugel® is used as a support for EC culture. The major goal of this thesis is to identify and understand the mechanism of action of the molecules secreted by Vascugel®-associated EC.

This thesis presents novel findings about the anti-inflammatory activity of an EC-conditioned medium, which is partly responsible for the therapeutic effect of Vascugel®-associated EC on intimal hyperplasia. The studies described here provide evidence for the potential therapeutic application of this anti-inflammatory activity for treating inflammation-driven vascular pathologies such as choroidal neovascularisation (CNV) in the eye. The following introduction provides information on how EC control vascular physiology and homeostasis, and how they behave during and modulate inflammation. Since intimal hyperplasia is a pathology in which inflammation has a major role, a description of intimal hyperplasia and its current treatment are provided. Next, a short description of the anatomy of the eye and the pathophysiology of CNV is given in order to introduce the animal model chosen for functional testing and *in vivo* experiments performed with the EC-conditioned medium. Lastly, details about cytokines, chemokines and adhesion

molecules involved in inflammation in EC are presented, since these molecules were analysed extensively to study the effects and mechanism of action of the EC-derived conditioned medium.

1.1 General structure of blood vessels

Arteries and veins are composed of three layers: the intima, media and adventitia (figure 1) (Davies MG 1993; Mitra AK 2006). The outermost layer is known as the adventitia and is composed of fibroblasts in a loose fibrous connective tissue that also contains small blood vessels (*vaso vasorum*), nerves and adipose tissue. Internal to this layer is the tunica media, which is made up of densely packed smooth muscle cells (SMC) and elastic tissue (Mitra AK 2006). This thesis focuses on the function of the innermost layer, the intima. This layer is in direct contact with the blood flow, and is comprised of a continuous monolayer of flat EC packed closely together and seated on a specialized and highly organized extracellular matrix (ECM), the subendothelium (Davies MG 1993; Newby AC 2000; Mitra AK 2006). The subendothelial layer provides structural integrity, mechanical strength and elasticity to the vessels and is composed of type IV collagen, laminin, elastin, fibronectin, thrombospondin, von Willebrand factor (vWF) and heparin sulphate proteoglycans (HSPG) such as perlecan and syndecan (Davies MG 1993; Timpl R 1996; Mitra AK 2006). In the artery, the media and adventitia layers are markedly thicker than those found in veins (figure 1) (Mitra AK 2006).

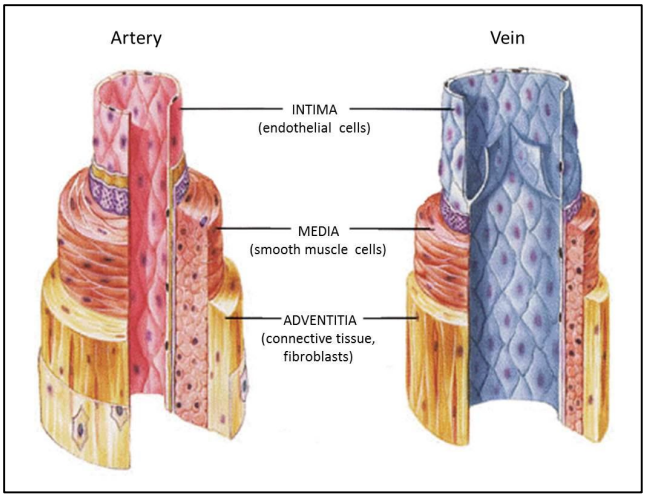

Figure 1: Structure of the vessel wall (adapted from *Human Physiology*, Fox Stuart, 4th ed., Brown Publishers)

1.2 Regulation of vascular homeostasis by the endothelium

The blood vessel is an extremely sophisticated structure in which the endothelial monolayer of the intima not only provides a structural integrity to the blood vessel by forming a continuous, selectively permeable barrier between the blood and the vessel wall (Rubanyi GM 1993), but also regulates vascular physiology by producing a variety of factors and metabolites that affect the underlying SMC and circulating blood elements. In this way, EC control thrombosis, coagulation, vasomotor tone, blood flow and cell growth. They also function as a mechanical sensor and responder, and modulate inflammatory and immunological responses to regulate vascular homeostasis.

Thrombosis

The basic barrier function of the endothelium exerts an important anti-thrombotic and anti-coagulation activity simply by separating platelets and coagulation factors present in the blood from pro-aggregating constituents of the subendothelial layer such as collagen and vWF. Under basal conditions the endothelium also maintains this non-thrombogenic blood-tissue interface by producing and releasing a variety of molecules. These anti-thrombogenic molecules, which include prostacyclin (also called prostaglandin I_2, PGI_2) and nitric oxide (NO), inhibit platelet aggregation and cause vasodilation (Davies MG 1993; Sumpio BE 2002). The reduction of reactive oxygen species (ROS) in EC also contributes to the non-thrombogenic status of the endothelium. Moreover, ectonucleotidases present on the endothelial surface can metabolize ADP to adenosine, which in turn increases cyclic AMP (cAMP) levels in platelets and inhibits their reactivity (Davies MG 1993). The endothelial surface

regulates fibrinolysis by binding the native form of plasminogen (glu-plasminogen) and converting it to its proteolytic derivative (lys-plasminogen), which is more easily activated into plasmin, the enzyme responsible for degrading fibrin clots (Gong Y 2001). In addition, EC synthesize plasminogen activators and inhibitors (Davies MG 1993). Finally, HSPG expressed on the EC surface localise and increase the activity of anti-thrombotic factors such as anti-thrombin III and lipoprotein-associated coagulation inhibitor (Davies MG 1993; Pober JS 2007).

Coagulation

In normal situations, the endothelium releases the inhibitors of the tissue factor pathway (TFPI), which blocks the initiation of coagulation (Pober JS 2007); it also releases activated protein C and protein S, which inactivate various components of the clotting cascade (Pober JS 2007). However, in response to injury, the endothelium can initiate coagulation rapidly by producing pro-coagulation co-factors such as high molecular weight kininogen (HMWK), factor V, factor VIII, tissue factor and vWF (Davies MG 1993; Sumpio BE 2002).

Vascular tone and blood pressure

Over the past two decades many vasoactive factors produced by EC have been discovered and characterised. These factors can be categorised in five families: prostanoids, NO and NO-containing compounds, ROS, endothelins and angiotensins (Davies MG 1993; Luscher TF 2000; Sumpio BE 2002; Campbell WB 2007; Pober JS 2007; Vignon-Zellweger N 2012).

Prostanoids are a class of eicosanoids produced during the metabolism of arachidonic

acid. In EC cyclooxygenase-1 and -2 (COX-1 and -2) convert arachidonic acid into prostaglandin (PG) D_2, E_2, $F_{2\alpha}$ and I_2 and thromboxane (TX) A_2 (Sumpio BE 2002; Campbell WB 2007). PGE_2 is a pyretic agent and vasodilator involved, together with PGD_2, in acute inflammation resolution (Sumpio BE 2002). PGI_2 is a potent vasodilator, inhibitor of platelet aggregation and pro-fibrinolytic agent (Davies MG 1993; Sumpio BE 2002; Pober JS 2007). Its effects are mediated by an increase of cAMP in SMC and platelets (Davies MG 1993; Campbell WB 2007). In contrast, TXA_2 and $PGF_{2\alpha}$ cause vasoconstriction and platelet aggregation (Davies MG 1993).

In EC NO is synthesized from the conversion of L-arginine to L-citrullin by two enzymes: a constitutively active NO synthase (eNOS) and an inducible NO synthase (iNOS), both of which are calcium- and calmodulin-dependent (Davies MG 1993). NO is a potent vasodilator (Davies MG 1993; Sumpio BE 2002; Pober JS 2007). It acts by activating guanylate cyclase in SMC, producing an increase in cyclic GMP (cGMP) that, in turn, stimulates protein phosphorylation and ultimately relaxation (Davies MG 1993).

Free radicals in the endothelium derive from the activity of xanthine oxidase and from direct transfer from the extracellular space. ROS are reduced by superoxide dismutase, catalase and the glutathione redox cycle in EC. Low ROS levels stimulate vasodilation by enhancing COX-1 and -2 activity and prostacyclin formation. High levels of ROS inactivate NO and inhibit prostacyclin production. They can also destroy the enzymes responsible for prostacyclin synthesis, while having no effect on those synthetizing TXA_2. The end result of these interactions is vasoconstriction (Davies MG 1993).

Endothelins are a small family of peptides (ET-1 to -4) in which ET-1 is the predominant isoform. ET-1 acts through the activation of G_i-protein-coupled receptors ETA and ETB (Luscher TF 2000). ETA receptors, found mainly on SMC (Vignon-Zellweger N 2012), mediate vasoconstriction, whereas ETB receptors, present in EC as well as in SMC (Vignon-Zellweger N 2012), stimulate the release of NO and prostacyclin, thereby mediating vasodilation (Luscher TF 2000).

The renin-angiotensin system in the vessel wall is important in controlling blood pressure (Davies MG 1993; Sumpio BE 2002). Angiotensin I synthesised by EC stimulates prostaglandin release and therefore vasodilation. On the other hand, angiotensin II, produced from angiotensin I by angiotensin-converting enzyme, acts as a potent vasoconstrictor.

Cell growth

The endothelium produces a variety of factors that affect both EC and SMC growth. Platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), transforming growth factor-α (TGF-α), oxygen free radicals, endothelins and angiotensin II promote cell growth, whereas TGF-β, NO and PGI_2 act as cell growth inhibitors together with various ECM molecules such as collagen (type V), glycosaminogycans and glycoproteins (Davies MG 1993). Endothelial cells also produce granulocyte- and granulocyte-macrophage colony stimulating factors (G-CSF and GM-CSF) which affect phenotype, proliferation and migration of macrophages, granulocytes, SMC and EC themselves (Bobik A 1993).

Mechanical sensing and response

The endothelium can transduce the physical forces produced by blood flow into biochemical signals that affect the vessel wall, therefore acting as a mechanosensor (Davies PF 1993). Rapid responses are mediated by changes in G-protein activity, adenylate cyclase activity and NO production. Slower responses are mediated by mitogen-activated protein kinase (MAPK) phosphorylation and subsequent changes in the gene expression of growth factors, vasoactive peptides and enzymes (Davies MG 1993).

1.3 The endothelium in inflammation

Due to its strategic position, the endothelium plays a key role in immune and inflammatory reactions by regulating leukocyte adhesion, activation and migration into the tissue (Davies MG 1993; Sumpio BE 2002). EC are usually in a quiescent, anti-coagulant and anti-thrombogenic state during which they do not interact with leukocytes (Davies MG 1993; Pober JS 2007). This is because chemokines and leukocyte-interacting proteins (such as P-selectin, interleukin-8 and eotaxin-3 in the Weibel Palade body (WPB) vesicles) are sequestered inside the cell and transcription of leukocyte adhesion molecules is suppressed (Øynebråten I 2004; Rondaij MG 2006; Pober JS 2007). The endothelium can be readily activated by different stimuli, including cytokines, thrombin, histamine and physical injury. Endothelial activation is characterised by a switch in the synthetic profile towards a pro-thrombogenic, pro-proliferative, pro-inflammatory and vasoconstrictive state (Davies MG 1993; Pober JS 2007). Once activated, EC express different classes of adhesion molecules that mediate the increased interaction with leukocytes: selectins, such as E-selectin, which bind to

carbohydrate determinants on leukocytes to facilitate rolling; and members of the immunoglobulin superfamily such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which bind to integrins to mediate firm adhesion and transendothelial migration of leukocytes (Davies MG 1993; Carlos TM 1994). Among the adhesion molecules expressed by the activated endothelium, E-selectin and VCAM-1 are of particular interest to this thesis and will be discussed in more detail later in this chapter. Activated EC also produce platelet activating factor, which enhances expression of the adhesion molecules and cooperates with P-selectin to amplify platelet and neutrophil aggregation at the endothelium (Sumpio BE 2002). Additionally, stimulation of the endothelium leads to a rearrangement of the cell-cell adhesion and tight junction proteins, which results in the opening of gaps between adjacent EC and subsequent extravasation of neutrophils and leukocytes (Pober JS 2007). The endothelium exerts further control of the immune and inflammatory response by producing and releasing cytokines such as interleukin (IL)-1, -6, -8 and other inflammatory mediators (Davies MG 1993; Sumpio BE 2002).

Acute inflammation

Acute inflammation is a rapid response to infectious microbes or injured tissues that involves local recruitment and activation of neutrophils. This recruitment depends on the activation of EC, a process that can be divided into rapid responses that do not require new gene expression (type I activation) and slower responses that depend on new gene expression (type II activation) (Pober JS 2007).

Type I activation is typically mediated by ligands (e.g. histamine) that bind to G protein-coupled receptors (GPCRs), which signal through the intracellular G-protein

α_q subunit. This subunit mediates the release of inositol-1, 4, 5- triphosphate ($InsP_3$), which then stimulates the release of Ca^{2+} from endoplasmic reticulum stores. The increase in Ca^{2+} activates phospholipase A_2 (PLA_2), an enzyme responsible for the production of arachidonic acid, the fatty acid that is converted by COX-1 and prostacyclin synthase into PGI_2, a potent vasodilator (see above). Cytosolic Ca^{2+} also forms a complex with calmodulin, activating NOS to produce NO, which synergises with PGI_2. This Ca^{2+}-calmodulin complex mediates the contraction of actin filaments through the activation of myosin light-chain kinase (MLCK) and subsequent phosphorylation of myosin light chain. Actin filaments are attached to the tight and adherens junction proteins, and their contraction results in the opening of gaps between EC and subsequent leakiness of the vessel. Moreover, intracellular Ca^{2+} is responsible for the exocytosis of WPBs, exposing P-selectin to the luminal surface for adhesion of neutrophils (Pober JS 2007).

Signalling through GPCRs lasts for 10-20 minutes, after which the receptors become de-sensitised, preventing further stimulation (Gainetdinov RR 2004). A more persistent EC activation, namely type II activation, is required for a more sustained inflammatory response. Type II activation is typically mediated by cytokines such as tumor necrosis factor-α (TNFα) (which will be described in more detail later) and IL-1. The end result of the action of these cytokines on EC is the activation of the transcription factors activator protein 1 (AP1) and nuclear factor-kB (NF-kB). These transcription factors mediate the expression of various genes important in the inflammatory process, including E-selectin, VCAM-1, intercellular adhesion molecule-1 (ICAM-1), COX-2 and various chemokines. Since the expression of adherence proteins on EC is enhanced, leukocyte recruitment is much more effective

during type II activation.

Chronic inflammation

If acute inflammatory reactions fail to eradicate the initiating stimulus, the inflammatory process will evolve into a chronic and more specialized form. EC might contribute to the process by acting as antigen presenting cells for the circulating T cells; however, this hypothesis is still controversial (Pober JS 2007). Other changes within the endothelium, such as cytokine production and adhesion molecule expression, seem to depend on the prevailing T helper cells (type 1 or 2) mediating the immunological response (Pober JS 2007).

1.4 Inflammation and intimal hyperplasia

The description of the numerous functions of EC highlights the pivotal role of the endothelium in regulating the homeostasis and biochemical potential of blood vessels. It is not surprising, therefore, that uncontrolled EC response and discordant stimulation of EC are common events in many pathological processes, including intimal hyperplasia, atherosclerosis, allograft vasculopathies, hypertension, congestive heart failure, primary pulmonary hypertension, sepsis and inflammatory syndromes (Nugent HM 1999; Sumpio BE 2002). This thesis focuses on characterizing a novel, secreted, anti-inflammatory activity of EC as a potential treatment for pathologies driven by vascular inflammation, including intimal hyperplasia and choroidal neovascularization associated with aged-related macular degeneration.

Intimal hyperplasia (IH) strictly signifies an increase in the number of cells in the intima. It is a distinctive state of vascular remodelling in which vascular smooth muscle cells (VSMC) proliferate and migrate to the intima, accompanied by an increase in the amount of ECM (Newby AC 2000; Mills B 2012). For this reason the phenomenon is generally referred to as “intimal thickening” (Newby AC 2000) and results in the gradual diminution of the vessel lumen diameter and flow and, ultimately, in occlusion (Collins MJ 2012; Mills B 2012).

Physiological intimal hyperplasia occurs during closure of the *ductus arteriosus* after birth and during involution of the uterus (Slomp J 1992). It is also observed in the aging human aorta (Orekhov AN 1984). The most common pathological process causing intimal hyperplasia is atherosclerosis, which is characterized by an expansion of the VSMC population in the intima, together with an infiltration of inflammatory cells such as monocytes, T-cells and other leukocytes from the plasma (Newby AC 2000). Pathological intimal hyperplasia also occurs in hypertensive pulmonary arteries, homograft transplanted organs, venous and prosthetic bypass grafts and after injury of the luminal surface of arteries during percutaneous transluminal angioplasty (PTA), stent placement or surgical repair (Nugent HM 1999; Newby AC 2000; Collins MJ 2012).

The origin of neo-intimal SMC observed in pathological IH conditions is still unclear, although several theories have been proposed. One model is that of the phenotype switch (Newby AC 2000; Collins MJ 2012). According to this model fully differentiated medial SMC of adult vessels are normally in a “contractile” phenotype, which shows low rates of proliferation or death. During pathological processes EC,

VSMC, macrophages and platelets produce and release the growth factors PDGF, EGF and bFGF and cytokines such as IL-6 and IL-8 that cause a change in the gene expression profile in VSMC. This leads to the "synthetic" dedifferentiated phenotype, characterized by a high proliferation rate, increased cell migration and increased synthesis of cytoskeletal and contractile proteins (Newby AC 2000; Collins MJ 2012). Another scenario for the origin of SMC in IH is that fibroblasts residing in the adventitia transform into myofibroblasts that migrate to the media to become SMC and then move within the intima, contributing to IH (Roy-Chaudhury P 2009; Havelka GE 2011). It has also been proposed that circulating bone marrow-derived progenitor cells may be incorporated into the affected vessel wall and differentiate into EC, myofibroblasts or synthetic SMC (Sata M 2002).

Though the key characteristic of intimal hyperplasia is the proliferation of VSMC and their accumulation in the tunica intima, it is important to highlight the fact that the loss of normal EC function and inflammatory reactions are mainly responsible for triggering and supporting these events. Pathological IH is generally caused by three key stimuli: inflammation, injury, and increased mean wall stress (figure 2) (Newby AC 2000).

Inflammation is a common and complicated feature of most vascular injury models. In response to inflammation platelets are activated and start releasing PDGF, which in turn activates EC. These PDGF-activated EC together with macrophages produce cytokines, growth factors and MMP that trigger SMC activation, migration and proliferation from the media (Newby AC 2000; Collins MJ 2012). As inflammation is an important component of many vascular diseases and a response to physical

injury to the vessels, it is considered the key stimulus responsible for development of intimal hyperplasia in most clinical cases.

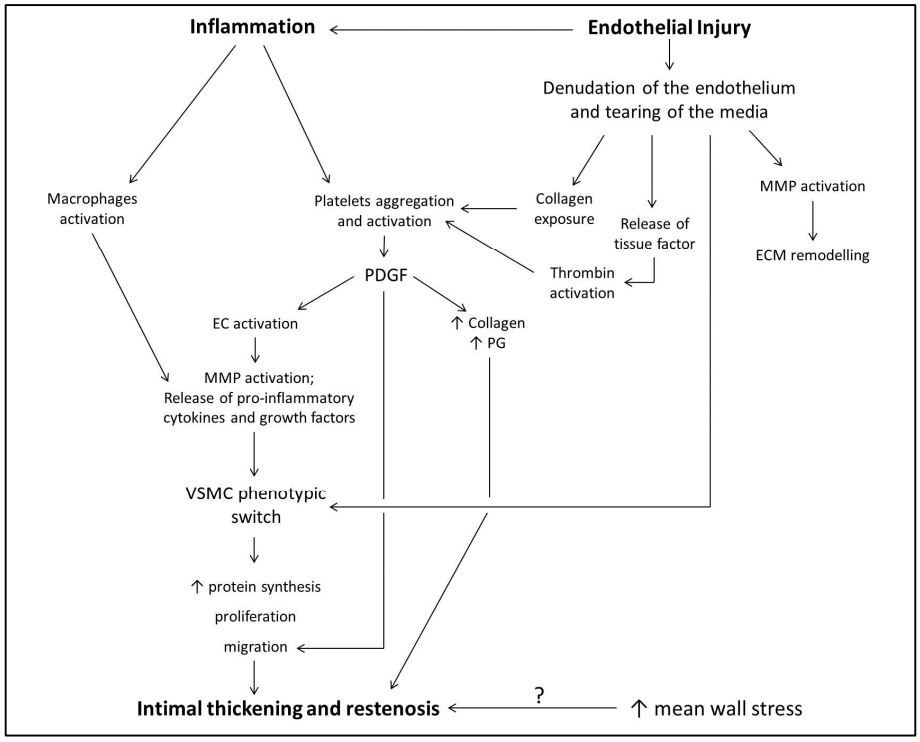

Figure 2: Schematic representation of the molecular mechanisms underlying intimal hyperplasia development.

Injury can be restricted to the endothelium or stretched to the media. A typical example of endothelial injury is observed when percutaneous transluminal angioplasty (PTA) is performed. This technique is used to mechanically widen a narrowed or obstructed blood vessel, usually an atherosclerotic coronary artery. A balloon catheter, namely a collapsed balloon on a guide wire, is placed into the narrowed vessel and inflated to a fixed size, so to open up the vessel and improve the blood flow. The balloon is then deflated and withdrawn. In some cases a mesh tube (stent) is inserted to ensure the vessel remains open. It has been shown that this procedure cause complete endothelial denudation of the treated area and a tear that in 50% of cases extends through the internal elastic lamina and into the media to a

variable depth (Steele PM 1985). Endothelial denudation and/or medial tearing activate heparanases and other proteases that destroy and loosen the ECM around the SMC in the tunica media. Injury causes the contractile-to-synthetic phenotypic switch in SMC, which leads to a change in intracellular proteins and to altered regulation of several ECM molecules (Newby AC 2000; Collins MJ 2012). Moreover, endothelial injury exposes collagen, which causes platelet adhesion, activation and aggregation. Activation of tissue factor also occurs, leading to thrombin production. Thrombin further activates platelets, which produce PDGF that acts as chemoattractant, causing SMC from the media to migrate towards the intima and proliferate there. Matrix metalloproteinases (MMP) facilitate the process by remodelling the ECM. PDGF also causes an increase in collagen and proteoglycan (PG) synthesis. Other growth factors are probably also involved in the process (Newby AC 2000).

The role of increased mean wall stress in the development of intimal hyperplasia is supported by the fact that pulmonary hypertension and vein grafting show a close relationship between intimal hyperplasia and wall stress (Newby AC 2000; Havelka GE 2011). However, the exact mediators and mechanisms involved in the process are still not clear.

1.5 Current treatment of intimal hyperplasia

Endothelial injury typically occurs after PTA and in approximately 30-50% of the treated vessels it activates a process of IH that leads to critical narrowing (restenosis) within 6-12 months (Nugent HM 2009). Restenosis is also responsible for 30-60% of

vascular graft failures and proliferative vascular disease in both transplanted organs and arteriovenous fistulae created for haemodialysis access (Nugent HM 1999; Collins MJ 2012). Although much effort and many resources have been dedicated to the development of an effective approach to treat and prevent IH, restenosis is still a major problem. The issue is complicated by the fact that the mechanisms leading to IH and affecting treatment are influenced by the location of the injury in the vascular system.

For the past four decades PTA has been the most important and widely used treatment for patients with coronary obstruction. Intravascular stents were introduced to prevent early complications of PTA, such as elastic recoil and arterial dissection, but rates of restenosis remained consistently high (Jukema JW 2012). For this reason in 2003 drug eluting stents (DES) were introduced in clinical practice. These metal stents are coated with a pharmacological agent known to interfere with VSMC activation and proliferation, sometimes embedded in a polymer for slow release (Hao H 2011). Sirolimus (rapamycin)- and paclitaxel-eluting stents were the first DES to be approved for use in humans (Jukema JW 2012). Currently DES are the best approach for restenosis prevention after coronary angioplasty. Various clinical studies have showed their efficacy in reducing restenosis and the incidence of repeated PTA (Windecker S 2007; Sastry S 2010); however, significant long-term safety concerns have arisen. Problems associated with DES include stent malposition, delayed re-endothelization of the treated vessel, hypersensitivity to the polymer releasing the drug or the metal used for the stent, late stent thrombosis, inflammatory reactions and some rare cases of myocardial infarction and death (Hao H 2011; Jukema JW 2012). These problems have fuelled research in stent

development and alternative approaches such as gene-based therapy (Jukema JW 2012). Additionally, systemic delivery of anti-proliferative or anti-inflammatory drugs has been explored (Kakio T 2004; Jukema JW 2012). However, this approach presents serious problems of toxicity and off-site effects, which make it inferior to DES.

Although for the past decade DES have been used extensively and with overall success in the treatment of arterial restenosis, it is important to remember that using drugs, either locally or systemically, in order to treat and/or prevent IH strongly alters the normal function of EC and therefore affects vessel homeostasis. Another important aspect to consider with regards to IH is that the mechanisms responsible for its development might differ depending on the location in the vascular system. Vascular vein grafts in particular are exposed to unique factors that are not experienced by arterial vessels, such as surgical manipulation and trauma, mechanical forces at the anastomotic site. In the case of prosthetic conduits, bioincompatibility contributes to IH development (Collins MJ 2012). These aspects make the treatment of IH in vascular grafts quite complicated. Similarly to arterial IH, many interventions have been tried to treat and prevent venous IH, but despite all these efforts, such strategies seem only to delay, not to reduce or prevent, the development of IH (Collins MJ 2012). Clearly, novel approaches are needed in order to effectively treat IH in all its forms and locations.

1.6 Cell-based therapeutic approaches to treat intimal hyperplasia

More promising attempts to treat IH have been made using a cell-based therapeutic approach. Innovative EC-based tissue engineering studies to repair injured blood vessel, especially by promoting re-endothelization of the injured endothelium have been reported (Wilson JM 1989; Conte MS 1994). Indeed, VSMC proliferation in the intima is inhibited by restoration of the endothelium (Davies MG 1993; Rubanyi GM 1993). However, tissue engineering attempts aimed at re-endothelization of injured vessels have failed to prevent IH development, mostly because of technical issues with the delivery and viability of the EC. Moreover, animal studies suggest that restoration of an intact EC layer may not be necessarily required to block IH (Nathan A 1995; Nugent HM 1999; Nugent HM 2009; Nugent HM 2012). What appears to be more important is the restoration of the biochemical regulatory activity of the endothelium, which may be provided even by EC that do not reside at the luminal interface.

The first evidence of tissue-engineered EC being able to regulate vascular injury when placed at a distance from the lumen was provided almost 20 years ago in a rat model (Nathan A 1995). Bovine aortic EC engrafted in a collagen matrix sponge (EC/matrix) were placed around the carotid artery after balloon denudation. After 14 days animals treated with the EC/matrix showed reduced cell proliferation and hyperplasia compared to controls; immunostaining showed no recovery of endogenous EC or migration of the engrafted EC towards the injured area. Similar results were later reproduced in a porcine model at 28 days post-angioplasty in the carotid arteries (Nugent HM 1999). The EC/matrix was able to inhibit intimal thickening and thrombosis. Immunostaining with EC-specific markers showed

complete re-endothelization only in two control arteries (one treated with a heparin-releasing device and one with a cell-free collagen matrix), suggesting once again that repairing the intact architecture of the endothelium is not sufficient to stop IH development (Nugent HM 1999). This EC/matrix approach has been further developed, and in more recent years a less invasive, injectable collagen particles/EC matrix has been tested in porcine stent models (Nugent HM 2009; Nugent HM 2012). When injected into the perivascular space, the EC/matrix particles were able to significantly decrease the percentage of stenosis and the intimal area, while increasing the lumen area of the treated vessel at 4 weeks (Nugent HM 2009) and 90 days post injury (figure 3) (Nugent HM 2012).

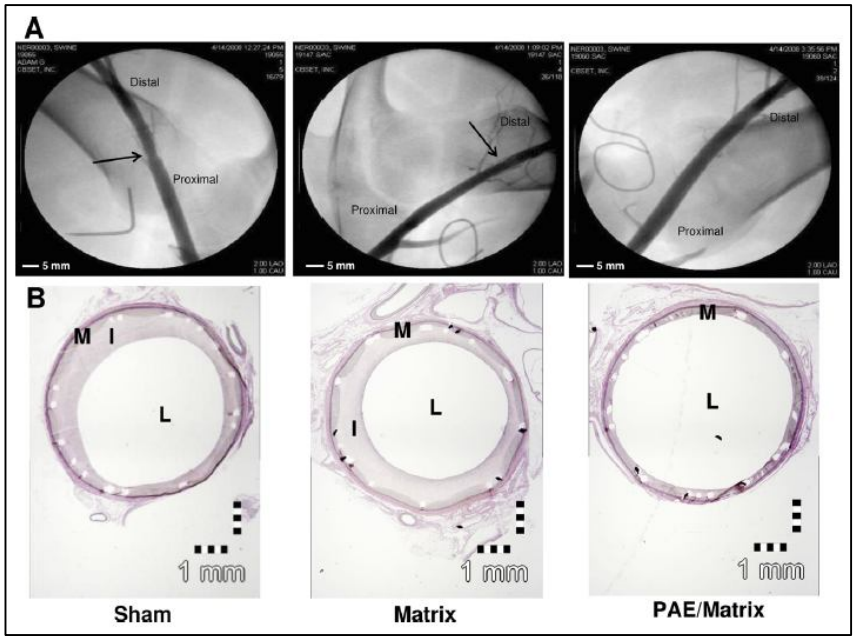

Figure 3: Representative day-90 angiograms (A) and photomicrographs of Verhoeff's elastin-stained arterial cross-sections (B). Comparison of the angiograms shows an increase in stenosis in the stented region of control arteries (left and middle panels, black arrows) compared with arteries treated with porcine aortic EC (PAE)/matrix particles (right panel). Histologic sections show significantly greater intimal area in control sham (left panel) and matrix (middle panel) arteries compared with arteries treated with PAE/matrix. I, Intima; L, lumen; M, media (Nugent, 2012).

Furthermore, the perivascular EC/matrix particles were able to significantly decrease the levels of local inflammation in the injured vessels, suggesting this anti-

inflammatory activity is likely to also play a role in reducing IH (Nugent HM 2012). These data suggest that the therapeutic effects provided by the engrafted cells placed outside the injured vessels were not mediated by a mechanical barrier function, but were, at least in part, the result of secreted, EC-derived products that provided biochemical regulation.

As described above, EC control vascular homeostasis through the production and release of a wide range of molecules, and soluble factors released from the EC/matrix (placed at a distance from the vascular lumen, in the perivascular space) can restore function to an injured vessel that has lost its biochemical and homeostasis regulatory activity. The possibility of inhibiting IH through soluble factors produced by engrafted EC is very important for the development of a novel effective treatment. Indeed, it suggests an EC/matrix-conditioned medium might be produced to use as a therapeutic agent in place of the cell-based formulation. Cell-based therapy has great potential, but it also has many issues: possible rejection; difficult reproducibility and inconsistency; problems with the delivery, shelf-life and storage of the product and ethical issues regarding the source and use of human cells. For these reasons the possibility of using cell-conditioned medium is of great advantage. In fact, preliminary experiments performed by Pervasis (an American biotech company that sponsored part of this PhD project) showed that the conditioned medium collected from EC grown on collagen sponge has a broad and strong anti-inflammatory activity and effectively reduces the effects of PF4 on EC *in vitro* (Nugent HM 2012). Moreover, it has been reported that supernatant from EC/matrix decreases the maturation of dendritic cells and significantly increases their production and release of IL-10 and TGF-β1 (Methe H 2007). Since maturation of dendritic cells is pivotal

in priming the immunological reaction (Banchereau J 2000), and since IL-10 and TGF-β1 are known for their anti-inflammatory activity (see Chapter 3.3), these results support the anti-inflammatory therapeutic potential of EC-conditioned medium and its possible application for the treatment of IH and other vascular inflammatory diseases, such as those affecting the eye.

1.7 Anatomy of the eye

The *in vivo* experiments presented in this thesis are based on experimental and genetic models of eye inflammation in the mouse. These models were chosen because they provide a localized and isolated ocular inflammation that can be easily monitored before, during and after treatment and they allow the exploration of the systemic effects of novel drugs, which can be administered by intraperitoneal injection, at a big distance from the eye. Moreover, various techniques are available to study different aspects of the inflammatory reaction and the effect of treatment on the eye. To better understand the *in vivo* experiments and their results, a brief description of the eye anatomy is provided, with particular focus on the retina and the choroid.

The eye is a highly specialised organ of photoreception. Specialized nerve cells in the retina, the rods and cones, undergo physiological changes in response to light from the environment; these changes produce nerve action potentials that are transmitted to the optic nerve and then to the brain, where the information is processed and perceived as vision. Other structures in the eye participate in the support of this basic physiological process: the cornea, lens, iris and ciliary body

focus and transmit light onto the retina, while the choroid, aqueous outflow and lacrimal systems nourish and protect the tissues of the eye (Forrester J 1996). Figure 4 shows the main components of the eye.

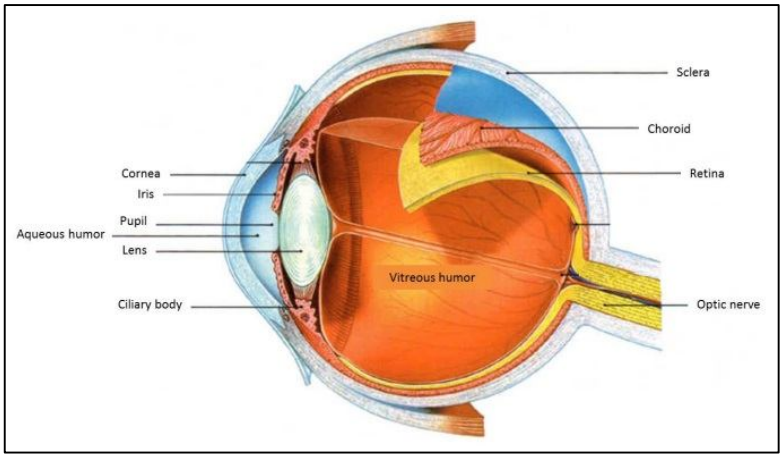

Figure 4: Anatomy of the eye (adapted from virtualmedicalcentre.com). See text for details.

Retina

The retina comprises light-sensitive layers of neurons positioned at the back of the eye. It converts relevant information from the image of the outer environment into neural impulses that are transmitted to the brain for decoding and analysis. The retina consists of two main layers: an inner neurosensory retina and an outer epithelium called the retinal pigmented epithelium (RPE). The neurosensory retina is a thin, transparent layer of neuronal tissue composed mainly of specialized neural cells such as photoreceptors, bipolar cells and ganglion cells, but it also includes glial cells, vascular endothelium, pericytes and microglia. These retinal cells are highly organized in eight distinct layers. The RPE is a continuous monolayer of hexagonally shaped, tightly packed epithelial cells containing pigment granules. Functions of the RPE include regeneration of the visual cycle, preserving adhesion of the neurosensory retina, providing a selectively permeable barrier between the choroid and the neurosensory retina, light absorption, phagocytosis of photoreceptor outer

segments, synthesis of interphotoreceptor matrix, transport and storage of metabolites and vitamins, spatial ion buffering and immune modulation (Forrester J 1996).

Choroid

The choroid is a thin, highly pigmented and highly vascularised connective tissue situated between the sclera and the RPE. Its main function is to nourish the highly metabolically active outer layers of the retina containing the photoreceptors. Absorption of light by choroidal pigment also contributes to vision, and regulation of blood flow by choroidal vessels influences photoreceptor metabolism. The choroid consists of five layers: the Bruch's membrane, the choriocapillaris, two vessel layers and the suprachoroid. Bruch's membrane lies beneath the RPE. It is a modified multi-layered ECM membrane that includes the RPE basal lamina, an inner collagenous zone, a middle elastic layer, an outer collagenous zone and the basement membrane of the EC in the choriocapillaris. The choriocapillaris is a vast assembly of wide-bore, fenestrated capillaries that deliver nutrition to the outer retina. SMC are usually absent. Beneath the choriocapillaris is the vascular layer, which can be divided into an outer part of major arteries and veins (Haller's layer) and an inner layer of intermediate-sized arterioles and venules (Sattler's layer). The suprachoroid represents a thick transition zone between choroid and sclera. It is formed by interconnected melanocytes, fibroblasts and connective tissue fibres and does not contain blood vessels. Importantly for the work presented in this thesis, the choroid comprises resident populations of immunocompetent cells including plasma cells, lymphocytes, macrophages, dendtritic cells and perivascular mast cells (Forrester J 1996; Spencer WH 1996).

1.8 Choroidal neovascularisation as a model of inflammation-driven angiogenesis

Choroidal neovascularization (CNV) is defined as growth of new blood vessels from the choroid, between the basement membrane of the RPE and the remainder of Bruch's membrane, and often invading into the subretinal space (Spencer WH 1996). It is the major complication associated with many retinal degenerative or inflammatory pathologies, especially age-related macular degeneration (AMD) (Spencer WH 1996; Espinosa-Heidmann DG 2003). In fact, about 10% of AMD patients develop CNV, and yet CNV is responsible for 90% of severe vision loss in AMD (Vinores SA 2006). Currently the molecular mechanism for CNV development is not fully understood. CNV represents a non-specific wound repair response to an underlying disease and, although different in intensity and cellular components depending on the specific causal pathology and stage of development, all CNV lesions involve inflammation (Grossniklaus HE 2004).

CNV can grow between the RPE and Bruch's membrane (type 1), between the retina and the RPE (type 2) or a combination of both (combined type). The new blood vessels are capillary-like and, with time, they display arterial and venous characteristics (Grossniklaus HE 2004). During the first, dynamic stages of development, CNV lesions are characterised by high levels of vascular endothelial growth factor (VEGF), IL-8 and monocyte chemoattractant protein (MCP) produced by the RPE, the retinal photoreceptors and the EC of the choroid vessels. Macrophages are attracted from the choriocapillaris and release TNFα, which in turn stimulates additional production of VEGF, IL-8 and MCP. TNFα also stimulates integrins expression in the RPE, with subsequent activation of tyrosine kinase pathways that mediate migration of EC and macrophages (Grossniklaus HE 2004).

After this initial stage, CNV grows during the inflammatory active stage. In this phase EC and macrophages produce MMPs that enable the lesions to pass through tissue planes. Macrophages also express tissue factor, which stimulates the production of a fibrin scaffold on which the CNV lesion can grow. Angiopoietins and their receptors (Tie-1 and -2), FGF, TGF-β, VEGF, PDGF, pigmented epithelium derived factor (PEDF), plasminogen, fibrin and tissue inhibitors of MMPs (TIMPs) are produced by the RPE and the vascular endothelium during this process until at some point the balance shifts towards anti-angiogenic, anti-proteolytic and anti-migratory signals (Grossniklaus HE 2004). This is the inflammatory inactive or involutionary stage of CNV, during which the lesions may become collagenized and display a disciform shape (Grossniklaus HE 2004).

Inflammation is a key component in the growth of CNV. Significant macrophage infiltration is observed in surgically excised CNV lesions, and these macrophages appear to be synthesizing cytokines and growth factors (Grossniklaus HE 2000; Grossniklaus HE 2002). Moreover, macrophages migrate to experimentally-induced CNV lesions and seem to contribute to the neovascularization (Ishibashi T 1985). It has been suggested that macrophages might be a stimulus for CNV, since they are a common factor linking the many different pathologies associated with CNV. In fact, it has been shown that macrophage depletion decreases the size and severity of the lesions in a mouse model of CNV (Espinosa-Heidmann DG 2003). This evidence suggests there is great therapeutic potential for anti-inflammatory drugs to correct CNV, which until about a decade ago was usually treated with laser photocoagulation, ionizing radiation or surgical removal (Grossniklaus HE 2004). A more recent therapy uses the drug verteporfin in photodynamic therapy, which

causes local EC damage, platelet aggregation and eventually thrombosis with occlusion of the vessels (Couch SM 2011). Several anti-VEGF therapeutics have also been approved for use in the treatment of CNV (Campochiaro PA 2013). These drugs act as anti-angiogenic and anti-permeabilization agents and, since they have shown a significant short-term benefit to patients' vision, they have become the standard of care for most patients with CNV (Couch SM 2011). It is of interest to note that overexpression of VEGF results in pro-inflammatory effects such as expression of endothelial adhesion molecules and monocyte chemoattraction (Takahashi H 2005; Luo J 2011). Anti-VEGF therapies might therefore have anti-inflammatory effects in addition to their direct effects on vascular cells.

1.9 Inflammatory cytokines and chemokines and their effect on endothelial cells

Platelet factor 4 (PF4), TNFα and IL-6 are known pro-thrombotic and pro-inflammatory molecules. Because of these properties they were used in the experiments described in this thesis, providing an *in vitro* model of inflammation to study the effects and mechanism of action of the EC-conditioned medium. A description of these cytokines and chemokine is provided.

PF4

PF4 was the first member of the chemokine family to be discovered (Kasper B 2011). This large group consists of cytokines that share the common ability to induce a direct cellular migration, especially of immune cells. PF4 is a member of the CXC subfamily of chemokines, which is characterized by four conserved cysteines, where the first two are divided by an amino acid residue (CXCCC) (Handin RI 1976;

Stringer SE 1997). Biosynthesis of PF4 is almost exclusively in megakaryocytes that will later mature into platelets, with only a very small portion produced in activated monocytes (Kowalska MA 2010). This chemokine accounts for almost 2% of the total α-granule storage in platelets (Kowalska MA 2010). Normal plasma levels of PF4 are below 1 nM (Kasper B 2011), but upon platelet activation the concentration in serum reaches 2 µM, with an even higher estimated concentration of 25 µM at localized sites of injuries and 280 µM within a thrombus (Fukami MH 2001; Kowalska MA 2010). At physiological pH PF4 exists as a tetramer (Kowalska MA 2010) and binds with high affinity to heparin and other negatively charged molecules, such as the cell surface glycosaminoglycans heparan sulphate, chondroitin sulphate and dermatan sulphate (Handin RI 1976; Stringer SE 1997; Kowalska MA 2010). Although a definite receptor for PF4 has yet to be identified, there is evidence that PF4 can bind with nM affinity to the receptor CXCR3B, which is expressed in the human heart, liver, kidneys and skeletal muscles, as well as on proliferative microvascular endothelium and EC in neoplastic tissues (Lasagni L 2003). It has also been reported that PF4 binds to lipoprotein receptor-related protein 1 (LRP1), a member of the low-density lipoprotein receptor superfamily. Interaction with this receptor in human umbilical vein endothelial cells (HUVEC) induced expression of E-selectin in a NF-kB-dependent manner (Yu G 2005).

Even though it was identified as the first chemokine, today it is clear that purified PF4 lacks chemotactic activity for neutrophils and monocytes (Kowalska MA 2010). The specific biological functions of PF4 and the pathways activated by the molecule are still not fully understood; however, various *in vitro* and *in vivo* studies suggest roles in thrombosis, atherosclerosis, angiogenesis and immune modulation (Sachais BS 2004; Kowalska MA 2010; Kasper B 2011).

TNFα

TNFα is a pro-inflammatory cytokine that belongs to the TNF superfamily and is mainly produced by macrophages (Aggarwal BB 2003; Chu WM 2013). Most of the TNF family members are physiologically expressed as transmembrane proteins to act locally, but during disease and/or injury they can be shed and released in the blood stream (Aggarwal BB 2003). Specifically, a metalloproteinase called TNFα-converting enzyme can derive soluble TNFα from the transmembrane cytokine (Black RA 1997).

There are two receptors for TNFα: TNF receptor 1 (TNFR1), expressed in most cells of the body; and TNFR2, found mainly on lymphocytes and the endothelium (Aggarwal BB 2003; Chu WM 2013). When binding to these receptors TNFα can induce tumor cell necrosis, fever and acute and chronic inflammatory reactions. The TNFα pathway is also involved in autoimmune diseases and in tumor progression, invasion and metastasis (Chu WM 2013). In EC, TNFα induces protein synthesis-dependent and -independent changes, which ultimately affect vascular permeability and tone, coagulation, apoptosis, leukocyte adhesion and leukocyte activation (Madge LA 2001). The effects of TNFα on EC are largely dependent on TNFR1 (Loetscher H 1993). None of the TNFRs has any intrinsic enzymatic activity (Madge LA 2001; Aggarwal BB 2003). The different cellular responses elicited by TNFα result from the recruitment of a wide range of adaptor proteins to the activated receptor (figure 5) (Madge LA 2001).

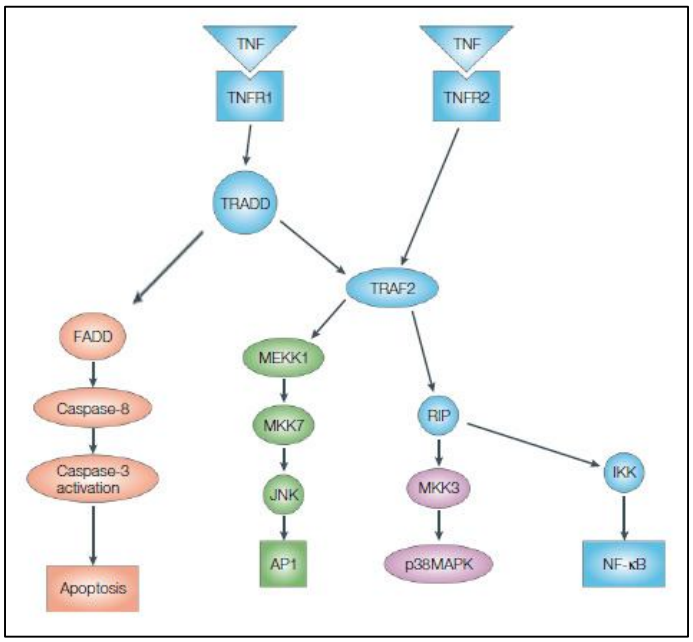

Figure 5: Intracellular signalling upon TNFα binding to TNFR1 or TNFR2 (adapted from Aggarwal BB, 2003)

In the case of TNFR1, binding of TNFα induces the recruitment of TNFR-associated death domain protein (TRADD) to the intracellular domain of the receptor (Madge LA 2001; Aggarwal BB 2003; Pober JS 2007). TRADD in turn recruits the serine/threonine kinase receptor-interacting protein 1 (RIP1) and the ubiquitin ligase TNFR-associated factor 2 (TRAF2) (Madge LA 2001; Aggarwal BB 2003; Pober JS 2007). This complex mediates the activation of gene transcription through different signalling cascades, the most prominent of which involves NF-kB (Madge LA 2001; Aggarwal BB 2003; Hayden MS 2008). The NF-kB family of transcription factors consists of five members: p50, p52, p65 (RelA), c-Rel and RelB (Hayden MS 2008). In resting cells NF-kB dimers are retained in the cytosol due to interaction with the inhibitors IkB (IkBα, β and ε or p100 and p105), which mask the nuclear localization sequence of the transcription factors (Hayden MS 2008). Activation of the pathway leads to degradation or processing of IkBs and subsequent release of NF-kB dimers, which move to the nucleus, bind to the target DNA sequence and regulate gene

transcription through recruitment of co-activators and co-repressors (Hayden MS 2008). The responses activated by TNFα typically involve the NF-kB dimer p65:p50 and the inhibitor IkBα in what is called the "canonical NF-kB pathway" (Hayden MS 2008; Brasier AR 2010) (figure 6). When the complex TRADD/RIP1/TRAF2 is formed, it activates the inhibitor of NF-kB kinase (IKK) complex (made by the subunits IKKα, β and NEMO), which in turn mediates the phosphorylation of IkBα. Phosphorylated IkBα is targeted for rapid ubiquitination and degradation through the proteasome. Once the inhibitor is degraded, NF-kB (p65:p50) is free to shuttle to the nucleus to activate transcription (figure 6).

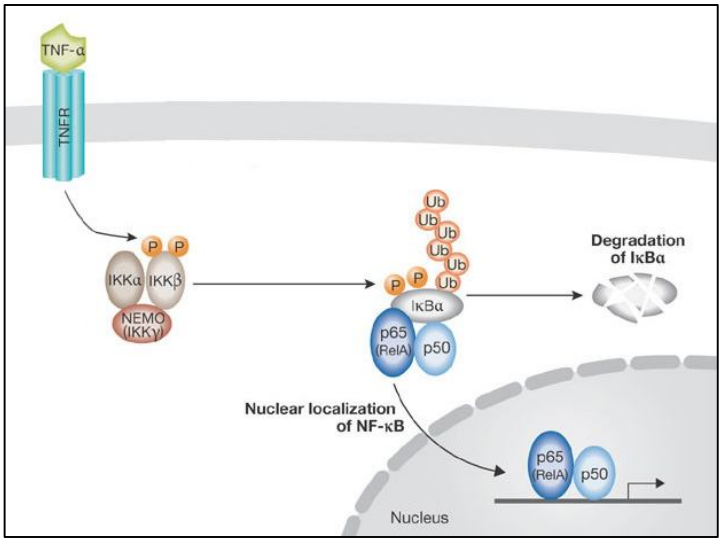

Figure 6: The canonical NF-kB pathway activated by TNFα (adapted from Iwai K and Tokunaga F, 2009).

Alternatively, formation of the TRADD/TRAF2 complex upon binding of TNFα to TNFR1 activates a MAP/ERK kinase kinase 1 (MEKK1)/MAPK kinase 7 (MKK7)/JNK kinase pathway that leads to activation of AP1 (figure 5) (Aggarwal BB 2003; Pober JS 2007); the TRADD/RIP1/TRAF2 complex also elicits MKK3 and p38 MAPK, but the mechanism is not yet fully understood (Aggarwal BB 2003). TRADD is also essential for the recruitment of proteins involved in the pro-apoptotic

activity of TNFα (figure 5) (Madge LA 2001; Aggarwal BB 2003). In fact, TRADD can interact with Fas-associated death domain protein (FADD) to form a complex that recruits and activates caspase-8. This results in a hierarchical cascade of caspase stimulation that culminates in the activation of caspase-3, which is responsible for cell apoptosis through the proteolysis of a large number of substrates (Madge LA 2001; Aggarwal BB 2003). Most cell types, including EC, are not sensitive to TNFα–induced apoptosis unless mRNA or protein synthesis is blocked (Madge LA 2001). This observation is attributed to the ability of TNFα to induce gene expression of anti-apoptotic products (Madge LA 2001). Binding of TNFα to TNFR2 activates the same signalling cascades as TNFR1 through direct recruitment of TRAF2 to the intracellular domain of the receptor (figure 5) (Aggarwal BB 2003).

Protein synthesis-independent effects of TNFα on EC have also been observed; these include alteration of the cytoskeleton and subsequent changes in cell shape, motility and permeability (Madge LA 2001). The specific mechanisms mediating those effects are still not clear, although they probably involve small G proteins, particularly of the Rac, cdc42 and Rho family, which are known to regulate actin organization (Madge LA 2001).

IL-6

IL-6 is a cytokine produced by activated monocytes, T- and B cells, endothelial cells, fibroblasts, keratinocytes, mesangial cells, adipocytes and some tumour cells (Mihara M 2012). IL-6 is a multifunctional cytokine that regulates inflammation, hematopoiesis, immune responses and acute phase inflammatory responses (Sprague AH 2009). It stimulates differentiation of lymphocytes and macrophages,

proliferation of thymocytes and peripheral T cells, expression of acute phase proteins in the liver and development of fever (Libermann TA 1990; Mihara M 2012). There is also evidence of IL-6 stimulating EC proliferation *in vitro* (Holzinger C 1993). IL-6 gene expression is induced by a plethora of inflammatory stimuli, including bacterial lipopolysaccharide (LPS), viral infection, TNFα, IL-1 and PDGF (Libermann TA 1990).

IL-6 signal transduction involves two receptors: gp130 and IL-6 receptor α (IL-6R) (Heinrich PC 1998), both of which are expressed in EC (Yuen DYC 2009). IL-6 specifically binds to IL-6R, which has no intrinsic kinase activity, but efficiently recruits two subunits of the corresponding signal-transducing receptor gp130 (Heinrich PC 1998). Hence, IL-6/IL-6R/gp130 is the functional receptor complex. The major mediators of IL-6 signalling are Jak kinases and the factor signal transducer and activator of transcription 3 (STAT3) (figure 7) (Heinrich PC 1998; Heinrich PC 2003). Specifically, Jak1, Jak2 and Tyk2 kinases are associated with the

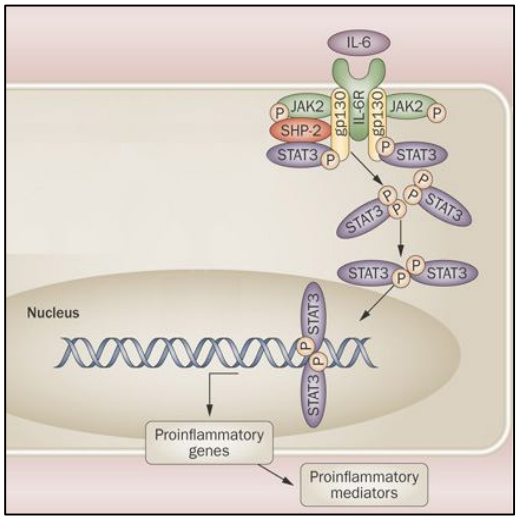

Figure 7: STAT3 activation through the IL-6/IL-6R/gp130 complex (adapted from Walters TD and Griffiths AM, 2009).

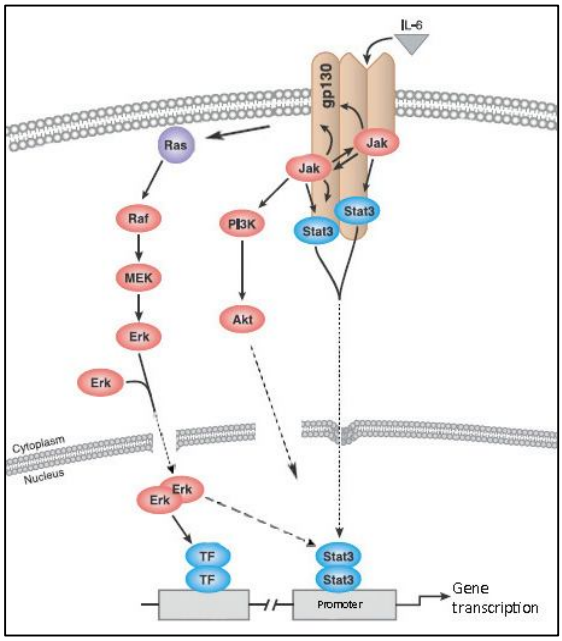

Figure 8: IL-6-activated pathways (adapted from the Cell Signalling pathways library).

intracellular domain of gp130. Upon gp130 stimulation the Jak kinases are activated and phosphorylate the cytoplasmic tail of gp130. The phospho-residues on gp130 serve as docking sites for the transcription factor STAT3, which also becomes phosphorylated, forms dimers and translocates to the nucleus to regulate transcription of target genes.

Stimulation of gp130 and the corresponding Jak kinases can also activate the Ras/Raf/MAPK pathway, which ultimately phosphorylates STAT3 (figure 8) (Heinrich PC 1998; Heinrich PC 2003). While the specific role of the kinase ERK1/2 in this process has been established, other findings have shown activation of different MAPK such as p38 and JNK, though this activity remains poorly understood (Heinrich PC 2003). IL-6 can lead to the activation of another signalling pathway involving phosphatidylinositol 3-kinase (PI3K) and Akt kinase (figure 8) (Heinrich PC 2003). However, this activation seems to happen in a cell-specific manner, mediating an anti-apoptotic effect in cardiomyocytes and carcinoma cells (Heinrich PC 2003). The molecular mechanism linking gp130 to the stimulation of PI3K/Akt signalling is still unknown.

1.10 Leukocyte adhesion molecules

Adhesive interactions between different cells, and between cells and the ECM are crucial to many tissue and cellular functions, including the inflammatory response (Crockett-Torabi E 1998). During inflammation leukocyte interaction with EC is essential for recruitment of the immune cells to the affected area. The specificity of leukocyte homing to inflamed tissues is defined by the chemokines released in the

microenvironment and by the adhesion molecules and counter-receptors expressed on EC and leukocytes, respectively (Cook-Mills JM 2011). The pro-inflammatory molecules that activate the endothelium determine the type of adhesion molecules expressed by EC (Cook-Mills JM 2011).

Vascular recruitment of leukocytes is a three-step process: 1) leukocytes roll on the endothelium, forming weak and transient interactions with the selectins expressed on EC; 2) high-affinity interactions with ICAMs and VCAM-1 allow the firm arrest of leukocytes on the endothelium; and 3) leukocytes transmigrate through the endothelium into the inflamed tissue (Carlos TM 1994; Cook-Mills JM 2011).

As mentioned previously, adhesion molecules expressed on the endothelium can be divided in three families: selectins, integrins and ICAMs. Each one is involved in a specific phase of leukocyte adhesion and migration through the endothelium, and the synchronization of their expression and function is crucial for the process. Inappropriate or abnormal sequestration of leukocytes at specific sites is involved in the development of many autoimmune and inflammatory diseases such as diabetes, ischemic and reperfusion injury, allograft organ rejection and hypersensitivity reactions (Tedder TF 1995). Since adhesive molecules play such an important role in the inflammatory response at the endothelium level, the experiments described in this thesis focus on expression of two adhesive molecules on EC induced by inflammatory cytokines: E-selectin and VCAM-1.

Expression of E-selectin and VCAM-1 largely depends on *de novo* synthesis of mRNA and protein (Carlos TM 1994). The promoters of both genes comprise short cytokine-responsive regions with multiple NF-kB binding sites that interact with

other elements to induce transcription. Indeed, the synergy between NF-kB and other transcription factors and activators seems to be a common feature of cytokine-induced gene expression (Collins T 1995).

E-selectin

The selectin family consists of three closely related cell-surface proteins designated by the prefixes E (endothelial), P (platelet) and L (leukocyte) (Carlos TM 1994). All selectins have an extracellular region composed of an amino-terminal, Ca^{2+}-dependent lectin domain, an epidermal growth factor (EGF)-like domain and two to nine short consensus repeat (SCR) units homologous to domains found on complement regulatory proteins (Tedder TF 1995). E-selectin has six SCR units (Tedder TF 1995). E-selectin binds the fucosylated tetrasaccaride sialyl Lewis X or closely related structures (on proteins or glycolipids) which are abundantly expressed on neutrophils, monocytes and natural killer (NK) cells (Carlos TM 1994).

E-selectin is not normally detected on the surface of resting EC, but its expression is strongly induced by various inflammatory mediators, including TNFα, IL-1β, LPS and interferon γ (IFNγ) (Collins T 1995; Tedder TF 1995). Therefore, E-selectin is highly present on endothelial cells at sites of injury and inflammation (Tedder TF 1995). The regulatory elements necessary for cytokine-induced E-selectin expression are localized in the first 170 base pairs (bp) upstream of the transcription start site (Whelan J 1991). In particular, the region between residues -170 and -85 is essential for induction by TNFα (Schindler U 1994). Figure 9 illustrates the four positive regulatory domains in the gene's promoter, named PDI-IV (Whitley MZ 1994).

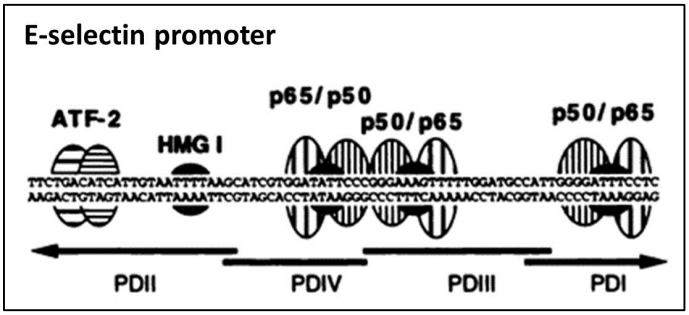

Figure 9: E-selectin promoter region, regulatory domains and transcription factors involved in its regulation. See text for details (Collins et al, 1995).

PDI, PDIII and PDIV are NF-kB binding sites; PDII is an activating transcription factor (ATF)-like site (Schindler U 1994; Collins T 1995). Induction of E-selectin expression requires NF-kB to bind to PDI, PDIII and PDIV (mainly in the forms of p50:p65 heterodimers) (Schindler U 1994; Collins T 1995). The high mobility group protein I (HMG I(Y)) is also required for interaction with PDIII and PDIV, enhancing NF-kB binding to those elements (Lewis H 1994; Whitley MZ 1994). PDII displays a sequence that is typically recognized by the ATF family of transcription factors. In fact, PDII has been shown to be bound by recombinant ATF-a, ATF-2, ATF-3, c-Jun and CREB (Kaszubaka W 1993; De Luca LG 1994). Moreover, members of the ATF family can interact with both NF-kB and HMG I(Y) (Kaszubaka W 1993).

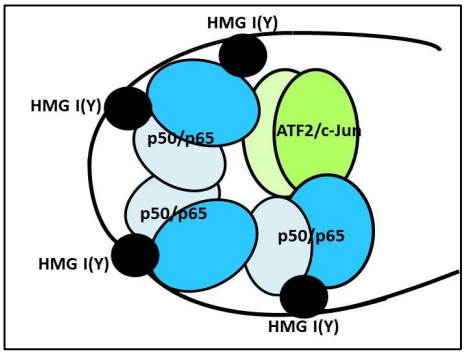

Figure 10: Model of the transcription complex regulating cytokine-induced E-selectin expression (adapted from Collins et al, 1995).

Transcription of E-selectin involves NF-kB transcription factor (Montgomery KF 1991; Collins T 1995), but this is not sufficient for cytokine-induced expression; HMG I(Y) and ATF-2 are also required to form a unique transcription-activating complex with NF-kB (Collins T 1995). Taking this into account, a model for enhanced cytokine-induced E-selectin expression has been proposed (figure 10) (Collins T 1995). Heterodimers of NF-kB (p50:p65), an ATF-2/c-Jun heterodimer or an ATF-2 homodimer bind to the promoter. HMG I(Y) binds on multiple sequences and increases the affinity of NF-kB and ATF-2 for their respective sites; it also bends the DNA to facilitate the physical interaction between factors and formation of a higher order complex necessary for transcription. This unique transcription complex then acts as a unit, possibly involving additional co-activators or transcription factors.

E-selectin protein half-life *in vitro* is quite short. The protein appears at 1-2 hours post-induction, peaks at 4-6 hours and returns to basal levels within 24 hours, even in the continuous presence of cytokine (Carlos TM 1994; Collins T 1995; Tedder TF 1995). Its short surface presence is largely due to a process of endocytosis (Carlos TM 1994).

A soluble version of E-selectin (sE-selectin) has been detected in the supernatants of cytokine-activated EC *in vitro* (Pigott R 1992) and in human serum, where its levels correlated with inflammatory states (Gearing AJH 1992; Tedder TF 1995). Since there is no evidence of a naturally-occurring soluble splice variant of the protein, shedding of E-selectin from its membrane form is more likely. Possible biological roles for sE-selectin include: clearing of adhesion molecules to control leukocyte

adhesion on EC, chemoattraction and/or activation of immunological cells.

VCAM-1

VCAM-1 belongs to the immunoglobulin (Ig) superfamily of proteins, which are characterized by several extracellular Ig-like domains. In the case of VCAM-1, the extracellular region comprises seven Ig-like domains (Cook-Mills JM 2011). VCAM-1 binds to integrins expressed by eosinophils, basophils, lymphocytes, mast cells and monocytes (Cook-Mills JM 2011). It is the activation state of these integrins that regulates their binding to VCAM-1 (Cook-Mills JM 2011). VCAM-1 is typically expressed on the cell surface of activated endothelium, but it can also be released in a soluble form (sVCAM-1) by the cleavage activity of disintegrin and metalloproteinase 17 (Cook-Mills JM 2011). This sVCAM-1 is thought to limit binding of leukocytes to VCAM-1 or act as chemoattractant for leukocytes (Cook-Mills JM 2011).

VCAM-1 expression is induced by cytokines, high levels of ROS, oxidized LDL, high glucose, turbulent shear stress and microbial infection (Cook-Mills JM 2011). Figure 11 shows the small region (~100 bp) upstream of the transcriptional start site that is involved in cytokine-induced VCAM-1 gene expression. The VCAM-1 promoter contains a Sp1 element (Neish AS 1995), an interferon regulatory factor-1 (IRF-1) element (Neish AS 1995) and two tandem NF-kB binding sites (Iademarco MF 1992; Ahmad M 1995; Collins T 1995).

Both NF-kB sites are necessary for transcription. Heterodimeric p50:p65 NF-kB binds to the VCAM-1 promoter and is responsible for cytokine-induced expression

of the gene (Neish AS 1995). The p65 unit acts as a powerful activator of transcription, while p50 seems to have more of a regulatory role (Ahmad M 1995; Neish AS 1995). IRF-1 can bind p50 and cooperates with NF-kB to increase VCAM-1 expression (Neish AS 1995). Sp1 is required for a maximal cytokine-induced response of the promoter (Neish AS 1995). The peak of expression for VCAM-1 protein *in vitro* is reached at 3-6 hours, and the expression persists for 72 hours after induction by cytokines (Carlos TM 1994).

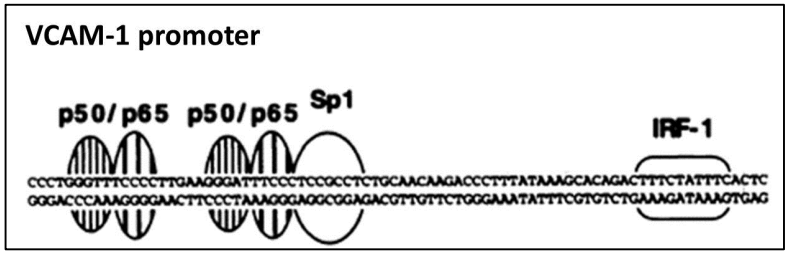

Figure 11: VCAM-1 promoter region and transcription factor binding sites involved in its regulation. See text for details (Collins et al, 1995).

VCAM-1 not only functions as an adhesion molecule, but also as an "outside-in" signal transducer. Through intracellular Ca^{2+} release and subsequent ROS generation VCAM-1 can activate p38 MAPK, protein kinase Cα and protein tyrosine phosphatase 1B and therefore regulate formation of cell junctions between EC, an important process for leukocyte transmigration (Cook-Mills JM 2011).

VCAM-1 represents an important therapeutic target in the treatment of vascular diseases due to its significant role during inflammation. VCAM-1 also plays prominent roles in embryonic development of the cardiovascular system and in cardiovascular diseases (Gurtner GC 1995). Various publications have shown that VCAM-1 is expressed in atherosclerotic lesions, and in advanced stages of

atherosclerosis VCAM-1 can be expressed by SMC (Collins T 1995; Iiyama K 1999). Furthermore, siRNA or antibodies against VCAM-1 reduced experimental neointima formation in rodents (Barringhau KG 2004; Qu Y 2009). VCAM-1 is also linked to calcification of aortic stenosis during coronary artery disease (Linhartova K 2009).

Hypothesis and aims of the PhD project

EC grown on a collagen matrix produce soluble factor(s) that can inhibit angioplasty-induced restenosis *in vivo*. Part of this beneficial effect seems to depend on the inhibition of the inflammatory reactions which are characteristic of IH development. Based on this observation, the conditioned medium obtained from EC grown on collagen particles (ECPCM) is expected to contain anti-inflammatory soluble factors. The goals of this PhD project are to confirm the presence of anti-inflammatory molecules in ECPCM, identify and/or partially purify the active factor(s), determine the molecular mechanism of action of the anti-inflammatory activity and evaluate its therapeutic potential *in vivo*.

Chapter 2: Materials and Methods

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2.1 Cell culture

Human aortic endothelial cells (HAEC; Lonza) were grown in endothelial growth medium 2 (EGM2; Lonza) and used at passages 7 to 8. Human umbilical vein endothelial cells (HUVEC) were grown in EGM2 (Lonza) and used at passages 6 to 7. Both lines of EC were plated in EGM2 without hydrocortisone (Lonza) and treated using the control collection medium (phenol red free-endothelial basal medium (EBM; Lonza), 0.5% fetal bovine serum (FBS), 50 µg/ml gentamicin) or ECPCM.

All cytokines and chemokines were purchased from Peprotech as human recombinant proteins and used at the following concentrations: 64.1 nM PF4, 2.39 nM IL-6, 0.1 nM TNFα, 120 pM TGF-β1.

2.2 Production of cells on particles-conditioned media

Collagen Gelfoam® powder for endothelial cell culture was prepared by wetting 1 g of powder (Pfizer) with 65 ml of EGM2 media, 10% FBS and by leaving it to fully hydrate at 37^0C, 5% CO_2 for 16-18 hours. About 5 ml (75 mg) of the gel was then aliquoted into 50 ml tubes (Corning). HAEC were seeded on the gel at the concentration of 5-7 x 10^5 cells/tube. Five to ten minutes after seeding 10 ml of EGM2, 10% FBS medium was added to each tube. The cells were cultured at 37^0C, 5% CO_2 in EGM2, 10% FBS for 10 days, changing the medium every other day. On the 10^{th} day of culture the medium was changed to regular EGM2 (containing 2% FBS). The cells were then grown for an additional 5 days at 37^0C, 5% CO_2, changing

the medium every 2 days. On day 15 of culture, one tube was use for cell counting. Cells on the gel were rinsed once with 10 ml HEPES buffered saline solution (Lonza) before addition of 5 ml collagenase type 2 (Worthington) at 37°C for 5-20 minutes. Once the collagen particles were completely dissolved, the cells were centrifuged at 106 x *g* for 5 minutes, trypsinized, diluted with trypan blue (Sigma) and counted in a hemacytometer. A value equal to or higher than 1.5 x 10^6 cells/tube was considered optimal for ECPCM production.

After evaluation of cell number, the remaining tubes were used to generate conditioned medium. After careful removal of the growth medium, cells on the gel were rinsed once with 30 ml of washing medium (phenol red free-EBM, 50 µg/ml gentamicin) and incubated at 37^0C, 5% CO_2 for 30 minutes. The washing medium was then removed and about 7 ml of collection media was added to each tube. The cells were cultured at 37^0C, 5% CO_2 for 24-27 hours before collection of ECPCM, which was then filtered (Durapore® 0.2 µm filter, Millipore), aliquoted and stored at -80^0C.

Cell-free particle-conditioned medium (PCM) was obtained by applying the above procedure to collagen gel only. Conditioned media from aortic SMC (passage 8; Lonza) and human embryonic kidney 293 (HEK293) cells were obtained by seeding ~ 5 x 10^5 cells/tube on the collagen gel. The cells were then cultured for 15 days in their respective growth media (smooth muscle growth medium 2 (SmGM2; Lonza) for SMC and Dulbecco's modified Eagle's medium (DMEM; Gibco) + 10% FBS for HEK293 cells). Cell counting and washing before conditioning was performed as for the EC. About 7 ml of collection medium was then added to each tube for 24-27 hours at 37^0C, 5% CO_2. SMC on particles-conditioned medium (SMCPCM) and

HEK293 on particles-conditioned medium (293PCM) were then collected, filtered (Durapore® 0.2 µm filter, Millipore), aliquoted and stored at -80^{0}C.

2.3 Cytokine and chemokine treatment for real-time polymerase chain reaction (PCR)

HAEC and HUVEC were seeded onto 24-well plates (50,000 cells/well) and grown at 37^{0}C, 5% CO_2 in EGM2 without hydrocortisone. When ~80% confluence was reached, the cells were serum-starved in collection medium for 16 hours before treatment. Cytokines and chemokines were added to collection medium (control) or ECPCM, then the cells were incubated for 4 hours (PF4) or 2 hours (IL-6 and TNFα) at 37^{0}C, 5% CO_2. HAEC were co-treated with TGFβ and IL-6 or TNFα in collection medium for some experiments. For pre-treatment of HAEC with ECPCM, serum-starved cells were placed in ECPCM for 2 hours, then the medium was changed to collection medium containing IL-6 or TNFα for another 2 hours. RNA samples were harvested after each treatment.

2.4 RNA extraction, reverse transcription and real-time PCR

RNA samples were harvested from each well using the RNeasy Mini spin column kit (Qiagen) according to the manufacturer's instructions. The resulting total RNA was treated with DNAse I to remove any contaminating genomic DNA and quantified by optical density at 260 nm. Around 300 ng of total RNA was then used for reverse transcription using the QuantiTect RT kit (Qiagen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using specific human

TaqMan probes (Applied Biosystems) for E-selectin (Hs00950401_m1) and VCAM-1 (Hs01003369_m1). A human hypoxantine-guanine phosphoribosyl transferase (HPRT1) TaqMan gene assay (4333768F) was used as the house-keeping gene.

2.5 Real-time PCR data analysis

The experiments were performed using triplicate samples, and the data represent the average of at least three experiments ± standard deviation. GraphPad Prism software was used for statistical analysis. All data from real-time PCR were normalized to the background control (collection medium) to determine E-selectin and VCAM-1 gene expression changes for each condition. Percentage of gene expression induction by PF4, IL-6 or TNFα and inhibition by ECPCM was determined by comparing the fold difference of chemokine/cytokine in ECPCM-treated samples to the chemokine/cytokine in collection medium-treated samples. Differences between multiple groups were compared using one-way ANOVA followed by the post hoc Tukey test. The criterion for statistical significance was p-value < 0.05, 0.01 or 0.001, depending on the experiment, as specified in figure legends.

2.6 ECPCM IC50 calculation

HAEC were seeded on 24-well plates (50,000 cells/well) and grown to confluence in EGM2 without hydrocortisone. Cells were serum-starved in collection medium for 16 hours before treatment. ECPCM was serially diluted 1:2 in collection medium, giving a total of four concentrations of ECPCM: 100%, 50%, 25% and 12.5%. Each concentration, as well as the collection medium control, was tested on HAEC in the

presence of IL-6 or TNFα for 2 hours at 37^0C, 5% CO_2. Cells were then processed for gene expression analysis by real-time PCR as described above. Relative E-selectin and VCAM-1 gene induction was expressed as percentages. GraphPad Prism software was used to produce a dose-response curve and calculate the IC50 value of ECPCM for each gene and cytokine treatment.

2.7 Electron microscopy

HAEC were seeded on a Gelfoam® sponge at the concentration of 5 x 10^5 cells/sponge. Two hours after seeding 10 ml of EGM2, 10% FBS medium was added to each tube. The cells were cultured at 37^0C, 5% CO_2 in EGM2, 10% FBS for 10 days, changing the medium every other day. On the 10^{th} day of culture the medium was changed to regular EGM2 (containing 2% FBS). The cells were then grown for an additional 5 days at 37^0C, 5% CO_2, changing the medium every 2 days. On day 15 of culture, one sponge was use for cell counting. On day 16 the remaining sponges were immersion fixed overnight in 3% glutaraldehyde and 1% paraformaldehyde buffered to pH 7.4 with 0.08 M sodium cacodylate/HCl buffer. The sponges were rinsed twice for 10 minutes with cacodylate buffer, post-fixed in 1% aqueous osmium tetroxide for 2 hours and then dehydrated by passage through ascending alcohols (50%, 70%, 90% and 100% ethanol, 10 minutes each). Gels were then immersed in propylene oxide (twice for 15 minutes) and infiltrated overnight with a 1:1 mixture of propylene oxide/araldite CY212. After an additional infiltration of 6 hours with the full resin, samples were embedded and cured overnight at 60°C.

Semi- and ultra-thin sections were cut using a diamond knife of the appropriate grade. Semi-thin sections were stained with a 1% mixture of toluidine blue-borax in

50% ethanol, and ultra-thin sections sequentially contrasted with lead citrate for imaging in a JEOL 1010 TEM operating at 80kV. Images were captured using a Gatan Orius CCD camera and the Digital Micrograph software.

2.8 Immunofluorescence

For the characterisation of EC growing on collagen particles, HAEC were seeded on Gelfoam® powder and grown for 13 to 14 days as for ECPCM production. For 4',6-diamidino-2-phenylindole (DAPI) staining, samples were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature, then incubated with 3µM DAPI (Invitrogen) in PBS for 5 minutes at room temperature and rinsed once with PBS. The Live/Dead Cell staining kit (Abcam) was used following manufacturer's instructions to visualise live EC on collagen particles. After each staining, a small amount of particle was mounted on glass slide with cover slip for imaging.

Glass coverslips were placed in a 24-well plate and covered with 1% Type B bovine collagen (Sigma) for 20 minutes at room temperature. Collagen was then removed and the coverslips allowed to dry at room temperature. EC were seeded on the coverslips and grown to confluence. Cells were then serum-starved in collection medium for 16 hours before being treated with 64.1 nM PF4, 2.39 nM IL-6 or 0.1 nM TNFα, in collection media (control) or ECPCM, for 5, 10 and 30 minutes and 1 or 2 hours at 37^0C, 5% CO_2. For analysis of phospho-Stat3, p65 and PECAM-1, cells were fixed in 4% PFA for 20 minutes at room temperature, washed three times in phosphate-buffered saline (PBS; 5 minutes per wash) and permeabilized with ice-cold methanol (MeOH) for 10 minutes at -20^0C. Cells were washed again three times

in PBS before addition of blocking buffer (5% bovine serum albumin (BSA), 0.3% Triton X100 in PBS) for 1-2 hours at room temperature. Immunofluorescence staining was performed in dilution buffer (1% BSA, 0.3% Triton X100 in PBS) for 16-18 hours at 4^0C using the following primary antibodies: rabbit anti-phospho-Stat3 (1:100; Cell Signalling), rabbit anti-p65 (1:100; Cell Signalling), sheep anti-PECAM-1 (1:40; R&D Systems). After three washes in PBS (5 minutes each), cells were incubated with the secondary antibodies AlexaFluor® 594 donkey anti-sheep and 488 donkey anti-rabbit (1:600; Invitrogen) for 1 hour at room temperature. Cells were washed three times in PBS (5 minutes each) before staining with 3µM DAPI (Invitrogen) for 3 minutes at room temperature. Cells were washed three times in PBS and once in water, then coverslips were mounted with ProLong® Gold anti-fade reagent (Invitrogen). Slides were analysed with an Olympus BX51 microscope and pictures were taken using the 60X objective.

For E-selectin staining, HAEC were treated for 6 hours in collection medium (control) or ECPCM containing PF4, IL-6 or TNFα. Cells were fixed in a 1:1 mixture of acetone:methanol for 15 minutes at -20°C. After three washes in PBS, blocking buffer was added to the cells for 2 hours at 4°C. Cells were then incubated with primary antibody mouse anti-E-selectin (1:50; R&D Systems) in blocking buffer for 16-18 hours at 4^0C. After three washes in PBS (5 minutes each), cells were incubated with the secondary antibody AlexaFluor® 488 donkey anti-mouse (1:600; Invitrogen) for 30 minutes at room temperature. Incubation with the secondary antibody only was also performed as a control for unspecific staining. The cells were washed three times in PBS (5 minutes each), then the coverslips were mounted with ProLong® Gold anti-fade reagent (Invitrogen). Slides were analysed with an Olympus BX51 microscope and pictures were taken using the 20X objective.

2.9 Fluorescence-activated cell sorting (FACS)

HAEC were seeded on a 6-well plate (4-5 x 10^5 cells/well) and grown to confluence in EGM2 without hydrocortisone. Cells were serum starved in collection medium for 16 hours before being treated with 0.1 nM TNFα or 2.39 nM IL-6 in collection medium (control) or ECPCM for 6 hours at 37^0C, 5% CO_2. Cells were washed twice with ice-cold PBS and trypsinized at room temperature for 10 minutes. Cells were then transferred to a 1.5 ml tube, centrifuged at 400 x *g* for 1 minute, and resuspended in PBS with 10% goat serum and 1% BSA at a volume of 1 x 10^6 cells/100 µl. The cells were then incubated for 30 minutes at 4°C on an end-to-end rotator. After two washes in PBS with 1% BSA, cells were stained with phycoerythrin-conjugated anti-VCAM-1 (0.125 µg/sample; eBioscience) or phycoerythrin-conjugated anti-E-selectin (0.5 µg/sample; eBioscience) in PBS with 10% goat serum and 1% BSA for 30 minutes at 4°C on an end-to-end rotator. Cells were washed twice in PBS with 1% BSA, centrifuged at 400 x *g* for 5 minutes and resuspended in 200 µl of PBS for immediate FACS analysis. Stained samples were compared to negative unstained controls (which underwent the same preparation, with the exclusion of the antibody incubation step). A FACScalibur (Becton Dickinson) was used for the analysis.

2.10 Cell viability assay

For the trypan blue assay, HAEC were seeded onto 24-well plates (50,000 cells/well) and grown at 37^0C, 5% CO_2 in EGM2 without hydrocortisone. For the calcein assay, HAEC were seeded on collagen-coated coverslips (50,000 cells/well) in 24-well plates as for immunofluorescent staining and grown at 37^0C, 5% CO_2 in EGM2

without hydrocortisone. When ~80% confluence was reached, the cells were serum-starved in collection medium for 16 hours and then incubated in collection medium (control) or ECPCM with or without IL-6 or TNFα for 2, 4 and 6 hours at 37^0C, 5% CO_2. Cell viability was determined at time 0 (starting time of treatments) and at each time point using the trypan blue or the calcein assay. For the trypan blue assay, cells in each well were trypsinized and then resuspended in 1ml of PBS + 5% FBS. The cell suspension was mixed 3:1 with trypan blue (Sigma) and cells were counted using a hemocytometer to determine the total number of live cells/ml. For the calcein assay, medium was removed, 250µl of 2 µM calcein AM (Invitrogen) in PBS were added to each well and then cells were incubated at 37^0C, 5% CO_2 for 10-20 minutes in the dark. Using a BX51 microscope, images of three non-overlapping fields of each well, on duplicate coverslips were taken at a magnification of 10X. Cells were counted using the automated cell counting software Image Pro Plus (Media Cybernetics). Cell viability at each time point was expressed as percentage of the total number of cells in control samples (time 0).

2.11 U937 attachment assay

For the fluorescence analysis, HAEC were seeded onto 96-well plates (5,000 cells/well) and grown at 37^0C, 5% CO_2 in EGM2 without hydrocortisone. For the counting of adherent cells, HAEC were seeded on collagen-coated coverslips (50,000 cells/well) in 24-well plates as for immunofluorescent staining and grown at 37^0C, 5% CO_2 in EGM2 without hydrocortisone. When confluence was reached, the cells were serum-starved in collection medium for 16 hours and then incubated in collection medium (control) or ECPCM with or without 2.39 nM IL-6 or 0.1 nM

TNFα for 5 hours at 37^0C, 5% CO_2. U937 cells (passage 4 to 7) were stained with 2 μM calcein AM (Invitrogen) in PBS for 20 minutes at 37^0C, 5% CO_2 and rinsed once in PBS before use. After 5 hours of cytokine treatment, the media on HAEC was removed and U937 cells resuspended in EGM2 without hydrocortisone were added on top of the EC (30,000 cells/well on the 96-well plate and 300,000 cells/well on the 24-well plate). After 30 minutes at 37^0C, 5% CO_2 the media was removed and EC were washed three times with PBS. Fluorescence quantification was performed immediately on the 96-well plate using a Modulus™ microplate reader (Turner Biosystems) (excitation at 490 nm; emission at 510-570 nm). Counting of the U937 cells attached to HAEC was performed using an Olympus BX51 microscope and a 10X objective: images of three non-overlapping fields of each well, on triplicate coverslips, were taken. The number of adherent U937 cells was determined using the automated cell counting software Image Pro Plus (Media Cybernetics). Variation in the number of cells was expressed as fold increase compared to the untreated control (collection medium). Differences between treatments were compared using Graph Pad software and one-way ANOVA followed by the post hoc Tukey test. The criterion for statistical significance was p-value < 0.05.

2.12 Treatment of ECPCM with proteinase K, RNase, agarose and heparin-conjugated agarose beads

For the proteinase K treatment 1 ml of ECPCM was heated at 95°C for 15 minutes. Approximately 0.2 units (5.5 mg) of the enzyme bound to agarose beads (Sigma) were added to the sample, which was then incubated at 55°C for 16 hours. The enzyme was removed from the medium by centrifugation at 2660 x *g* for 10 minutes

at 4°C. RNase A/T_1 mix (Fermentas) was used to eliminate single-strand RNA from ECPCM. For this treatment, 1 ml of conditioned medium was incubated with 20 µl of the enzyme mix at 37°C for 1 hour. After each treatment the ECPCM was cooled on ice and then stored at 4°C until use.

About 2 ml of agarose or heparin-agarose beads (Sigma) were washed twice with PBS and then incubated with 4 ml of ECPCM or collection medium at 4°C for 16 hours with gentle rotation. The beads were centrifuged at 106 x *g* for 3 minutes and the supernatant (agarose- or heparin-unbound collection medium or ECPCM) recovered and stored at 4°C until use.

2.13 Coomassie blue polyacrylamide gel staining

Forty µl of proteinase K-treated or untreated ECPCM were mixed 1:5 with a reducing lane marker sample buffer (Thermo Scientific) and boiled at 95^0C for 5 minutes. Samples were loaded on a 7.5% polyacrylamide gel for SDS-PAGE. The gel was then fixed for 30 minutes in 50:10:40 methanol:acetic acid:H_2O and stained another 30 minutes in Coomassie blue working solution (concentrated Coomassie blue solution (2 g brillant blue in 50 ml methanol + 6 ml acetic acid) diluted 3:58 in 5:40:10 methanol:acetic acid:H_2O). De-staining was performed in 45:10:45 methanol:acetic acid:H_2O until no background staining was observed.

2.14 Griess Reaction

Griess reagent kit for nitrite determination was purchase from Invitrogen and used according to manufacturer's instructions to calculate NO levels in collection medium, ECPCM, SMCPCM, 293PCM and PCM.

2.15 Enzyme-linked immunosorbent assay (ELISA)

ELISA kits for TGF-β1, IL-10, cAMP (all from R&D Systems) and PGI_2 (MyBioSource) were used, according to the manufacturer's instructions, to determine concentrations of these molecules in collection medium, ECPCM, SMCPCM, 293PCM and PCM.

An ELISA kit (eBioscience) was also used to evaluate the total and phosphorylated Stat3 levels in protein extracts. For this purpose HAEC were seeded on 6-well plates (4-5 x 10^5 cells/well) and grown to confluence in EGM2 without hydrocortisone. Cells were serum-starved in collection medium for 16 hours before being treated with 2.39 nM IL-6 in collection medium or ECPCM for 30 minutes, then ELISA was performed according to the manufacturer's instructions.

2.16 Dexamethasone and RU486 treatment

HAEC were seeded on 24-well plates (50,000 cells/well) and grown to confluence in EGM2 without hydrocortisone. Cells were serum-starved in collection medium for 16 hours before treatment. Dexamethasone (DEX) (Sigma) was resuspended in ethanol and used at a concentration of 100 nM; RU486 (Sigma) was also dissolved in

ethanol and used at a concentration of 1 µM. HAEC were treated with each drug or a combination of the two in collection media (control) or ECPCM with IL-6 or TNFα for 2 hours at 37^0C, 5% CO_2 and processed for gene expression analysis by real-time PCR as described above. A control treatment of 1 µl/ml ethanol in collection medium with IL-6 or TNFα was also included in the experiment to assess the effect of the solvent on gene expression.

2.17 Resolvins treatment

HAEC were seeded onto 24-well plates (50,000 cells/well) and grown to confluence in EGM2 without hydrocortisone. Cells were serum-starved in collection medium for 16 hours before treatment. Human resolvin D_2 and resolvin E_1 (Cayman Chemical) were resuspended in dimethyl sulfoxide (DMSO) and used at concentrations of 10 nM and 50 nM. HAEC were treated with resolvin D_2 or -E_1 in combination with IL-6 or TNFα in collection medium (control) or ECPCM for 2 hours at 37^0C, 5% CO_2. Cells were then processed for gene expression analysis by real-time PCR as described above. A control treatment of 1 µl/ml DMSO in collection medium with IL-6 or TNFα was also included in the experiment.

2.18 Protein extraction and western blot

EC were seeded onto 6-well plates (4-5 x 10^5 cells/well) and grown to confluence in EGM2 without hydrocortisone. Cells were serum-starved in collection medium for 16 hours before being treated with 2.39 nM IL-6 or 0.1 nM TNFα in collection medium (control) or ECPCM for 30 minutes or 6 hours –depending on the

experiment- at 37^0C, 5% CO_2. Cellular proteins were then extracted from each well. Cells were washed twice with PBS, scraped and centrifuged at 2660 x *g* for 1 minute. Pellets were resuspended in about 100 µl of radioimmunoprecipitation assay buffer (RIPA buffer; Sigma) with protease and phosphatase inhibitors (Thermo Scientific), left on ice for 10 minutes and cleared at 2820 x *g* for 10 minutes at 4^0C. Supernatant from each sample was collected, mixed 1:5 with a reducing lane marker sample buffer (Thermo Scientific) and heated at 98^0C for 5 minutes. SDS-PAGE was performed by loading 25-40 µl of each sample into a well in a 10% polyacrylamide gel. Proteins were then transferred onto a Hybond membrane. The membrane was incubated in a blocking solution (Tris-buffered saline (TBS) with 0.5% Tween and 5% BSA or non-fat dehydrated milk) at room temperature for 2 hours before incubation with the appropriate primary antibody dilution in blocking solution at 4^0C for 16-18 hours. The following primary antibodies were used: mouse anti-VCAM-1 (1:100, Santa Cruz Biotechnology); rabbit anti-E-selectin (1:200, Santa Cruz Biotechnology); mouse anti-β-actin (1:3000; Sigma); mouse anti-phospho-tyrosine (1:2000; Cell Signaling); mouse anti-annexin A2 (1:1000; AbCam); mouse anti-phospho-annexin A2 (1:1000, R&D Systems); rabbit anti-IkBα (1:5000; AbCam); rabbit anti-Stat3 (1:1000; Cell Signaling); rabbit anti-phospho-Stat3 (1:12,000; AbCam). After primary antibody incubation, membranes were washed at least three times for 10 minutes in TBS 0.5% Tween, then incubated for 1 hour at room temperature with the appropriate secondary antibody conjugated to horseradish peroxidase (GE Healthcare) diluted 1:1000 in blocking solution. Blots were developed using the GE Healthcare ECL+ System according to the manufacturer's instructions. Quantification of protein bands was performed using ImageJ software: for each sample, the density value of E-selectin and VCAM-1 bands was normalized

to the value of the β-actin loading control band and protein levels were expressed as relative density.

2.19 Phospho-tyrosines and mass spectrometry analysis

HAEC were seeded, grown and treated in collection medium or ECPCM with or without IL-6 as for the western blot analysis. The protein extract was divided in two and run simultaneously in the same chamber in separate polyacrylamide gels of the same percentage (10%). One gel was used for western blot anti-phospho-tyrosine analysis, whilst the other underwent coomassie blue staining. The anti-phospho-tyrosine blot was used to identify the differentially phosphorylated bands in ECPCM samples on the coomassie blue-stained gel. These bands were cut from the gel (using a clean blade for each one) and sent for mass spectrometry identification. Protein isolation from the gel and mass spectrometry analysis were performed by the proteomic facility at the UCL Institute of Child's Health, which uses an ESI-QTOF mass spectrometer.

2.20 Inhibition of NF-kB and Stat3

BAY 1170-85 (Enzo Life Sciences) and Stattic (Santa Cruz) were used to inhibit NF-kB and Stat3 activation, respectively. Each drug was dissolved in DMSO and used at a concentration of 2 μM.

For the western blot analysis, HAEC were seeded onto 6-well plates (4-5 x 10^5 cells/well) and grown to confluence in EGM2 without hydrocortisone. Cells were

serum-starved in collection medium for 16 hours before treatment. For NF-kB inhibition, HAEC were treated for 30 minutes with TNFα in collection medium with or without BAY 1170-85 or DMSO as vehicle control. To block Stat3 activation, HAEC were pre-treated for 1 hour with Stattic or DMSO in collection medium, then treated for another 30 minutes with IL-6 in collection medium with Stattic or DMSO as vehicle control. Protein extraction was performed after each treatment as described above.

To determine the effect of the inhibitors on E-selectin and VCAM-1 gene expression, HAEC were seeded onto 24-well plates (50,000 cells/well) and grown to confluence in EGM2 without hydrocortisone. Cells were serum-starved in collection medium for 16 hours before being treated for 2 hours with either TNFα in collection medium, with or without BAY 1170-85 or DMSO, or IL-6 in collection medium, with or without Stattic or DMSO. RNA extraction, reverse transcription and real-time PCR were then performed as described above. All treatments were performed at 37^0C, 5% CO_2.

2.21 mRNA stability assay

HAEC were seeded onto 24-well plates (50,000 cells/well) and grown at 37^0C, 5% CO_2 in EGM2 without hydrocortisone. When cells reached 80% confluence they were serum-starved for 16 hours in collection medium. HAEC were pre-treated for 2 hours with IL-6 or TNFα in collection medium before changing the medium to collection medium (control) or ECPCM with IL-6 or TNFα and 3 μg/ml of actinomycin D (Sigma). Cells were treated for 30 minutes, 1, 2 or 4 hours at 37^0C,

5% CO_2 and processed for gene expression analysis by real-time PCR as described above.

2.22 Chromatin immunoprecipitation (ChIP)

HUVEC were seeded onto 10 cm dishes (6 x 10^6 cells/dish) and grown to confluence in EGM2 without hydrocortisone. Cells were serum-starved in collection medium for 16 hours before being treated with 0.1 nM TNFα in collection medium (control) or ECPCM for 1 hour at 37^0C, 5% CO_2. Three 10 cm dishes were used for each treatment and cells were pooled together in one tube after scraping (see below). After cytokine treatment HUVEC were processed for chromatin fixation and extraction using the ChIP-IT Express kit (Active Motif) according to the manufacturer's instructions. Briefly, cells were fixed with 1% formaldehyde in washing medium (phenol red-free EBM, 50 µg/ml gentamicin) for 10 minutes at room temperature. The fixation reaction was stopped by adding the Glycine Stop-Fix Solution for 5 minutes at room temperature. Cells were then collected by scraping, centrifuged for 10 minutes at 660 x *g* at 4°C and lysed in 500 µl Lysis Buffer for 30 minutes on ice. To ensure cell lysis, cells were further homogenized with four strokes through a 32 gauge needle. Nuclei were pelleted by centrifugation for 10 minutes at 2660 x *g* at 4°C and then resuspended in 350 µl of Shearing Buffer. Sonication was performed on ice using a Sonopuls HD2070 ultrasonic homogenizer (Bandelin). Samples were subjected to four rounds of 11-second pulses at 30% power, with a 30-second rest on ice between pulses. Sheared chromatin was centrifuged for 10 minutes at 20820 x *g* at 4°C. Fifty µl of supernatant were used to assess the efficiency of the DNA

shearing and determine the DNA concentration (see below). The remaining chromatin was aliquoted and stored at -80°C until use in the immunoprecipitation.

DNA clean-up to evaluate DNA shearing and concentration was performed using phenol/chloroform extraction according to the ChIP kit manufacturer's instructions. DNA concentration was determined by measuring absorbance at 260 nm with a spectrophotometer.

Immunoprecipitation was performed using ~10 µg of DNA in all ChIP reactions and 4 µl of anti-p65 antibody (Active Motif) or control rabbit IgG (Cell Signaling). Samples were immunoprecipitated for 16-18 hours at 4°C on an end-to-end rotator. Final washing and DNA elution steps were performed according to the ChIP kit manufacturer's instructions. The obtained DNA was further purified using the Chromatin IP DNA Purification kit (Active Motif) and then used in real-time PCR. The primers and TaqMan probes used in the PCR reaction were designed by and purchased from Integrated DNA Technologies. The sequences are reported in Table 1.

E-selectin promoter	
Forward	5'-TTG TCC ACA TCC AGT AAA GAG G-3'
Reverse	5'-AGG CAT GGA CAA AGG TGA AG-3'
Probe	5'-/56-FAM/CCC CAA TGG CAT CCA AAA ACT TTC CC/36-TAMSp/-3'
VCAM-1 promoter	
Forward	5'-TTA ATA GTG GAA CTT GGCTGG G-3'
Reverse	5'-GGA GTG AAA TAG AAA GTC TGT GC-3'
Probe	5'-/56-FAM/TGT TGC AGA GGC GGA GGG AAA T/36-TAMSp/-3'

Table 1: Primers and probes used for ChIP analysis of the E-selectin and VCAM-1 promoters.

The immunoprecipitated DNA was quantified by creating a standard curve with known concentrations of the input DNA used in the ChIP analysis. Binding of the transcription factor to the specific promoter was calculated as fold-enrichment of the ChIP samples relative to the IgG samples.

2.23 *In vivo* experiments

Zymosan intra-peritoneal (IP) injection was performed in C57/BL6 female mice, 10-12 weeks old (Harlan laboratories) to induce peritonitis. 0.5 mg of zymosan (Sigma) resuspended in 1 ml of either collection medium or ECPCM were used for the treatment. After 1, 2, 4, 6, 8, 16, 18 or 24 hours mice were sacrificed and a peritoneal lavage was performed: a horizontal skin incision was made 1/3 of the distance from the anus and 5 ml of ice-cold PBS, 3% FBS were injected in the peritoneal cavity after the peritoneum had been lifted up to prevent bowel perforation. After about 1 minute of gentle abdominal massage the lavage liquid was recovered with a syringe. Inflammatory cells in the lavage were centrifuges at 100 x *g* for 5 minutes and resuspended in 10 ml PBS. Trypan blue exclusion cell counting was then performed. The cell number was adjusted to the volume recovered from each animal after peritoneal lavage to express the total cell number/ml.

The leukostasis assay was performed by perfusion-labelling with fluorescein isothiocyanate (FITC)-coupled concanavalin A lectin (Con A) (Vector). Deep anaesthesia was induced using ketamine/xylazine, then the chest cavity was opened and a 27 G cannula was introduced into the left ventricle. After injection of 10 ml of PBS to remove erythrocytes and non-adherent leukocytes, 10 ml of FITC-conjugated Con A lectin were perfused. The eyes were then enucleated and the retinas dissected

and flat-mounted. Retinas were imaged using a fluorescence microscope (Olympus BX51) and the total number of Con A-stained adherent leukocytes per retina was determined.

Experiments were performed on JR5558 mice on a C57Bl/6 background kept as a homozygous line (Jackson Laboratory). Animals were kept on a 12-hour light–dark cycle and all experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. At the age of postnatal day (P) 22 mice were assessed for CNV lesions through fundus fluorescein angiography. The pupils of the mice were dilated with 2.5% tropicamide (Bausch & Lomb), and 0.2 ml of 2% fluorescein sodium (Bausch & Lomb) diluted in water was administered by intraperitoneal injection. Fluorescein angiograms were obtained at early (~90 seconds after fluorescein injection) and late (~6 minutes after injection) phases of dye transit using a Kowa Genesis-Df fundus camera (Kowa). The animals received daily intraperitoneal administration of 0.5 ml of ECPCM for 7 days, from P22 to P28. At the age of P29 CNV lesions were analysed again by fluorescein angiography. The area of the lesions was calculated using Photoshop software: for each FA image, the CNV lesions were circled using the lasso tool and the area (expressed in pixels) was measured and recorded using the measurement log. Measurements of all CNV lesions from each eye were added together to provide a total area of CNV per retina. Macrophage quantification was performed by direct cell counting on masked samples: three CNV lesions were analysed on three separate eyes for each group, for a total of nine images for each treatment. Statistical analysis to compare the lesions' areas and the macrophages number in PBS- and ECPCM-treated mice was performed with GraphPad software using the Mann-Whitney t- test.

On P30 mice were euthanized in a CO_2 chamber. Eyes were enucleated and fixed with 4% PFA for 16-18 hours. Eyes were then dissected to isolate the eyecup (consisting of the RPE, choroid and sclera), permeabilized in 0.5% Triton X-100 for at least 2 hours at room temperature and incubated in blocking buffer (5% BSA in PBS) for another 2 hours. Staining was performed by incubating the eyecups for 16 or 18 hours at 4°C with rabbit anti-PECAM-1 and rat anti-F4/80 primary antibodies (both at 1:300; Abcam) in 0.5% Triton X-100 with 5% BSA. AlexaFluor® 488-conjugated goat anti-rabbit and AlexaFluor® 594-conjugated goat anti-rat secondary antibodies (1:600; Invitrogen) in 0.5% Triton X-100 with 5% BSA were then applied for 1½ hours at room temperature. Images were obtained with an Olympus BX51 microscope using the 20X objective.

Chapter 3: Results

Chapter 3.1: Characterisation of endothelial cells grown on collagen particles

3.1.1 Establishment of optimum culturing conditions for endothelial cells on collagen particles

Before starting the investigation of ECPCM anti-inflammatory effects, the optimal culturing conditions for EC grown on collagen particles were established, including the amount of FBS in the culture media, and the optimum length of time to allow the cells to grow to confluency and thus become quiescent.

Preliminary data from Pervasis showed that growth of HAEC on collagen particles in standard EGM2 medium did not produce the desirable cell yield in three weeks, as less than 1.5 million cells were produced per culture (data not shown). In order to improve the growth profile, additional fetal bovine serum (FBS) was added. HAEC were cultured on collagen particles in 50 ml tubes with growth medium containing 2% FBS (standard EGM2), 5% FBS, 7% FBS and 10% FBS for 14 days. Cell counts were performed at days 5, 8, 12 and 14 of culture. The resulting growth curves for each condition are shown in figure 12. In general, media containing higher percentages of FBS promoted greater cell growth; by day 14 the 10% FBS cultures yielded more than 2.5 x 10^6 cells, compared to less than 1 x 10^6 cells in the 2% FBS condition. The 5% and 7% FBS cultures had cell yields higher than that of the 2% cultures, but still lower than the 10% condition. Medium containing 10% FBS was subsequently used to support the robust growth of HAEC on collagen particles.

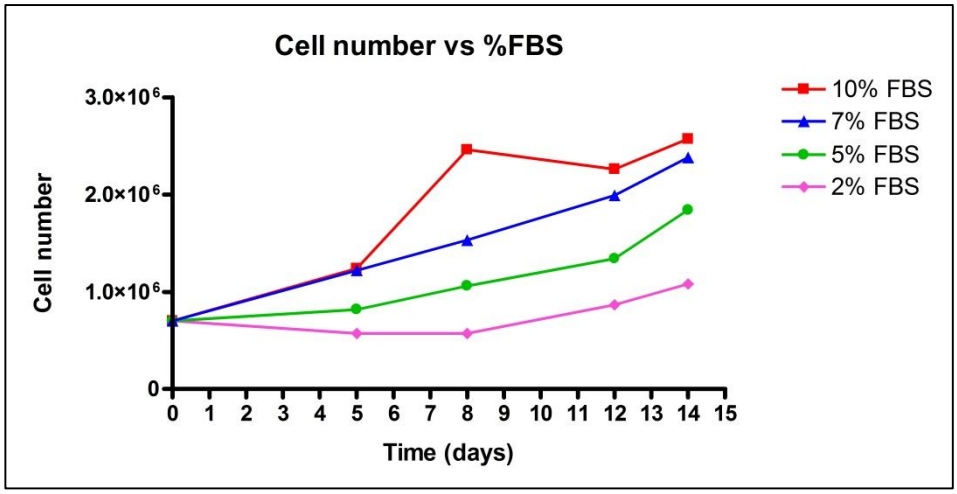

Figure 12: Viable HAEC number per tube at various days of culture on collagen particles using EGM2 medium supplemented with different concentrations of FBS. The experiment was repeated three times.

Since experiments on EC using conditioned media are usually performed in standard EGM2 medium (2% FBS) and/or EGM2 with only 0.5% FBS, the amount of FBS in the culture medium must be reduced from 10% to 2% before the EC/matrix culture can be used for ECPCM production. To facilitate this change, the optimal culture time with medium containing 10% FBS before switching to regular EGM2 media with 2% FBS was determined. Cells were transitioned from 10% FBS to regular 2% FBS EGM2 media at days 6, 8 and 10; then cells were cultured to day 13 in standard EGM2. The effects of the medium switch on EC growth and on growth factor levels were determined by counting the number of viable cells at each time point and by measuring transforming growth factor beta 1 (TGF-β1) concentration in the conditioned medium on the last day of culture. Figure 13 and table 2 show that medium switch on day 10 provided the best cell growth and appropriate TGF-β1 concentration at about 500 pg/mL. A minimum TGF- β1 concentration of 300 pg/mL has been established by Pervasis to be one of the criteria for establishing a mature and quiescent EC/matrix culture ((Nugent HM 2009). Therefore, for subsequent studies, EC/particles were cultured in medium containing 10% FBS for the first 10

days and 2% FBS for an additional three days prior to generating ECPCM. Numbers of viable cells and levels of TGF-β1 were determined from a selected tube from each lot of EC/particle cultures on day 14 to confirm the consistency of the cultures prior to ECPCM production.

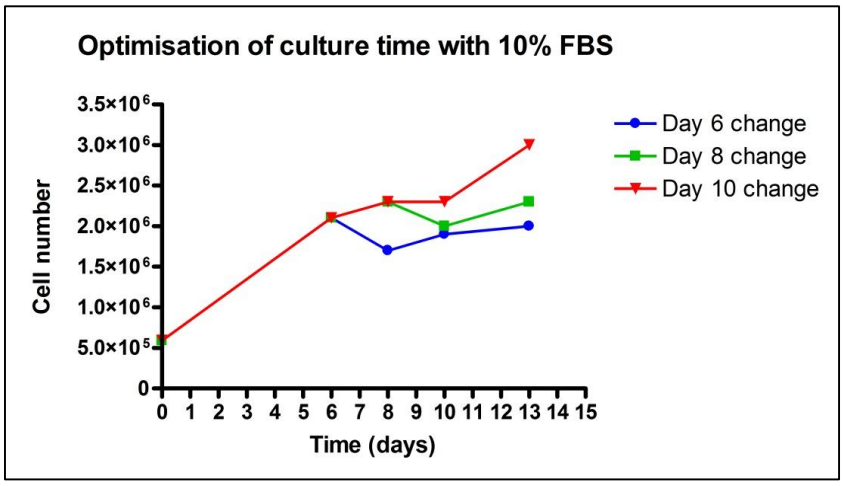

Figure 13: Number of viable cells in EC/particle cultures grown in EGM2 containing 10% FBS for 6 days, 8 days or 10 days before changing to EGM2 medium containing 2% FBS.

	D6 Day 13	D8 Day 13	D10 Day 13
TGF-β1 (pg/ml)	451	504	552

Table 2: Average concentration of TGF-β1 in culture medium at day 13 after switching from 10% to 2% FBS at day 6, day 8 or day 10 (D6, D8, D10).

3.1.2 Phenotype characterisation of endothelial cells growing on collagen particles

In order to study the phenotype of EC growing on collagen particles, the cellular morphology was analysed by immunocytochemistry and electronic microscopy (EM).

HAEC were grown on collagen particles for 14 days, and then stained with DAPI (nuclear stain) or a live/death kit to visualize the growth pattern of EC on the 3-D matrix. Figure 14 shows that HAEC growing on collagen particles were abundant, alive and well-distributed throughout the matrix. Cells appeared confluent and organized into a loosely connected network that followed the particle surface to give total coverage; however, there is no strong evidence of any tube-like vascular structures within the matrix and therefore no obvious evidence of angiogenesis by EC in the 3-D particle culture.

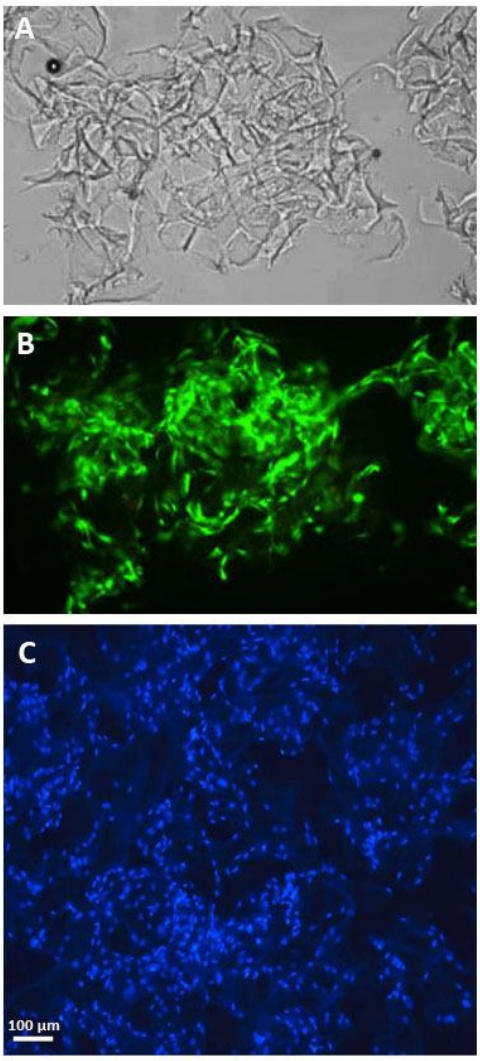

Figure 14: Collagen particles seeded with HAEC after 13 days of culture. A) light microscopy image; B) viable EC (green) after staining with the live/death kit; C) DAPI nuclear staining (blue).

Collagen particles seeded with EC have a soft, jelly-like consistency; their very low density prevented any examination by EM. However, the collagen sponges from which the collagen particles are made have a matrix that is significantly denser. Collagen sponges were therefore used to grow EC and were subsequently processed for EM analysis. The collagen sponge and the particles are made of identical materials and provide a similar 3-D environment for EC growth; therefore the sponge represents a valid surrogate to study morphology of EC grown on a 3-D collagen matrix.

HAEC were seeded on collagen sponges, grown for 14 days as on the particles and then processed for EM analysis. Figure 15 shows that EC largely adhered to the surface of the collagen matrix, and spread by following the contour of the matrix. Areas of wrinkled cell membrane that did not contact the matrix were visible (figure 15-B, C and E), and regions of apparent collagen degradation were also observed (figure 15-A, B and D); however, these phenotypes were observed only infrequently. In other areas, production of a basement membrane and ECM was evident (figure 15-G). Although in most images only a single EC was visible, examination of serial EM images demonstrated that EC in this environment extend protrusions to contact other EC in their proximity (figure 15-C, left corner), forming a network structure similar to that observed by light microscopy of collagen particles. Formation of tube-like structures resembling blood vessels was not observed. Overall, EM analysis shows that HAEC grown on a collagen matrix formed connecting networks, but not vessel-like luminal structures, and this unique morphology is significantly different from that of HAEC grown on tissue culture plastic, as cells cultured on plastic flatten and form a continuous sheet.

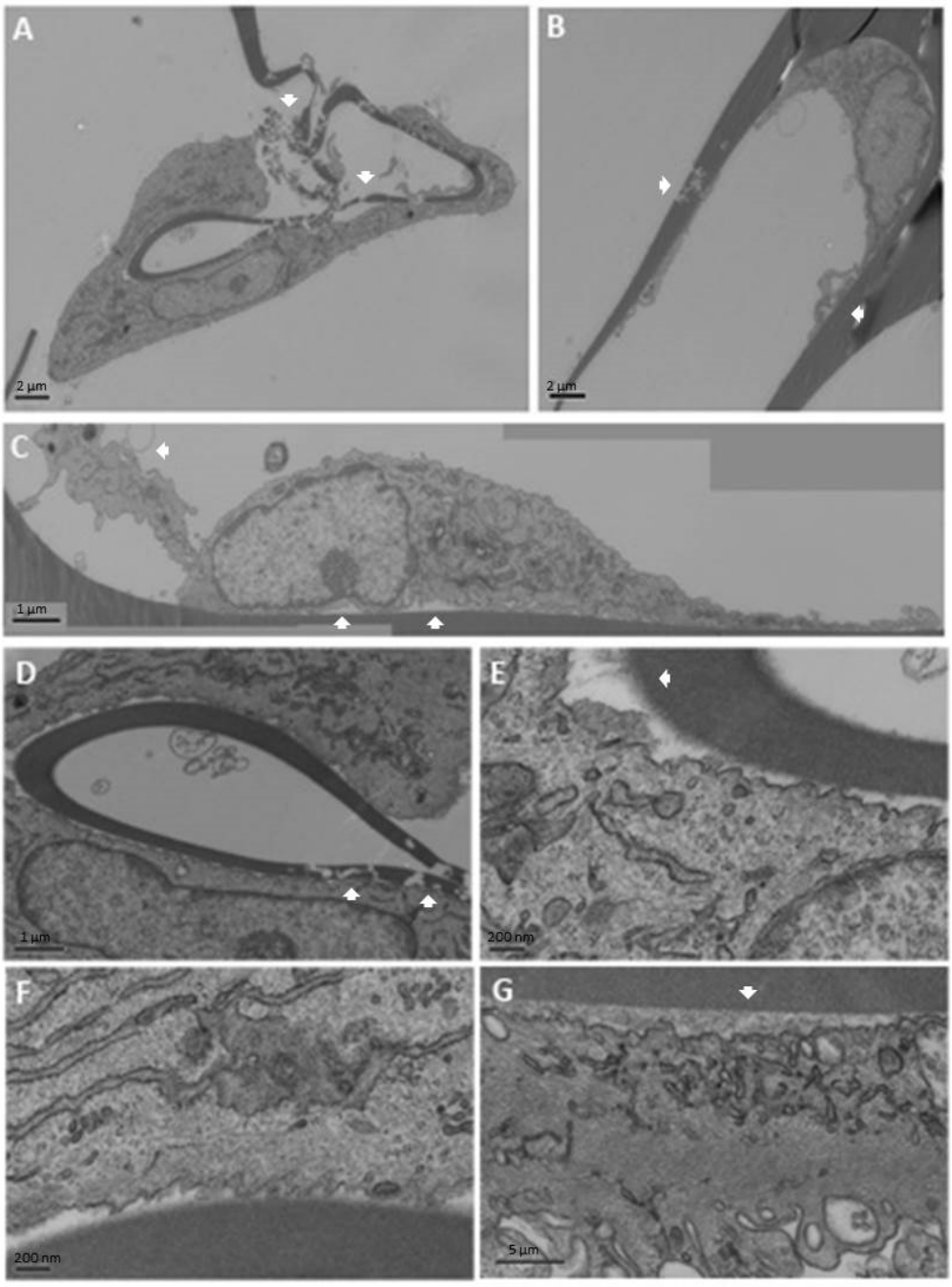

Figure 15: EM images of HAEC grown on collagen sponge for 14 days (10 days in medium containing 10% FBS followed by 4 days in medium containing 2% FBS). D-G are higher magnifications of A.

3.1.3 Characterisation of endothelial cells grown on collagen particles: conclusions and discussion

As mentioned in the introduction, normal EC are usually in a quiescent state that promotes vascular homeostasis. Endothelial quiescence is achieved and maintained through cell contact between confluent EC and through the production and release of a wide array of anti-inflammatory, anti-thrombotic and anti-coagulant factors (Davies MG 1993; Sumpio BE 2002; Pober JS 2007). Since ECPCM is obtained from quiescent EC, it should contain anti-inflammatory activity, activity that could account for the therapeutic effects of Vascugel® in supressing IH and might also suppress inflammatory neovascularization in the eye.

The results presented in this section show that collagen particles in a 50 mL tube provide an effective culturing system for the growth of EC and the production of ECPCM. HAEC grown on this 3-D matrix for up to 14 days were abundant and viable when EGM2 medium supplemented with 10% FBS was used for the first 10 days, and then switched to regular EGM2 with 2% FBS for the last 3-4 days of culture. EC displayed an interesting network morphology, with long cytoplasmic and plasma membrane extensions allowing interaction with neighbouring cells, that covered the available surface of collagen matrix. The culture reached confluency/quiescence in 13 days. Further evidence of the quiescent nature of the EC/particles was provided by the high levels of TGF-β1 detected in the conditioned media on day 13.

Over the past three decades 3-D matrices of various types have been used for *in vitro* studies of angiogenesis, a process in which new vessels develop from the pre-existing vasculature. These methods included Matrigel, a mixture of ECM and

basement membrane proteins; fibrin clots; and co-culture of EC with fibroblasts or other perivascular cells, with or without addition of ECM (Staton CA 2004). In these assays the 3-D matrix offers physical support to the EC, allowing the EC to form capillary-like tubule structures in a process that resembles the angiogenic events that take place *in vivo*. EC engaged in angiogenesis have different proliferation, migration, rearrangement and differentiation properties compared to quiescent EC (Staton CA 2004). The fact that no tube-like structures were observed in the images obtained from HAEC grown on collagen particles suggests that EC were not involved in an angiogenic program, but rather formed a network and became quiescent, in this 3-D culture system.

A successful method for culturing HAEC on collagen particles was established. EC in this system grow as a complex cellular network and achieve a quiescent state. This culturing method was used to generate EC/particle cultures for the production of ECPCM containing anti-inflammatory activity.

Chapter 3.2: Demonstration of ECPCM anti-inflammatory activity *in vitro*

3.2.1 ECPCM inhibits E-selectin and VCAM-1 expression in EC *in vitro*

In order to study the anti-inflammatory activity of ECPCM, an *in vitro* assay based on gene expression analysis was developed. Conditions of the assay were optimized by testing different kinds of primary human endothelial cells (aortic, microvascular and umbilical vein EC), different cytokines at various concentrations and different durations of treatment. The expression of various genes known to be involved in inflammation was tested under these conditions. The optimal *in vitro* assay was obtained by using HAEC or HUVEC stimulated with 64.1 nM PF4 for 4 hours or with 2.39 nM IL-6 or 0.1 nM TNFα for 2 hours (data not shown). Among the genes tested, E-selectin and VCAM-1 showed the highest induction by each of the three chemokines/cytokines, therefore they were chosen as inflammation markers in subsequent real-time PCR analysis.

Although IH affects both arteries and veins (see Introduction), most clinical cases are caused by angioplasty to treat atherosclerosis in coronary arteries and large arteries in peripheral artery disease (Nugent HM 1999; Newby AC 2000; Collins MJ 2012). In addition, this thesis is based on preliminary data obtained from HAEC in culture and animal models of angioplasty performed on large arteries (Nathan A 1995; Nugent HM 1999; Nugent HM 2009; Nugent HM 2012). For these reasons arterial EC were used for most of the *in vitro* experiments presented in this thesis, and HUVEC were used when large number of cells were needed for the experiment, for example in the chromatin immunoprecipitation (ChIP) assay.

In order to test the anti-inflammatory activity of ECPCM, HAEC were treated in collection (control) medium or ECPCM in the absence or presence of PF4, IL-6 or TNFα using the concentrations and times noted above. As shown in figure 16, ECPCM was able to strongly inhibit the expression of E-selectin stimulated by all three factors. Compared to the controls containing collection medium and the stimulatory factor, ECPCM reduced E-selectin expression in response to PF4 treatment by 88% ($p<0.001$), in response to IL-6 treatment by 56% ($p<0.001$), and in response to TNFα treatment by 50% ($p<0.001$). The inhibition of VCAM-1 expression by ECPCM was lower, but still significant: ECPCM reduced TNFα-induced VCAM-1 expression by 38% ($p<0.001$), PF4-induced expression by 44% ($p<0.001$) and IL-6-induced expression by 51% ($p<0.001$) compared to controls.

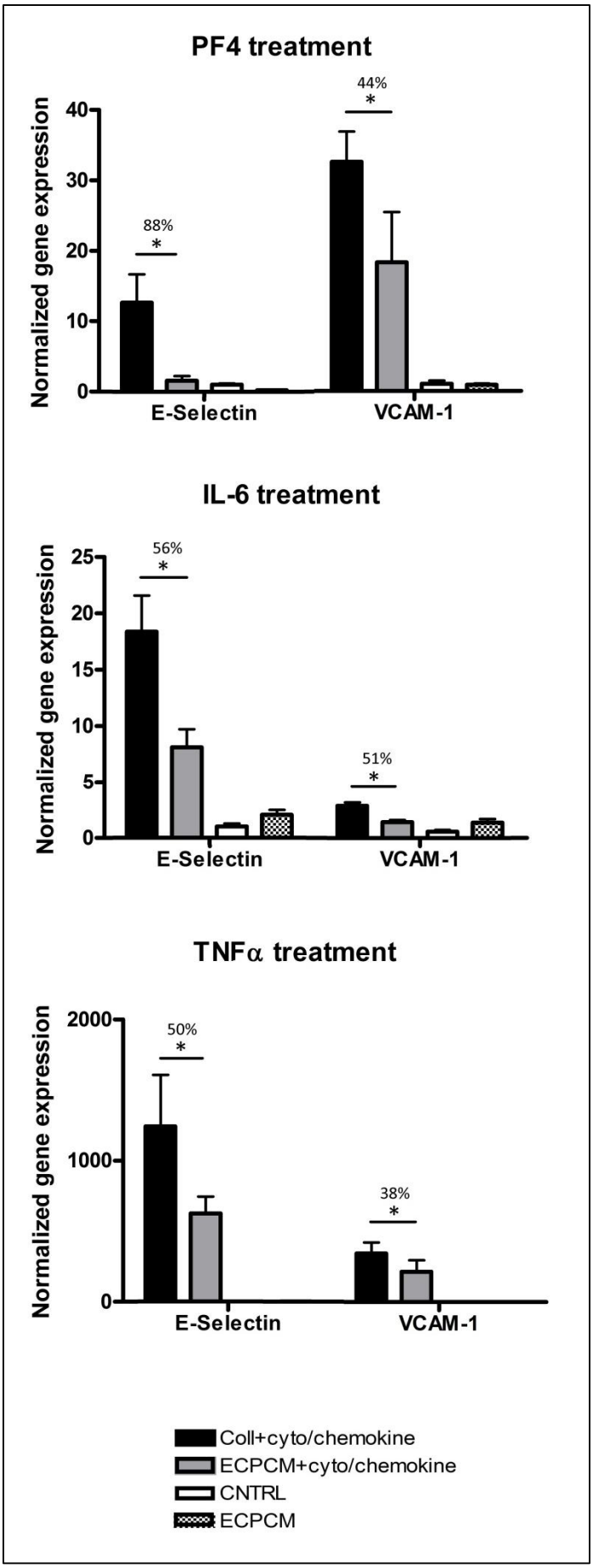

Figure 16: Inhibition of E-selectin and VCAM-1 gene expression by ECPCM. HAEC were treated with 64.1 nM PF4 for 4 hours or 2.39 nM IL-6 or 0.1 nM TNFα for 2 hours in collection medium or ECPCM. CNTRL: collection medium alone. Coll: collection medium. *p-value <0.001 compared to treatment with PF4, IL-6 or TNFα in collection medium. Data = mean ± SD; n (individual well) = 12 per group.

Expression of E-selectin and VCAM-1 was also analysed at the protein level in HAEC treated with PF4, IL-6 and TNFα, with and without ECPCM treatment. Immunofluorescent analysis showed that ECPCM suppressed E-selectin protein expression in response to all three of the pro-inflammatory molecules tested (figure 17).

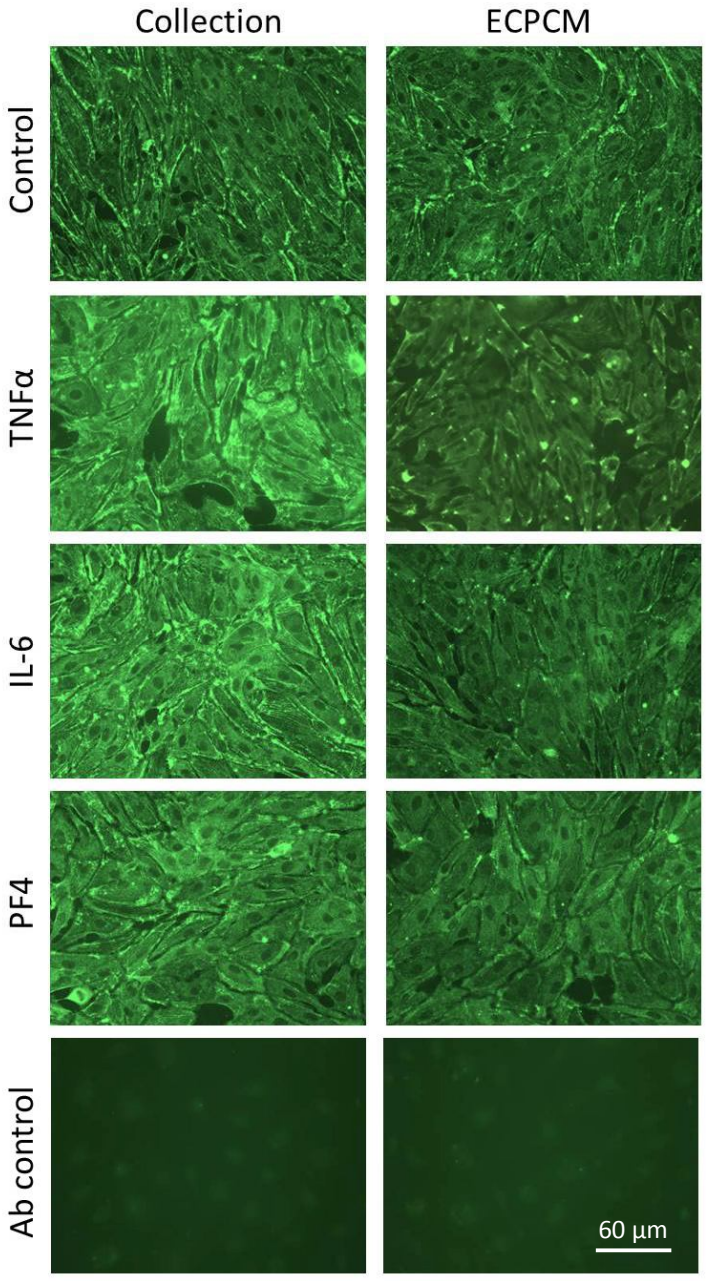

Figure 17: ECPCM decreases E-selectin protein expression induced by TNFα, IL-6 and PF4. Representative image of immunofluorescent staining of E-selectin (green) in HAEC treated for 6 hours with 0.1 nM TNFα, 2.39 nM IL-6 or 64.1 nM PF4, in collection medium or ECPCM. Control: cells not treated with chemokines/cytokines. Ab control: cells treated with TNFα and then incubated only with the secondary antibody for unspecific staining control. The experiment was repeated three times with similar results.

To further investigate the effect of ECPCM on cytokine-induced E-selectin and VCAM-1 protein expression in EC, FACS and western blot analyses were performed on HAEC treated for 6 hours with TNFα or IL-6 in collection medium or ECPCM. As shown in figures 18 and 19, ECPCM suppressed the cytokine-induced expression of E-selectin and VCAM-1 proteins compared to treatment in collection medium control.

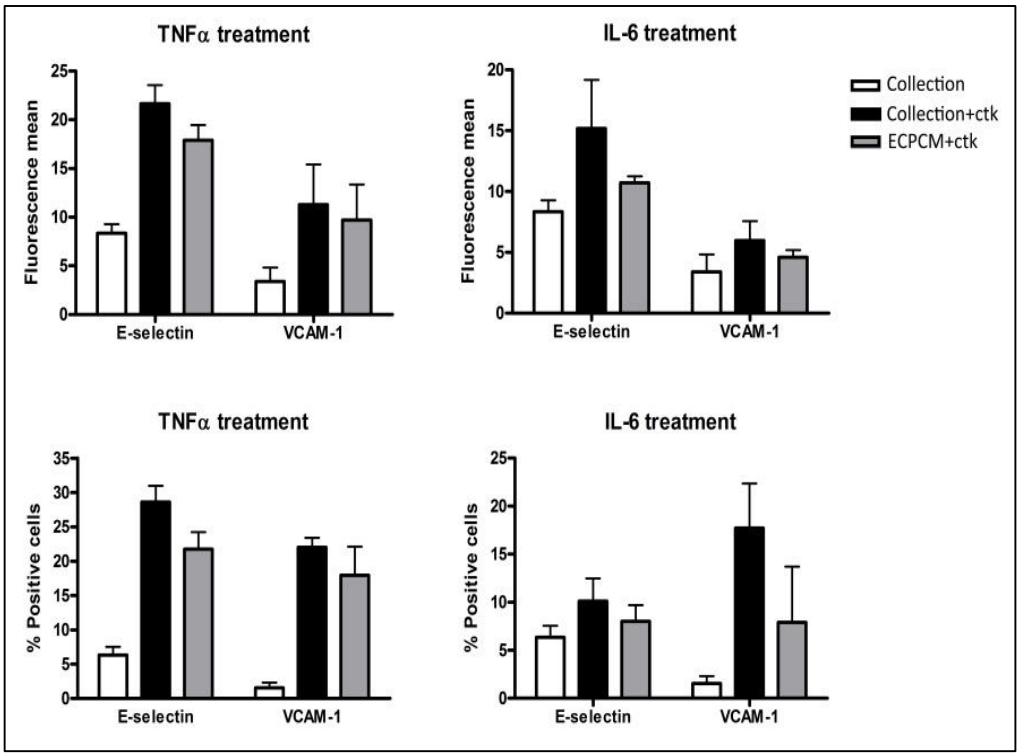

Figure 18: ECPCM decreases cytokine-induced E-selectin and VCAM-1 protein expression by FACS analysis. HAEC were treated for 6 hours with or without 0.1 nM TNFα or 2.39 nM IL-6 in collection medium or ECPCM and then processed for FACS analysis with anti-E-selectin or anti-VCAM-1 antibody. Protein expression is presented as fluorescence mean value (upper graphs) and percentage of positively stained cells (lower graphs). In all experiments, cytokine treatment significantly induced E-selectin and VCAM-1 protein levels, and ECPCM reduced TNFα- and IL-6-induced expression of both adhesion molecules. Data = mean ± SD, n (individual well) = 6 per group.

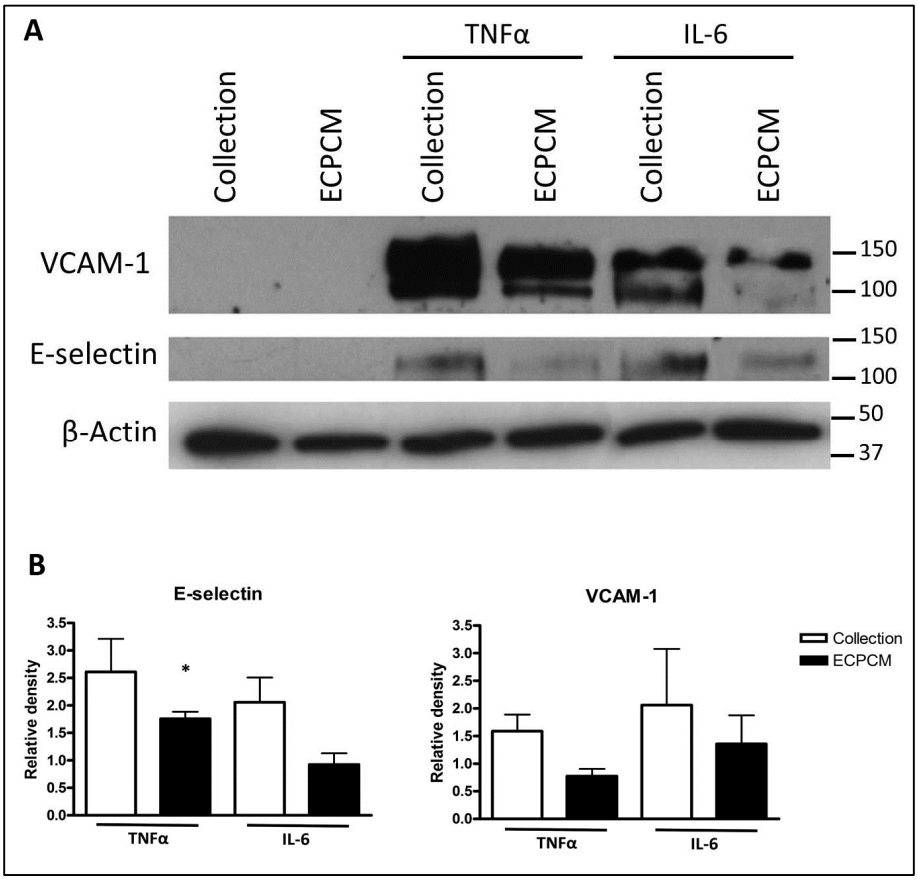

Figure 19: ECPCM decreases cytokine-induced E-selectin and VCAM-1 protein expression by western blot analysis. A) HAEC were treated for 6 hours with or without 0.1 nM TNFα or 2.39 nM IL-6 in collection medium or ECPCM. Western blot analysis was performed with anti-VCAM-1, anti-E-selectin and anti-β-Actin antibodies. The image is representative of three separate experiments. B) Relative density quantification of western blot bands performed using ImageJ software. E-selectin and VCAM-1 bands were normalised to the β-Actin loading control band. Data = mean ± SD; n = 3 per treatment. *p-value <0.05.

At the time point tested, cytokines-induced E-selectin and VCAM-1 protein expression quantification by FACS and western blot analyses showed a strong trend of reduction upon ECPCM treatment, and significant reduction in TNF-α-induced E-selectin protein expression was detected by western blot (Figure 19-B). The lack of significant results was mostly because of technical difficulties for detecting both E-selectin and VCAM-1 proteins by using commercially available antibodies. However, as a whole, a reduction of E-selectin and VCAM-1 protein levels in EC

treated with ECPCM was evident, validated the real-time PCR gene expression data and confirmed the anti-inflammatory effects of ECPCM *in vitro*. Therefore, monitoring of gene expression for E-selectin and VCAM-1 via real-time PCR analysis as a more sensitive and robust assay was used to assess the anti-inflammatory activity of ECPCM *in vitro*.

3.2.2 ECPCM treatment does not affect viability of EC

In order to verify that the ECPCM-mediated decrease in E-selectin and VCAM-1 expression was not caused by an increase in cell death, EC viability was analysed during treatment. HAEC were treated in collection medium or ECPCM for 2, 4 and 6 hours with or without IL-6 or TNFα, and the number of live cells was determined at each time point using two different stains: trypan blue and calcein AM. Trypan blue is a dye commonly used to count viable cells in a cell suspension and it is based on the principle that live cells with an intact cell membrane exclude the dye and show a clear cytoplasm, whereas dead cells appear blue under a light microscope. Calcein AM is a cell-permeable fluorogenic dye that in live cells is converted by intracellular esterases into calcein, the fluorescent product, whereas no fluorescent product will be made in dead cells.

As shown in figure 20, ECPCM did not affect cell viability compared to treatment in collection (control) medium. Although very small differences in cell viabilities were observed between the collection medium control and ECPCM group during IL-6 and TNFα treatments, they were not statistically significant.

These data show that ECPCM does not affect cell viability and prove that the reduction in E-selectin and VCAM-1 gene and protein expression during cytokine treatment in EC was a specific effect induced by ECPCM. These results also validate the *in vitro* gene expression assays as a tool to study ECPCM anti-inflammatory activity.

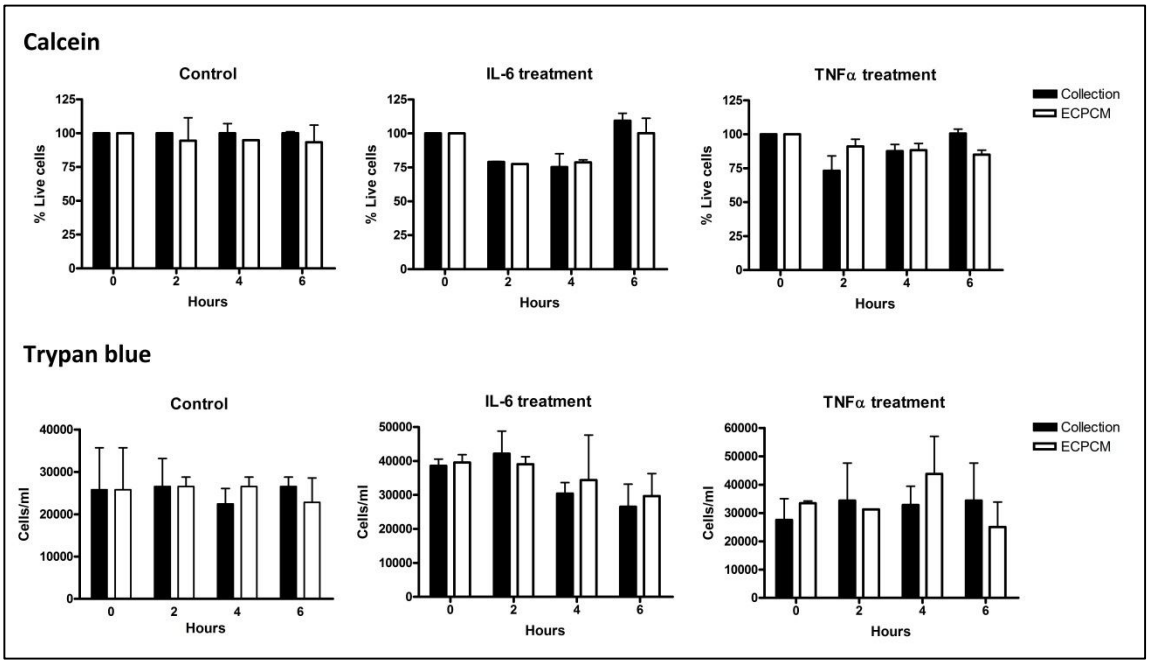

Figure 20: Cell viability during treatment in ECPCM or collection medium. HAEC were treated for 2, 4 or 6 hours in collection medium or ECPCM with or without IL-6 or TNFα. The number of live cells was determined at each time point using trypan blue exclusion or a calcein AM staining assay. The survival rate was expressed as percentage of the total number of live cells in control samples (time 0) in the calcein assay and as the total number of live cells/ml in the trypan blue assay. Data = Mean ± SD; n (individual well) = 3 for each group.

3.2.3 ECPCM reduces attachment of U937 monocyte-like cells to HAEC

In order to study the functional effects of ECPCM anti-inflammatory activity, an *in vitro* monocyte-EC attachment assay was developed. For this purpose U937 cells were used as the monocytes; these cells are derived from a leukemic monocyte

lymphoma line and display many characteristics of monocytes and macrophages, including attachment to EC in response to inflammatory stimuli.

HAEC were activated with inflammatory cytokines TNFα or IL-6 in collection medium (control) or ECPCM for 5 hours, then U937 cells previously stained with the fluorogenic dye calcein AM were added on top of the EC and allowed to attach for 30 minutes. After extensive washing with buffer to remove non-adherent cells, attachment of U937 cells to the activated EC was quantified both with a fluorometer and by counting the monocytes under the microscope.

Treatment in ECPCM caused a significant reduction in the number of U937 cells attached to cytokine-activated EC, based on both the fluorescence emission and cell counting methods (figures 21 and 22).

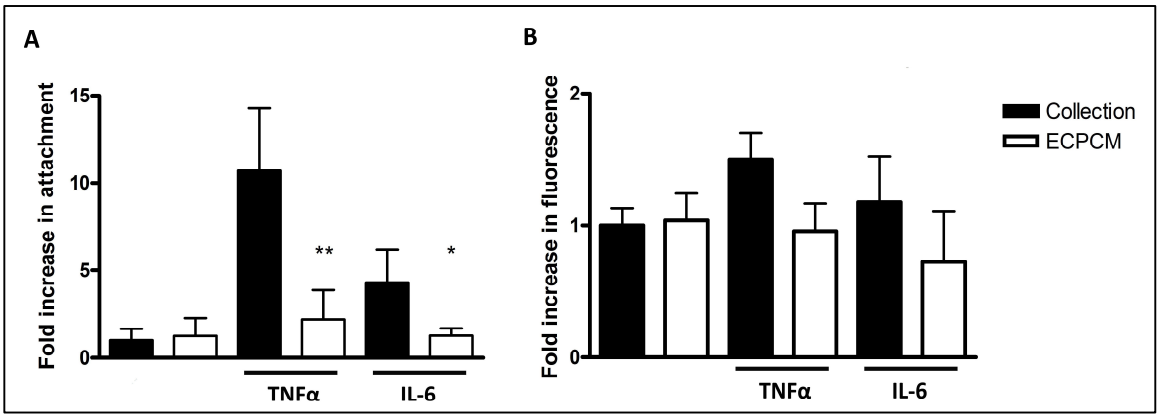

Figure 21: U937 attachment assay. HAEC were treated for 5 hours in collection medium or ECPCM with or without TNFα or IL-6. U937 cells were stained with calcein AM and then placed on top of HAEC for 30 minutes. After three rinses with PBS, U937 attachment to EC was determined by counting the fluorescent cells with a microscope (A) and by fluorescence quantification with a fluorometer (B). Variations in the number of attached cells or fluorescence intensity are expressed as fold changes over the control (treatment in collection medium).
p-value: *< 0.05; **< 0.001. Data = Mean ± SD; n (individual well) = 3 for each group.

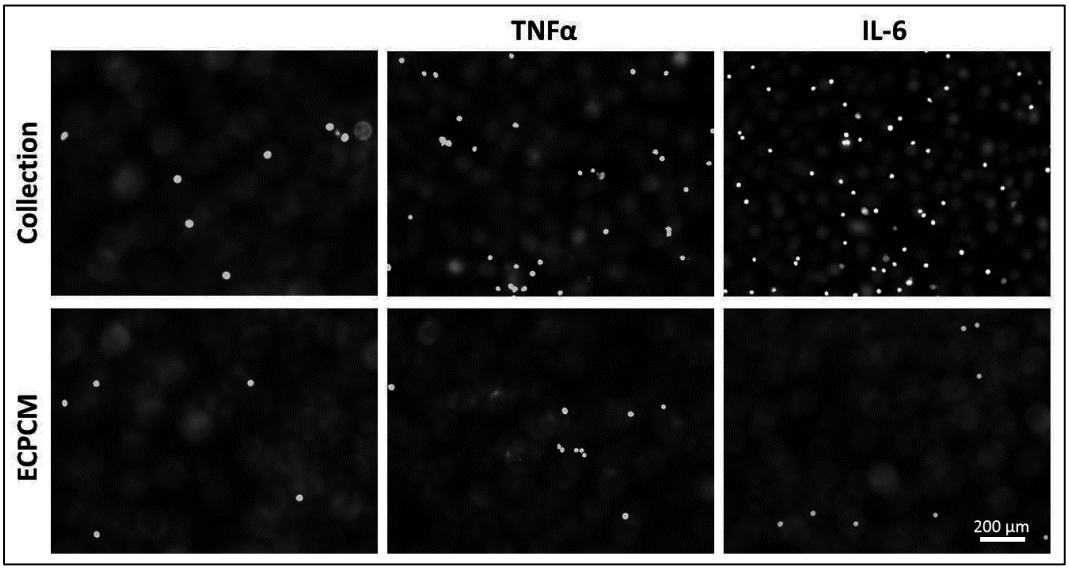

Figure 22: Representative images of U937 cells attached to cytokine-activated HAEC treated in collection medium or ECPCM.

These data suggest that ECPCM treatment inhibits immune cell attachment to cytokine-activated EC, likely by reducing expression of adhesion molecules such as E-selectin and VCAM-1 by the EC, and confirm an anti-inflammatory effect of ECPCM on the endothelium.

3.2.4 ECPCM anti-inflammatory effects are dose-dependent

In order to determine if the inhibitory effects of ECPCM on cytokine/chemokine-induced expression of E-selectin and VCAM-1 by HAEC are mediated by soluble factors, and to quantify this inhibitory activity, ECPCM was serially diluted in collection (control) medium and tested in the *in vitro* gene expression assay.

Data from the *in vitro* gene expression assay clearly showed a dose-dependent anti-inflammatory effect of ECPCM, in which IL-6- and TNFα-induced expression of both E-selectin and VCAM-1 was progressively lower with increasing doses of

ECPCM (figure 23). Inhibition of IL-6-induced gene expression by ECPCM was particularly strong. Indeed, during treatment with this cytokine half of the inhibition potency was delivered by a formulation containing between 23% and 33% ECPCM (table 3). The half-maximal inhibitory concentration (IC50) of ECPCM against TNFα treatment was slightly higher (~50% ECPCM) for both E-selectin and VCAM-1 genes (table 3). These data further support the idea that soluble factors in the ECPCM are responsible for its anti-inflammatory effects.

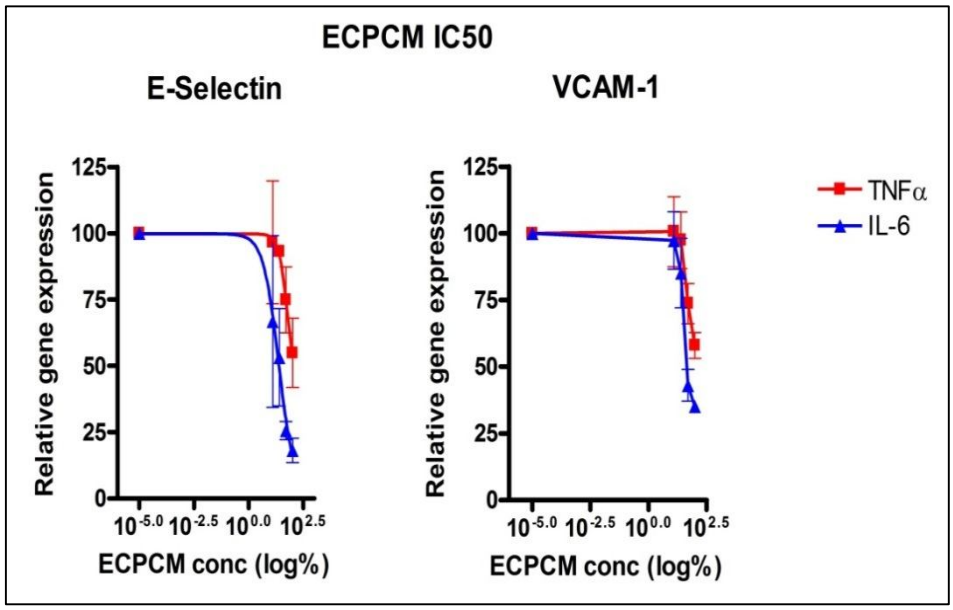

Figure 23: ECPCM anti-inflammatory effects are dose dependent. HAEC were treated for 2 hours with 2.39 nM IL-6 or 0.1 nM TNFα in collection medium, ECPCM or serial dilutions (1:2) of ECPCM in collection medium. The graphs show E-selectin and VCAM-1 % expression relative to treatment in the absence of ECPCM. Data = Mean ± SEM.

	ECPCM IC50	
	E-selectin	VCAM-1
IL-6	23%	33%
TNFα	57%	46%

Table 3: IC50 of ECPCM expressed as % of ECPCM in collection medium.

3.2.5 Pre-treatment with ECPCM reduces inflammatory gene expression in response to IL-6 but not TNFα

To better understand the kinetics and molecular mechanism of the anti-inflammatory effects of ECPCM, HAEC were incubated with EPCPM for 2 hours and then treated with IL-6 or TNFα in collection medium for an additional 2 hours. The expression levels of E-selectin and VCAM-1 were then determined by real-time PCR.

The results suggest that the effect of the pre-treatment in ECPCM was different for the two cytokines (figure 24): IL-6-induced expression of both E-selectin and VCAM-1 was significantly reduced after the pre-incubation in ECPCM, although this treatment strategy was less potent than direct treatment with ECPCM. On the other hand, pre-treatment with ECPCM had no inhibitory effect on the gene expression induced by TNFα.

The results suggest the anti-inflammatory factor(s) in ECPCM has a short-term and reversible effect on both IL-6- and TNFα-induced expression of E-selectin and VCAM-1, although its anti-inflammatory effect on IL-6-induced gene expression seems to last longer.

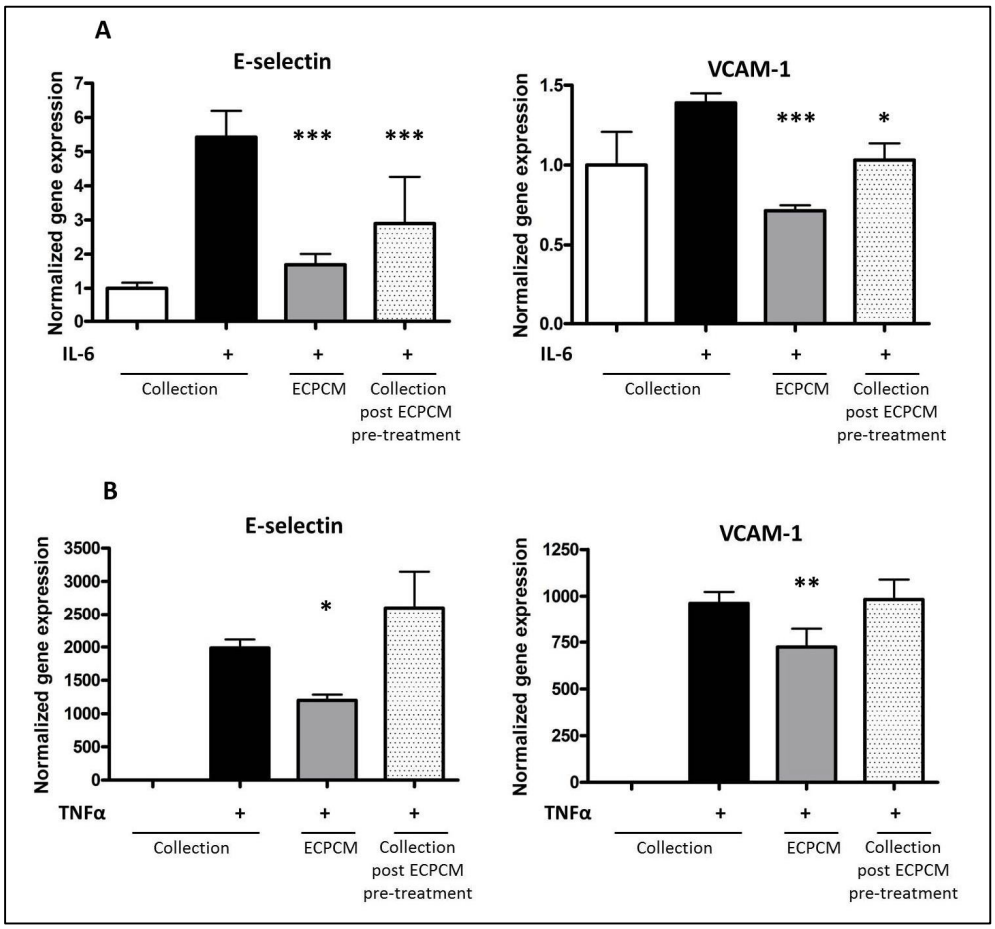

Figure 24: E-selectin and VCAM-1 expression in HAEC with or without 2 hours pre-incubation in ECPCM followed by 2 hours treatment in collection medium with 2.39 nM IL-6 (A) or 0.1 nM TNFα (B). p-value: *<0.05; **<0.01; ***<0.001 compared to treatment in collection medium+IL-6 or TNFα. Data = mean ± SD; n (individual well) = 6 for each group.

3.2.6 Conditioned medium derived from human aortic SMC has anti-inflammatory effects.

In order to determine if the anti-inflammatory activity of ECPCM is dependent on EC-specific products, human aortic SMC and human embryonic kidney (HEK) 293 cells were grown on collagen particles to produce the respective conditioned media (SMCPCM and 293PCM). These conditioned media were tested in the *in vitro* gene expression assay using HAEC treated with IL-6 or TNFα; conditioned medium from cell-free collagen particles (PCM) was used as a negative control.

Upon IL-6 treatment SMCPCM showed an anti-inflammatory activity comparable to that of ECPCM on E-selectin expression, whereas VCAM-1 inhibition was not statistically significant (figure 25). For TNFα treatment the inhibitory effect on both E-selectin and VCAM-1 expression was greater with SMCPCM than with ECPCM, but this difference was not statistically significant. The 293PCM had no anti-inflammatory effects on gene expression induced by IL-6 or TNFα. As expected, PCM also had no detectable effect on cytokine-induced expression of E-selectin or VCAM-1.

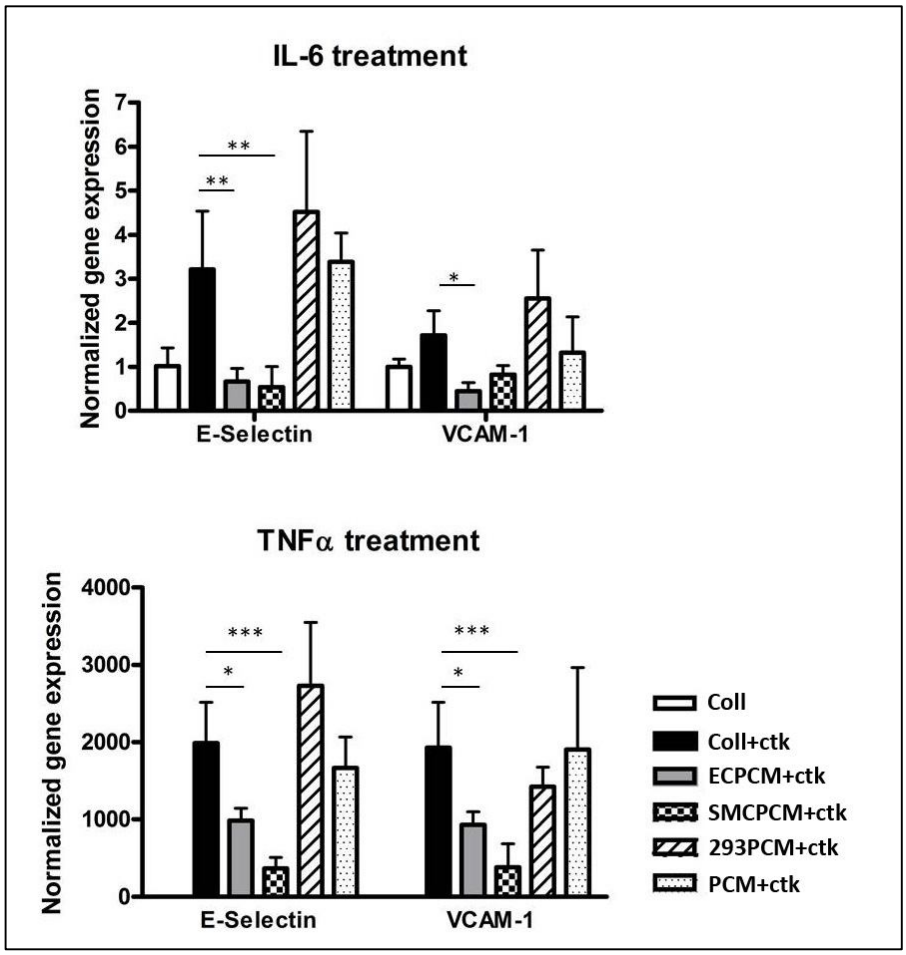

Figure 25: E-selectin and VCAM-1 gene expression in HAEC treated for 2 hours with 2.39 nM IL-6 or 0.1 nM TNFα in collection medium (control), ECPCM, SMCPCM, 293PCM or PCM. Ctk = cytokine. p-value *<0.05; ** <0.01; *** <0.001. Data = Mean ± SD; n (individual well) = 6 per group.

These results suggest that not only HAEC, but also aortic SMC grown on a 3D matrix are able to produce and release soluble anti-inflammatory factors that affect the endothelium and possibly vascular homeostasis. Since conditioned medium from HEK 293 cells grown on 3D does not provide anti-inflammatory activity against IL-6 and TNFα effects on gene expression, the anti-inflammatory activity of cell-PCM may be limited to the cells of the vessels. The anti-inflammatory activity is clearly cell-based, since PCM did not have any detectable activity.

3.2.7 ECPCM anti-inflammatory activity *in vitro:* conclusions and discussion

The data presented in this chapter show that HAEC grown on a 3D collagen matrix release anti-inflammatory molecules that inhibit PF4-, TNFα- and IL-6-induced expression of the inflammatory adhesion molecules E-selectin and VCAM-1 in HAEC. The data confirm previously published data, in which Nugent et al showed that the conditioned medium collected from EC grown on collagen sponge has anti-inflammatory activity and effectively reduces PF4-induced gene expression of VCAM-1, ICAM-1, E-selectin, tissue factor and IL-8 in EC *in vitro* (Nugent HM 2012). The conditioned medium was also able to reduce growth factor-stimulated SMC proliferation *in vitro* (Nugent HM 2012). In addition, the experiments presented herein show that the effect of ECPCM on gene expression in cytokine-stimulated HAEC was not only potent (38% to 88% inhibition of gene expression), but also multi-targeted. PF4, TNFα and IL-6 belong to different classes of pro-inflammatory molecules that act on different receptors and activate different signalling pathways in EC, and the fact that ECPCM inhibited the gene expression induced by all three of these pro-inflammatory molecules suggests the factor (or

factors) responsible for the observed effects inhibits the inflammatory activity of the three different molecules in a similar way, perhaps by activating a common anti-inflammatory pathway. This anti-inflammatory mechanism might not be limited to aortic EC; similar ECPCM anti-inflammatory activity is also observed when HUVEC are treated with TNFα and IL-6 (see chapter 3.5.10). Although HAEC and HUVEC are both EC, they come from different types of vessels (artery vs. vein) and from different locations (aorta vs. umbilical vein). These different characteristics can influence the phenotype of the EC and their reaction to various stimuli. However, TNFα and IL-6 activate expression of E-selectin and VCAM-1 in HAEC and HUVEC, and ECPCM inhibited these pro-inflammatory effects in both cell types with similar potencies. Together, the data suggest that EC of different origin are similarly activated by pro-inflammatory cytokines, and that ECPCM likely exerts its anti-inflammatory effects via the same mechanism in arterial and venous EC.

Interestingly, it has been reported that supernatant from EC/matrix decreases the maturation of dendritic cells and significantly increases the production and release of IL-10 and TGF-β by these dendritic cells (Methe H 2007). Since maturation of dendritic cells is pivotal in priming the immunological reaction (Banchereau J 2000), and since IL-10 and TGF-β are known for their anti-inflammatory activity (see chapter 3.4), these results support the anti-inflammatory therapeutic potential of EC-conditioned medium.

Treatment of HAEC with serially diluted ECPCM showed that the anti-inflammatory activity is dose-dependent. The IC50 data strongly suggest a soluble nature of the molecule(s) responsible for the anti-inflammatory effects and introduce the possibility of concentrating the anti-inflammatory molecule(s) for increased activity

and therapeutic use. However, the IC50's of ECPCM were different against TNFα and IL-6, and for E-selectin expression vs. VCAM-1 expression. ECPCM seemed to be more effective in inhibiting IL-6 effects than TNFα effects. This is likely because TNFα can trigger a much higher gene expression response in EC than does IL-6. Another possibility is that the anti-inflammatory factor (or factors) in ECPCM is simply more potent in inhibiting IL-6-induced inflammation.

Pre-treatment of HAEC with ECPCM prior to treatment with pro-inflammatory cytokines did not suppress TNFα induction of E-selectin or VCAM-1, but was sufficient to mediate inhibition of inflammatory gene expression induction by IL-6. These results could be due to anti-inflammatory molecules in ECPCM that act with dissimilar potencies against the different signalling pathways and the transcription factors activated by IL-6 and TNFα. Whereas IL-6 activates a Stat3-dependent response, TNFα effects are mediated by activation of NF-kB. The anti-inflammatory factors in ECPCM could have differential effects on Stat3 and NF-kB transcriptional activity, which could explain the different inhibitory effect of the pre-treatment on the two cytokines. As mentioned above, the broad-spectrum activity of ECPCM on different pro-inflammatory cytokines and different types of EC suggests a common mechanism of action. Perhaps the molecular mechanism responsible for ECPCM anti-inflammatory activity is the same for both IL-6 and TNFα-induced inflammation, but the kinetics are different. ECPCM may affect the pathways activated by IL-6 and TNFα with different timing and/or magnitude, thereby affecting the potency and duration of the anti-inflammatory effects. The data from the pre-treatment experiment support this possibility.

Conditioned medium from aortic SMC, but not HEK 293 cells derived from the embryonic kidney, grown on a 3D collagen matrix also showed anti-inflammatory activity. Indeed, SMCPCM significantly inhibited IL-6-induced expression of E-selectin and TNFα-induced expression of both E-selectin and VCAM-1 in HAEC. It is possible that the molecule (or molecules) responsible for these anti-inflammatory effects in SMCPCM is the same as in ECPCM. In this case the difference in the potency of the formulations is likely caused by different concentrations of the soluble factor(s) in ECPCM and SMCPCM. Alternatively, HAEC and SMC could produce different anti-inflammatory molecule(s) that act via different mechanisms but mediate a similar inhibitory effect on the expression of endothelial adhesion molecules. It is interesting to note that both the EC and the SMC used to produce the anti-inflammatory conditioned media were isolated from the aorta. Since the phenotype of vascular cells is strongly affected by their location in the vascular system, this is an important aspect to take into consideration for future development of therapeutics derived from ECPCM and SMCPCM and for the analysis of their molecular mechanisms.

The data presented here raise the possibility that cells such as EC and SMC of the healthy vessel may naturally produce soluble anti-inflammatory molecules to maintain the homeostasis of the vasculature. This hypothesis is consistent with the observation that systemic cardiovascular diseases are associated with increased risk of developing vascular complications in the eye. These comorbidities may occur because the vessels in the disease state are less able to produce anti-inflammatory molecules, among other important beneficial factors, to maintain the overall homeostasis of the vasculature.

In summary, HAEC in 3D culture produce a soluble, potent and broad-spectrum anti-inflammatory activity that can significantly suppress the activation of EC by PF4, IL-6 and TNFα and reduce the adhesion of immune cells to the EC following treatment with these inflammatory factors. Further study into the mechanism of action for this anti-inflammatory activity and identification of the molecules responsible may result in development of novel therapeutic strategies for vascular pathologies associated with local inflammation.

Chapter 3.3: Characterization of the molecule(s) responsible for the anti-inflammatory activity of ECPCM

3.3.1 The anti-inflammatory activity of ECPCM is proteinase K- and RNase-resistant

Proteins, acting as adaptors, co-factors or active enzymes, are responsible for most of the signalling between and within cells. To determine if the anti-inflammatory activity of ECPCM is protein-mediated, the ECPCM was treated with proteinase K, a broad-spectrum protease. Coomassie blue staining of untreated and proteinase K-treated ECPCM separated on a polyacrylamide gel confirmed the full digestion of proteins in the media (Figure 26).

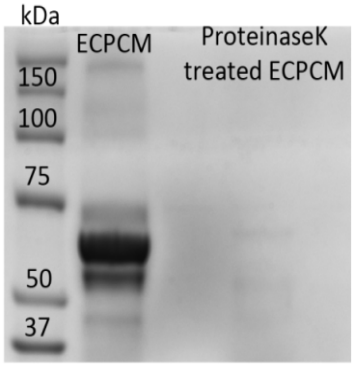

Figure 26: Representative image of coomassie blue staining of untreated and proteinase K-treated ECPCM. The band of ~65kDa in the ECPCM lane corresponds to serum albumin. Absence of this band in the proteinase K-treated ECPCM confirmed complete protein digestion.

When tested using the *in vitro* gene expression assay, the proteinase K-treated ECPCM significantly inhibited IL-6-, TNFα- and PF4-induced gene expression of both E-selectin and VCAM-1 (Figure 27). More specifically, during treatment with IL-6 and TNFα, the inhibition of E-selectin and VCAM-1 expression by the proteinase K-treated ECPCM was similar to that observed with the untreated ECPCM. Proteinase K-treated ECPCM was not as effective as the untreated ECPCM at inhibiting the PF4-induced E-selectin expression, although the inhibition was still significant compared to control. On the other hand, inhibition of PF4-induced VCAM-1 expression was better with proteinase K-treated ECPCM.

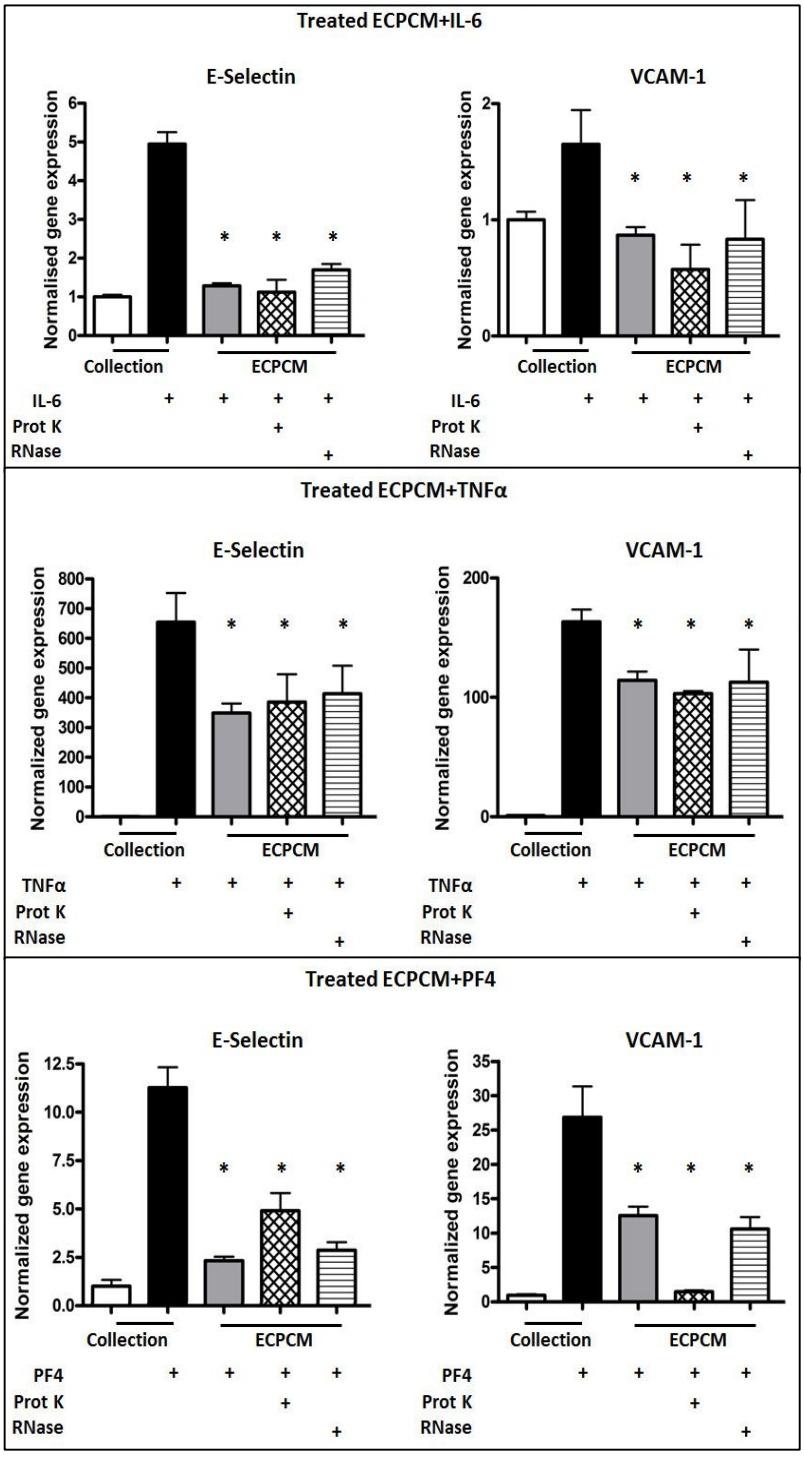

Figure 27: ECPCM anti-inflammatory activity is proteinase K- and RNase-resistant. Expression of E-selectin and VCAM-1 was analysed after treatment for 2 hours with 2.39 nM IL-6 (upper panel) or 0.1 nM TNFα (middle panel) or for 4 hours with 64.1 nM PF4 (lower panel). HAEC were treated in collection medium (control), ECPCM or ECPCM previously treated with proteinase K or RNase. *p value<0.001 compared to treatment with IL-6, TNFα or PF4 in collection media. Data = mean ± SD; n = 6 per group.

In recent years, it has been demonstrated that RNA molecules play important roles in mediating signals and controlling specific functions in cells, as described in detail below. To determine the role of RNA molecules in the anti-inflammatory effect, ECPCM was treated with an RNase A/T_1 mix. RNase A and T_1 enzymes are exo-ribonucleases that degrade single strand RNA (ssRNA). Upon IL-6, PF4 or TNFα treatment the RNase-treated ECPCM inhibited E-selectin and VCAM-1 expression in HAEC to levels comparable to those observed with untreated ECPCM (figure 27).

Based on these experiments it can be concluded that the factor (or factors) mediating ECPCM anti-inflammatory activity is not ssRNA and, if it is a protein, it is proteinase K-resistant.

3.3.2 Agarose affects ECPCM activity

Heparin is a highly sulphated glycosaminoglycan produced by mast cells (Bjorn I 1982). When it is released in the blood it acts as an anti-coagulant and anti-thrombotic agent by activating anti-thrombin III, which in turn inactivates thrombin, factor Xa and other protease involved in the clotting cascade (Bjorn I 1982). Because of its highly negative charge, heparin is typically used in affinity chromatography to isolate molecules (proteins in particular) based on their electric charge. For this reason, in the attempt to identify the molecule(s) responsible for ECPCM anti-inflammatory activity and to obtain some information about its chemical and physical properties, ECPCM was incubated with heparin-conjugated agarose beads to remove positively charged molecules and tested in the *in vitro* gene expression assay. As a control, ECPCM was also incubated with empty agarose beads.

As shown in figure 28, ECPCM that was treated with heparin-conjugated agarose beads lost its inhibitory activity on IL-6-induced E-selectin expression. It also showed less inhibition of VCAM-1 induction by IL-6 and E-selectin induction by TNFα. However, after heparin bead treatment ECPCM showed increased inhibition of TNFα-induced VCAM-1 expression. These results would suggest the presence in ECPCM of multiple anti-inflammatory factors with different properties, with the one(s) responsible for E-selectin expression inhibition being heparin-binding and positively charged molecule.

HAEC were also treated with IL-6 and TNFα in ECPCM previously incubated with agarose beads. Surprisingly, this control treatment affected ECPCM activity as well as did the heparin-conjugated agarose (figure 28), suggesting that the effects of heparin-conjugated agarose are due to the agarose rather than the heparin.

These data demonstrate the non-specific effects of the heparin-agarose and agarose beads. Because of this, heparin and agarose could not be used further in molecular identification of the anti-inflammatory factor(s) in ECPCM – a limitation that greatly diminished the array of treatments and procedures that could be applied in the identification process.

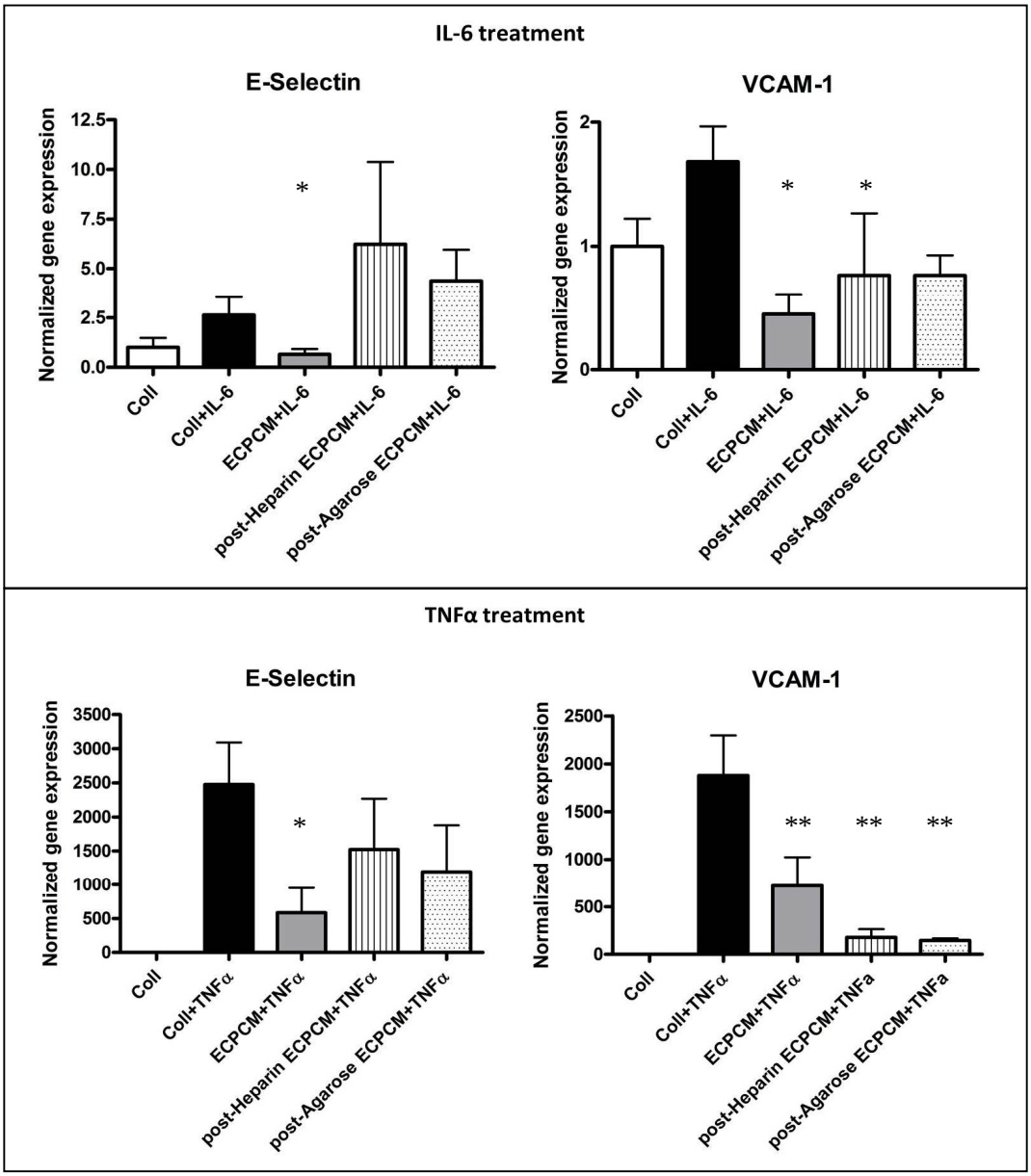

Figure 28: Incubation of ECPCM with agarose beads affects E-selectin and VCAM-1 expression in HAEC. ECPCM was incubated with heparin-agarose beads or agarose beads and tested on HAEC. Cells were treated for 2 hours in collection media or with 2.39 nM IL-6 or 0.1 nM TNFα in Collection media, ECPCM or ECPCM post incubation with heparin-agarose or agarose beads. Data = mean ± SD; n = 3 per group. p-value: * <0.05; ** <0.01 compared to treatment in collection media + IL-6 or TNFα.

3.3.3 Molecular characterization: conclusions and discussion

Identification of the soluble anti-inflammatory molecule(s) responsible for ECPCM activity was not possible given the technical limitations in this study, but certain characteristics could be defined: namely that the factor(s) partly bind to agarose, are resistant to proteinase K digestion, and are not ssRNA-based.

The agarose beads used as a control for the heparin-agarose binding experiment are highly inert, but proteins such as the pentraxin serum amyloid P (SAP) have been shown to bind to agarose (Hind CRK 1984). Pentraxins are a superfamily of serum multifunctional proteins with a pentameric structure that are involved in foreign antigen and altered self-antigen recognition and activate the innate immune response (Du Clos TW 2013). SAP is produced in the liver and is present at constitutive levels in human plasma. It inhibits fibrocyte differentiation after tissue damage (Pilling D 2003) and it acts as an acute phase protein induced by IL-6 in mice, but not in humans (Du Clos TW 2013). Although a role in inflammation is possible, at the moment there is no evidence of SAP's anti-inflammatory activity in humans, therefore it can be excluded as main mediator of ECPCM activity.

Other generally inert materials were used to try to isolate the anti-inflammatory factor(s) in ECPCM. These included size-fractionation tubes and dialysis cassettes, both made of regenerated cellulose membranes in a range of molecular weight cut-offs. Like agarose, cellulose is characterized by high stability and low chemical complexity; therefore it is not expected to interact with many other molecules. However, the effects on EC using cellulose filter-treated ECPCM and cellulose filter-treated collection medium were comparable (data not shown). Surprisingly, both media showed pro-inflammatory effects even in the absence of cytokines (data not

shown). The fact that inert materials such as agarose and cellulose alone were able to change the activity of ECPCM and of the basic EC-growing medium that is the collection medium suggests that EC growing *in vitro* require a very delicate balance of molecules for physiological quiescence.

Another process that was attempted was the Folch method for lipid isolation. This is a well-established protocol that uses organic solvents to isolate lipid molecules in a solution (Folch J 1957). Application of this method to ECPCM led to the production of an aqueous and a lipid phase, which were then tested on HAEC with or without cytokines (data not shown). Although much care was put in the removal of any residual organic solvent, a small quantity of it remained in solution and greatly affected the *in vitro* assay. In fact, both aqueous and lipid phases disturbed EC phenotype and survival even in the absence of cytokines. For these reasons this method of lipid isolation could not be applied to identify the anti-inflammatory factor(s) in ECPCM.

Similarly, in an attempt to investigate the role of carbohydrates in the anti-inflammatory activity, ECPCM was treated with glycosidases (sialidase A and β–N-acetylhexosaminidase). For these treatments, the small volume of buffer necessary for proper enzyme activity was added to the ECPCM. Unfortunately, even in the absence of enzyme, the buffers impaired ECPCM activity (data not shown). This was probably because of the high salt concentration in the buffers. Filters could not be used to eliminate the extra salt after glycosidase treatment because typical cellulose filters used for this purpose affect ECPCM activity, as mentioned above. Since enzyme treatments could not be used on ECPCM, exploration of the possible role of carbohydrates in ECPCM activity was ended.

The above studies eliminated the possibility of using solvents, buffers, cellulose and agarose in further characterization, severely reducing the amount of protocols applicable in the identification process. Fortunately, RNase and proteinase K treatments do not require addition of buffer for activity.

Proteinase K is a broad-spectrum serine protease that digests proteins in their native and denatured forms and is stable over a wide range of temperature and pH (Ebeling W 1974). ECPCM was treated with proteinase K and then tested on EC in the presence of IL-6 or TNFα. Since after this treatment ECPCM was still able to inhibit the cytokine-induced expression of E-selectin and VCAM-1, the molecule(s) mediating the effect are either non-protein or proteins that are resistant to proteinase K. Besides proteinase K itself, to date some forms of α-synuclein, β-amyloid and prion proteins are known to be (at least partially) resistant to the protease digestion (Forloni G 1996; Miake H 2002). These proteins are not expressed in EC and have no anti-inflammatory activity but are, instead, localized in the brain and implicated in neurodegenerative diseases (Forloni G 1996; Miake H 2002). For these reasons they can be excluded as potential mediators of ECPCM anti-inflammatory effects. SAP has also been shown to be resistant to proteinase K degradation (Manning M 2004). Given the lack of evidence of anti-inflammatory activity of SAP in humans, this protein can also be excluded as the one responsible for ECPCM effects.

It could be argued that the observed results were caused by a residual proteinase activity that digested the cytokines used to stimulate EC *in vitro* during the assay, thus degrading the cytokines before they could mediate their effect on EC. However, proteinase K treatment was performed using the enzyme attached to agarose beads, which were then carefully removed by centrifugation. Therefore, residual proteinase

K activity at the time when the *in vitro* assay was performed is highly unlikely. In this case incubation with agarose bead carriers did not seem to affect ECPCM's activity, probably because a very small quantity was used compared to the experiments using heparin-conjugated agarose beads.

ECPCM was also treated with a mix of RNase A and -T_1, which are both exo-ribonucleases specific for ssRNA. Since this treatment did not eliminate the anti-inflammatory activity, ssRNA molecules can be excluded as mediators of ECPCM effects. This is not surprising, given that there is no evidence of ssRNA being released from cells and directly mediating biological effects. On the other hand, double strand RNA in the form of microRNA (miRNA) is known to be an important player in gene and protein expression. Indeed, miRNA can negatively regulate post-transcriptional events by inducing mRNA degradation and/or inhibition of translation (He L 2004). No enzyme is currently available to directly remove double strand RNA in solution; therefore, it cannot be excluded that miRNAs have a role in the anti-inflammatory activity of ECPCM. Indeed, various miRNAs that regulate EC adhesion molecule expression have been discovered. These include miR126, which downregulates VCAM-1 (Harris TA 2008); miR-31, which affects E-selectin (Suarez Y 2010); and miR-10a, which mediates inhibition of both molecules (Fang Y 2010).

To date, there is no evidence for direct secretion of free miRNA from mammalian cells, nor is there knowledge of a specific pathway that would allow these molecules to reach the cytosol of an eventual target cell. However, what has been reported is the release from stem cells of microparticles containing miRNA (Ratajczak J 2006; Deregibus MC 2007; Hunter MP 2008; Yuan A 2009; Koh W 2010). Microparticles are small vesicles with variable diameter (0.1-1 µm) that can be found in human

plasma and are released by various types of cells. Microparticles are released through remodelling of the phospholipids in the cellular membrane and express phosphatidylserine and antigens typical of their parent cell (Leroyer AS 2010; Dignat-George F 2011). On their surface, endothelial microparticles (EMP) present oxidised bioactive lipids, membrane receptors involved in coagulation (endothelial protein C receptor, thrombomodulin and tissue factor) and adhesion molecules (E-selectin, VCAM-1, ICAM-1, PECAM-1). They also harbour MMPs, growth factor receptors and receptors of the plasminogen activation system (urokinase plasminogen activator and its receptor) (Leroyer AS 2010). Some microparticles can express immunoglobulins and major histocompatibility molecules on their surface and enclose transcription factors, mRNA and miRNAs (Leroyer AS 2010). As can be easily deduced from their composition, EMP regulate vessel homeostasis by affecting thrombosis, coagulation, angiogenesis, vascular tone, cell survival, inflammation and transmission of cell information (Leroyer AS 2010; Dignat-George F 2011). Depending on the specific composition, pathological context and stimulus, and mechanism and site of release, EMP can either promote or disrupt endothelial function (Dignat-George F 2011).

It is conceivable that EC grown on a collagen matrix release microparticles and that these mediate the anti-inflammatory activity of ECPCM, for example by carrying the miR-126, -31 or -10a. However, most of the data published up to now show that EMP have pro-inflammatory effects. In fact, increased levels of EMP are associated with IL-6 release, increased expression and shedding of ICAM-1 and, more in general, with diseases such as diabetes, acute coronary syndrome, stroke and hypertension (Leroyer AS 2010; Dignat-George F 2011). Other microvescicles released by EC include exosomes and apoptotic bodies. Exosomes are less than 100

nm in diameter, contain RNA and miRNA, and seem to have a role in vascular development (Dignat-George F 2011). Apoptotic bodies are larger than microparticles (1-5 μm diameter) and deliver oncogenes, DNA or miRNA (Dignat-George F 2011). These varied, EC-derived microvescicles have ambivalent biological effects that are still debated and need further investigation. With regards to this thesis, it will be of great interest to evaluate the role of EMP in the anti-inflammatory activity of ECPCM.

Chapter 3.4: The roles of known anti-inflammatory molecules in mediating ECPCM effects

3.4.1 Exploration of known anti-inflammatory mediators

As noted in the introduction, EC produce and release many molecules that control vascular physiology and homeostasis. Among these are many with anti-inflammatory effects. NO and PGI_2 have already been mentioned. Other molecules that affect inflammation include TGF-β, IL-10, steroids, resolvins and cyclic AMP (cAMP). Given the importance of these molecules in inflammation, their levels in ECPCM were determined and various experiments performed to verify if any might mediate the ECPCM effects.

Levels of TGF-β1, IL-10, cAMP, NO, and PGI_2 in the ECPCM were compared to the levels detected in collection medium, SMCPCM, 293PCM and PCM (Table 4). Levels of TGF-β1 were far greater in ECPCM than in the collection medium or any of the other conditioned media. Levels of IL-10, on the other hand, were lower in ECPCM than in collection medium or the other conditioned media. Levels of cAMP and PGI_2 did not vary among the samples tested, and levels of NO were lower in all conditioned media compared to collection (control) medium.

	Collection media	ECPCM	SMCPCM	293PCM	PCM	Analysis conc. range Min	Max
TGF-β1 (pg/ml)	61.81	749.4	227	246.05	61.81	0	2000
IL-10 (pg/ml)	77.62	44.96	112.20	123.03	120.23	0	500
cAMP (pmol/ml)	6.5	6.5	6.5	6.5	6.5	3.25	240
NO (μM)	21.72	0.072	1.305	0	1.151	0	50
PGI_2 (ng/ml)	<2.5	<2.5	<2.5	<2.5	<2.5	0	50

Table 4: Average concentration of known anti-inflammatory molecules in collection medium, ECPCM, SMCPCM, 293PCM and PCM, as assessed by ELISA (TGF-β, IL-10, cAMP, PGI_2) or the Griess reaction (NO).

3.4.2 TGF-β1 can inhibit cytokine-induced expression of E-selectin and VCAM-1

The TGF-β family of cytokines plays a role in a wide range of events: cell growth, differentiation and apoptosis, fibrosis, angiogenesis, immune response and inflammation (Massague J 1990; Pintavorn P 1997; Akhurst RJ 2012). TGF-β cytokines consist of three highly homologous isoforms: TGF-β1, expressed in epithelial cells, EC, SMC, hematopoietic cells and fibroblasts; TGF-β2, localized on epithelial cells and neurons; and TGF-β3, expressed primarily in mesenchymal cells (Ghosh J 2005). TGF-β is synthesized within the cell and released in a dimeric large latent complex consisting of TGF-β, a latent TGF-β-binding protein and a latency-associated peptide (Suwanabol PA 2011; Akhurst RJ 2012). Once in the ECM, active TGF-β is released from the large latent complex by specific enzymes, mechanical stress, extreme pH and heat (Suwanabol PA 2011; Akhurst RJ 2012).

Quiescent EC produce basal low levels of TGF-β1. Its synthesis and activation are enhanced in cytokine-activated cells and in EC stimulated by hemodynamic forces (Pintavorn P 1997). Up-regulation of TGF-β1 was also observed at sites of vascular injury in both human and animal studies (Suwanabol PA 2011). Various *in vitro* models have showed that TGF-β is able to inhibit leukocyte adhesion and transmigration across cytokine-activated endothelium (Gamble JR 1988; Cai JP 1991; Gamble JR 1993; Smith WB 1996). This is due, at least in part, to inhibition of E-selectin expression (Cai JP 1991; Gamble JR 1993).

As shown in table 4, the average concentration of TGF-β1 was more than 10 times higher in ECPCM compared to collection medium (749.4 pg/ml and 61.81 pg/ml, respectively) and about double that of SMCPCM and 293PCM (227 pg/ml and

246.05 pg/ml respectively). These values suggest that the cytokine might have a part in mediating the anti-inflammatory effects of ECPCM.

To investigate the role of TGF-β1 in the anti-inflammatory activity of ECPCM, HAEC were treated with IL-6 or TNFα in collection medium (control) with the addition of activated TGF-β1 at various concentrations. It is important to note that TGF-β1 effects on the vasculature are context- and concentration-dependent, being influenced by type and status of cells, genetic variations, conditions of culture *in vitro*, phase of disease or development *in vivo*, and interaction with other signalling pathways (Pintavorn P 1997; Akhurst RJ 2012). Gene expression analysis showed that at low concentrations (300 pg/ml) TGF-β1 did not inhibit IL-6- or TNFα-induced expression of E-selectin or VCAM-1; similar results were obtained when cells were treated with a level of TGF-β1 comparable to that observed in ECPCM (1000 pg/ml; figure 29). Since the anti-inflammatory effects of ECPCM are dose dependent (figure 15), a concentration of TGF-β1 four times higher than that measured in ECPCM (3000 pg/ml) was also tested in the *in vitro* assay. At this concentration the cytokine inhibited both IL-6- and TNFα-induced expression of E-selectin and VCAM-1 to levels similar to those obtained during ECPCM treatment (figure 29).

These results show that TGF-β1 can inhibit cytokine-induced expression of E-selectin and VCAM-1 in a dose-dependent manner in HAEC; the molecule might therefore have a role in mediating ECPCM anti-inflammatory activity. However, TGF-β1 did not inhibit gene expression of the adhesion molecules at a concentration similar to that measured in ECPCM. These data, together with the proteinase K findings presented in chapter two, suggest a different molecule in the formulation is mainly responsible for the anti-inflammatory effects.

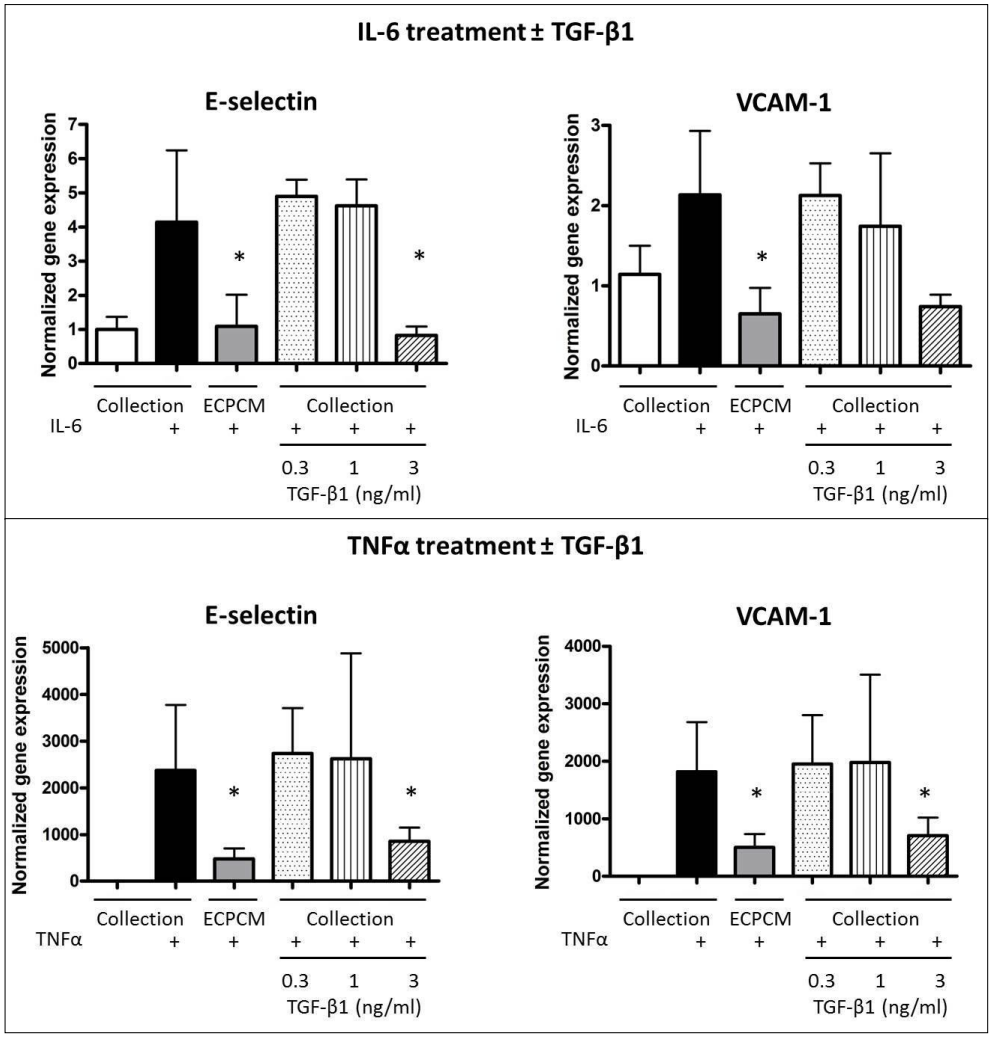

Figure 29: TGF-β1 effects on cytokine-induced E-selectin and VCAM-1 gene expression. HAEC were treated for 2 hours with 2.39 nM IL-6 (upper panel) or 0.1 nM TNFα (lower panel) in ECPCM, collection medium or collection medium with increasing amounts of TGF-β1. *p-value <0.05 compared to treatment in collection medium with IL-6 or TNFα. Data = mean ± SD; n = 5 per group.

3.4.3 IL-10 does not mediate the anti-inflammatory effects of ECPCM

IL-10 was first described for its ability to inhibit activation of and cytokine production by T_H1 cells (Fiorentino DF 1989). Later studies showed a much broader anti-inflammatory activity of IL-10. In fact, the protein not only can inhibit cytokine synthesis by both T cells and natural killer cells, but it also affects expression of cytokines, soluble mediators and cell surface molecules by myeloid cells, thereby modulating their ability to stimulate and sustain immune and inflammatory responses (Moore KW 2001). Similarly, IL-10 inhibits cytokine and chemokine production by neutrophils (Moore KW 2001). IL-10 affects the generation and maturation of immature dendritic cells and induces survival, proliferation and isotype switching of B cells (Moore KW 2001). It was recently shown that IL-10 decreases TNFα-induced inflammation in EC by reducing TNFα-dependent ROS production, ICAM-1 expression and leukocyte adhesion to the endothelium (Huet O 2013). IL-10 has also been reported to reduce endothelial dysfunction in vessels stimulated with various pro-inflammatory molecules (Kang H 2008; Zemse SM 2008; Didion SP 2009; Zemse SM 2010).

Table 4 shows that IL-10 concentration was actually lower in ECPCM compared to collection medium (45 pg/ml and 78 pg/ml, respectively). Concentration of the cytokine in SMCPCM was quite high (~110 pg/ml), nearly three times the amount detected in ECPCM. Cell-free PCM and 293PCM, which exhibited no anti-inflammatory activity, showed the highest levels of IL-10 (both around 120 pg/ml). Based on these findings IL-10 was excluded as the molecule potentially responsible for ECPCM anti-inflammatory activity.

3.4.4 Cyclic AMP does not mediate the anti-inflammatory effects of ECPCM

Cyclic adenosine monophosphate (cAMP) is a ubiquitous regulator of inflammatory and immune responses. It blocks lymphocyte activation, inhibits the release of pro-inflammatory cytokines from macrophages, and suppresses the expression of adhesion molecules in peripheral EC and astrocytes (Pober JS 1993; Balyasnikova IV 2000). Indeed, pharmacologic agents that elevate cAMP in HUVEC and cAMP analogues inhibit the TNFα-induced expression of E-selectin and VCAM-1, but not ICAM-1 (Pober JS 1993).

As reported in table 4, ECPCM and collection medium showed the same very low concentration of cAMP (< 7 pmol/ml). SMCPCM and 293PCM contained a similarly small amount of the molecule. These data suggest that cAMP is not responsible for the anti-inflammatory effects of ECPCM.

3.4.5 NO does not mediate the anti-inflammatory effects of ECPCM

NO inhibits platelet aggregation and has relaxing effects on vascular tone (Davies MG 1993; Sumpio BE 2002; Pober JS 2007). It also inhibits the expression of adhesion molecules. Since platelet activation, aggregation, and ROS production characterize inflammatory reactions, NO can be classified as an anti-inflammatory molecule for its ability to block these events.

Given the volatile and unstable nature of NO, which is rapidly oxidized to nitrite and/or nitrate by oxygen (Sun J 2003), it was unlikely that this is the factor mediating ECPCM beneficial effects. Nevertheless, the concentration of NO was verified in various samples of ECPCM through the Griess reaction colorimetric assay. This is a

method which allows estimation of NO levels in a biological sample by determining the concentration of nitrite end products (Sun J 2003). Table 4 shows that collection medium contained nearly 22 µM of NO, whereas the concentration of the molecule in ECPCM was almost zero. SMCPCM, which showed anti-inflammatory activity similar to that of ECPCM, also showed very low levels of NO. Based on these findings, NO was excluded as the factor mediating ECPCM anti-inflammatory effects.

3.4.6 PGI_2 does not mediate the anti-inflammatory effects of ECPCM

PGI_2 is a potent vasodilator, platelet anti-aggregator and pro-fibrinolytic agent (Davies MG 1993; Sumpio BE 2002; Pober JS 2007). The anti-inflammatory effects of PGI_2 analogs have also been linked to the inhibition of maturation, cytokine production and T cell stimulatory function of dendritic cells (Zhou W 2007; Yeh CH 2011).

PGI_2 has a very short half-life both *in vivo* and *in vitro*, ranging from 30 seconds to a few minutes. For this reason it was highly improbable that this molecule was responsible for ECPCM anti-inflammatory activity. Indeed, table 4 shows that concentration of PGI_2 was very low (< 2.5 ng/ml) in ECPCM and also in collection medium. In SMC- and 293PCM the levels of the molecule were similarly low. These data suggest that PGI_2 does not mediate the anti-inflammatory activity of ECPCM.

3.4.7 Glucocorticoids do not mediate the anti-inflammatory effects of ECPCM

Glucocorticoids are a class of potent anti-inflammatory steroid hormones that are released from the adrenal cortex within minutes in response to stress and tissue injury in order to control the severity of the inflammatory response (Newton R 2000). Most of the effects of glucocorticoids on cells are mediated via the glucocorticoid receptor (GR), a member of the superfamily of ligand-regulated nuclear receptors (Newton R 2000; Rhen T 2005), which is usually found in the cytoplasm. Thanks to their hydrophobic nature, glucocorticoids can easily pass through the plasma membrane and enter the cells following release into the blood stream. Once in the cytoplasm, glucocorticoids bind with high affinity to GR (Rhen T 2005). This binding promotes the dissociation of molecular chaperones from the receptor. The glucocorticoid-GR complex then moves to the nucleus and binds as a homodimer to specific glucocorticoid-responsive DNA sequences, resulting in activation of anti-inflammatory genes and/or repression of pro-inflammatory genes (Newton R 2000; Rhen T 2005); alternatively, the complex can also interact with other transcription factors, such as AP1 and NF-kB, and block their transcriptional activity (Newton R 2000; De Bosscher K 2006).

To test if glucocorticoids might be responsible for the anti-inflammatory effects of ECPCM, experiments were performed using RU486 (also called mifepristone), a powerful GR antagonist. Dexamethasone (DEX), a synthetic steroid drug that acts as anti-inflammatory agent and immunosuppressant, was used as a positive control. To determine if gene expression of E-selectin and VCAM-1 is responsive to glucocorticoid-mediated inhibition, HAEC were treated with IL-6 or TNFα in collection medium with 100 nM DEX. To determine if the anti-inflammatory effects

of the formulation were mediated by glucocorticoids, HAEC were treated with IL-6 or TNFα in ECPCM with 1 μM RU486. To check the ability of RU486 to antagonize glucocorticoid-mediated inhibition of gene expression, cells were also treated with IL-6 or TNFα in collection medium with both 100 nM DEX and 1 μM RU486. The experimental concentration of RU486 was determined empirically by dose curve treatments (data not shown). Since both drugs were dissolved in ethanol, a control treatment of IL-6 or TNFα in collection medium with ethanol was included in the experiment.

Figure 30 shows that DEX inhibited E-selectin and VCAM-1 expression induced by IL-6 or TNFα and that RU486 reverted this inhibition of gene expression, except from the VCAM-1 expression induced by TNFα. Even higher concentrations of RU486 were still not able to reverse the DEX inhibition in the presence of TNFα (data not shown). Since reversal of DEX/GR trans-repression of TNFα-induced E-selectin by RU486 was also partial and not as good as the reversal observed for the same gene during IL-6 treatment, this suggests that DEX/GR affects Stat3 and NF-kB differently. Most likely, the DEX/GR complex exerts a strong and more stable repression of NF-kB than Stat3. Moreover, for TNFα-induced VCAM-1 expression, the timing of analysis might not be ideal to detect the impact of RU486 on the DEX/GR complex; later or earlier time points could be a better choice to observe the effect. Importantly, RU486 did not reverse the anti-inflammatory effects of ECPCM against both TNFα and IL-6 treatments, suggesting the anti-inflammatory effects of ECPCM are not mediated by GR. Indeed, ECPCM inhibited the expression of both E-selectin and VCAM-1, with and without RU486, with comparable potencies. These results suggest that glucocorticoids are not responsible for the anti-inflammatory activity of ECPCM.

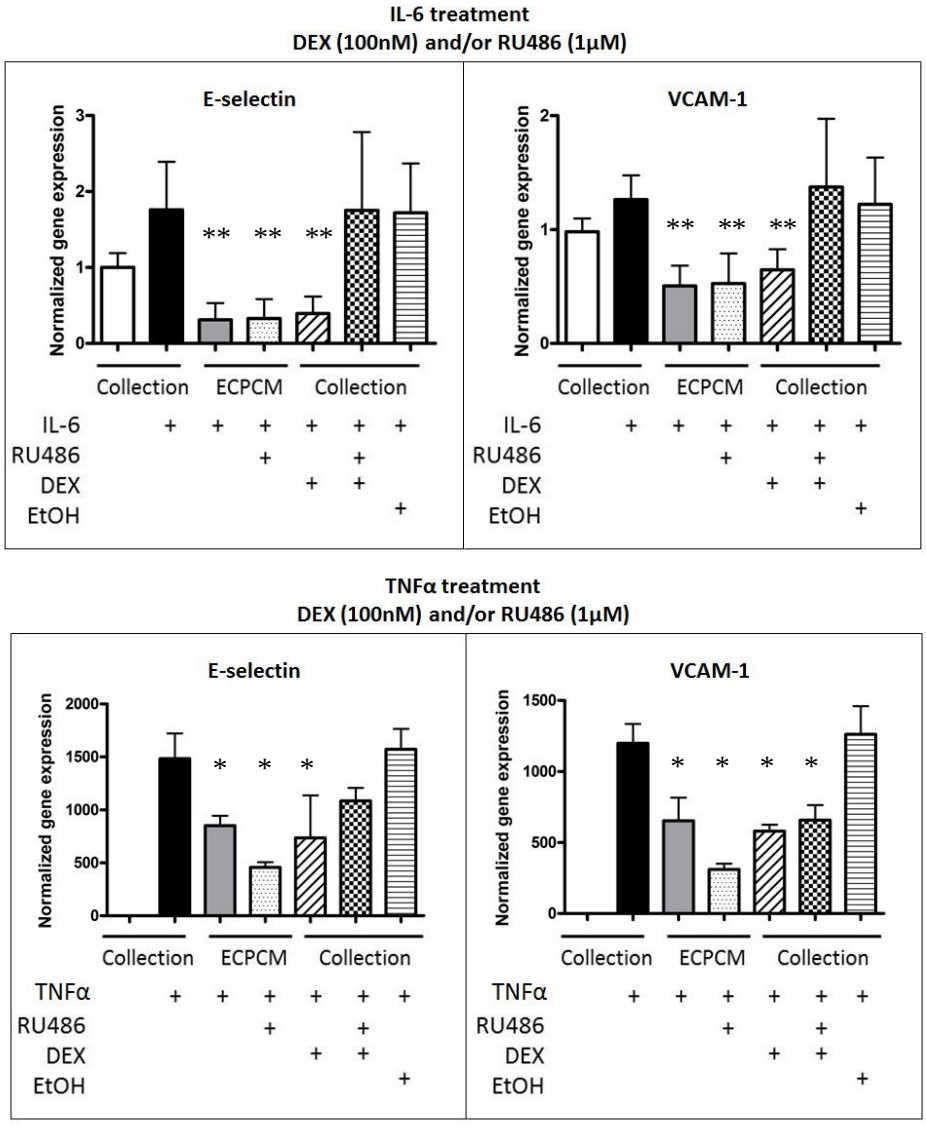

Figure 30: The GR antagonist RU486 does not suppress the anti-inflammatory activity of ECPCM. Gene expression of E-selectin and VCAM-1 was analysed in HAEC treated for 2 hours with 2.39 nM IL-6 (upper panel) or 0.1 nM TNFα (lower panel) in collection medium (control), collection medium with 100 nM DEX, collection medium with 100 nM DEX and 1 µM RU486, ECPCM, ECPCM with 1 µM RU486 or collection medium with ethanol. p-value **<0.001; *<0.05 compared to treatment in collection medium with IL-6 or TNFα. Data = mean ± SD; n = 6 per group.

3.4.8 Resolvins do not mediate the anti-inflammatory effects of ECPCM

Resolvins are a novel family of lipid mediators that show beneficial actions in inflammatory diseases (Serhan CN 2008). They are produced from the precursor essential omega-3 polyunsaturated fatty acids eicosapantaenoic acid (EPA) and docosahexaenoic acid (DHA). Depending on which polyunsaturated fatty acid they derive from, resolvins are divided into the E-series (from EPA) and D-series (from DHA). Each series show a chemically unique structural form (Serhan CN 2008). Both *in vivo* and *in vitro,* vascular EC treated with aspirin convert EPA to the intermediate fatty acid 18R-HEPE, which is released and then converted to the bioactive form of resolvins E1 and E2 (RvE1 and -2) by the action of polymorphonuclear leukocytes (PMN) (Serhan CN 2007). RvE1 reduces PMN infiltration and general inflammation in animal models; RvE2 stops PMN infiltration *in vivo* (Serhan CN 2007). When treated with aspirin and DHA, EC release a different intermediate fatty acid (17R-HDHA), that is then converted by COX-2 in neutrophils to resolvins D1-4 (RvD1-4) (Serhan CN 2007). RvD1-4 inhibit PMN infiltration, reduce inflammation in various animal models and inhibit the TNFα-induced production of IL-1β (Serhan CN 2008).

Given the potent anti-inflammatory action demonstrated by resolvins on leukocytes *in vivo*, their possible role in mediating the beneficial effects of ECPCM on EC was investigated. To this end HAEC were treated with IL-6 or TNFα in collection (control) medium in the presence of one resolvin from each series: either RvE1 or RvD2. Two concentrations of the molecules were tested (10 nM and 50 nM). Since resolvins were dissolved in ethanol, HAEC were also treated with IL-6 or TNFα in collection medium with ethanol as vehicle control.

Upon treatment with IL-6, neither RvD2 nor RvE1 had any significant effect on the cytokine-induced gene expression of E-selectin and VCAM-1 by HAEC (figure 31). During treatment with TNFα, RvD2 was able to significantly inhibit both E-selectin and VCAM-1 expression at a concentration of 50 nM, whereas RvE1 caused a small decrease in E-selectin induction when used at 10 nM (figure 31). However, treatment with ethanol gave a similar pattern of inhibition (figure 31). This suggests the inhibitory effects observed during TNFα treatment in the presence of resolvins were due to the solvent and not to the specific action of the resolvins. Based on these observations, resolvins were excluded as major mediators of the anti-inflammatory activity of ECPCM.

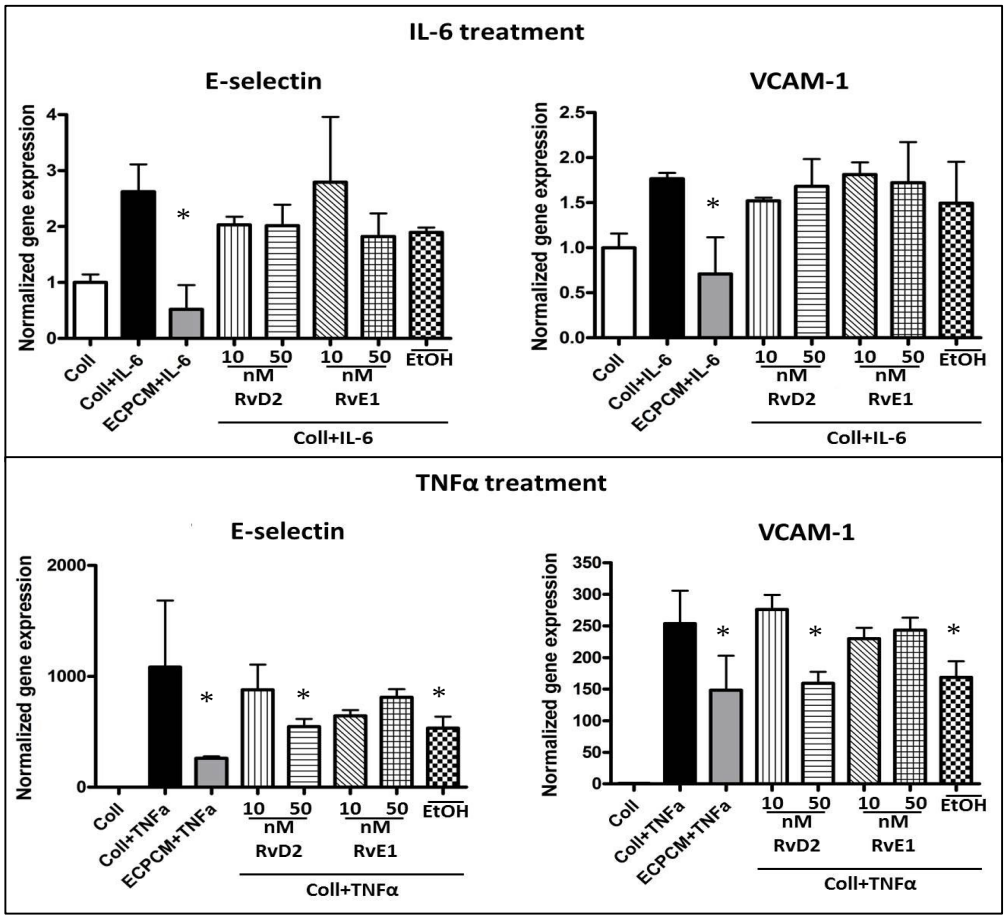

Figure 31: Gene expression of E-selectin and VCAM-1 is not affected by resolvins. HAEC were treated for 2 hours with 2.39 nM IL-6 or 0.1 nM TNFα in ECPCM, collection medium or collection medium with RvD2 or RvE1 (10 nM or 50 nM). Vehicle control treatment: collection medium with IL-6 or TNFα and ethanol. *p-value <0.05 compared to treatment in collection medium with IL-6 or TNFα. Data = mean ± SD; n = 3 per group.

3.4.9 Exploration of known anti-inflammatory mediators: conclusions and discussion

Multiple and varied molecules known to mediate anti-inflammatory effects were studied to determine their possible roles in the activity of ECPCM. Proteins such as TGF-β and IL-10 were tested, though these protein mediators were unlikely candidates based on the proteinase K findings. The small quantity of IL-10 in ECPCM was sufficient to exclude it as the main anti-inflammatory factor in the formulation, however TGF-β levels were higher in ECPCM than in collection medium. Moreover, administration of TGF-β together with IL-6 or TNFα inhibited cytokine-dependent expression of E-selectin and VCAM-1. Nevertheless, inhibition of the cytokine-induced gene expression to the same extent as ECPCM required a concentration of TGF-β four times higher than that detected in ECPCM; use of TGF-β at a similar concentration to that found in ECPCM did not inhibit gene expression of E-selectin or VCAM-1. These data suggest TGF-β might be partly responsible for ECPCM anti-inflammatory activity, but the results from proteinase K treatment argue against a prominent role for this protein.

Quantification of NO, PGI_2 and cAMP levels revealed very low concentrations for each of these molecules in ECPCM. This suggests none of those factors is responsible for the anti-inflammatory activity of ECPCM. For NO and PGI_2 this is not surprising, given the highly unstable nature and short half-life of these molecules.

In vitro experiments with RvE_1 and RvD_2 showed some anti-inflammatory effects, but these were not comparable to those obtained with ECPCM in terms of concentration-dependence, intensity, extent and specificity; therefore, these molecules are probably not involved in mediating the beneficial effects of ECPCM.

Lastly, pharmacological experiments showed that glucocorticoids are not responsible for the anti-inflammatory activity of ECPCM.

Overall, the experimental evidence described in this thesis suggests that the soluble anti-inflammatory factor (or factors) in ECPCM is not ssRNA. If it is a protein, it is proteinase K resistant, and it is not any of the well-known anti-inflammatory molecules tested in the studies described above. It is possible that other, as-yet unidentified factors, possibly of lipid or saccharide nature or proteinase K-resistant peptides, are responsible for the anti-inflammatory activity of ECPCM.

Chapter 3.5: Molecular mechanism underlying the anti-inflammatory effects of ECPCM

3.5.1 ECPCM affects protein tyrosine-phosphorylation

Phosphorylation is an important post-translational modification that controls many proteins' function and localisation and mediates cell signalling. As a systematic approach to study the molecular mechanism behind ECPCM anti-inflammatory effects on EC, the protein tyrosine-phosphorylation (p-Tyr) profiles for HAEC treated with ECPCM or collection (control) medium, with or without IL-6, were analysed. Figure 32 shows that the pattern of p-Tyr in EC treated with ECPCM was different from that observed with collection medium treatment. More specifically, both in the presence and absence of the cytokine, ECPCM treatment increased the intensity of a p-Tyr band of ~40 kDa, accompanied by the appearance of one extra band just above it. Moreover, in EC treated with ECPCM+IL-6 another very faint band just below 37 kDa appeared.

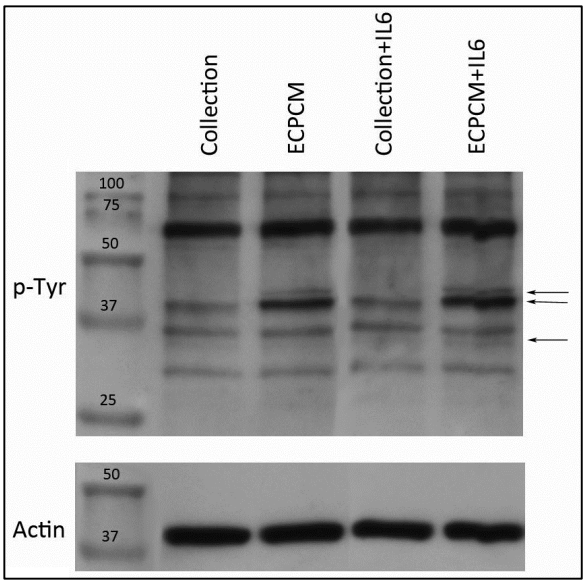

Figure 32: ECPCM affects the protein tyrosine phosphorylation pattern in EC. HAEC were treated for 30 minutes with collection medium (control) or ECPCM with or without 2.39 nM IL-6. Western blot analysis was performed using anti-phospho-tyrosine and anti-β-Actin antibodies. The arrows indicate the bands showing a different pattern in ECPCM samples compared to collection medium treatment. The image is representative of three independent experiments.

3.5.2 Analysis of the differentially tyrosine-phosphorylated proteins observed during treatment with ECPCM

ECPCM induced an increase in intensity or the appearance of three tyrosine-phosphorylated proteins in EC. In order to identify these proteins and study their potential roles in ECPCM anti-inflammatory activity, the specific bands were isolated and analysed by mass spectroscopy. This approach identified annexins A1, A2 and A5 as the proteins potentially affected by ECPCM.

Annexins are a large family of Ca^{2+}-dependent, phospholipid-binding proteins which are involved in a variety of cellular functions: membrane organization and trafficking, endo- and exocytosis, apoptosis, inflammation and fibrinolysis (Gerke V 2002; Gerke V 2005). The molecular weight of annexins varies between 36 and 39 kDa. Annexin A1 promotes membrane fusion and multi-vesicular endocytosis; promotes apoptosis of neutrophils and Jurkat-T lymphocytes; regulates phospholipase A2 activity and displays anti-inflammatory activity similar to that mediated by glucocorticoids when administered in the extra-cellular environment (Gerke V 2005). Annexin A2 is involved in Ca^{2+}-dependent exocytosis, including those events involving WPB and P-selectin exposure in EC; binds certain species of RNA, suggesting a role in controlling their transport or export from the nucleus; participates in the organization of lipid-raft-like domains at sites of actin recruitment; and stimulates fibrinolysis by acting as co-factor for tissue plasminogen activator and plasminogen (Gerke V 2005). An anti-coagulant role has also been proposed for annexin A5 (Gerke V 2005). Tyrosine phosphorylation has been reported for annexin A1 and A2 (Gerke V 2002; Gerke V 2005). This post-translational modification makes annexin A1 more susceptible to proteolysis (Gerke V 2002), whilst in the case of annexin A2 it decreases the annexin's phospholipid binding affinity and stimulates

translocation to the cell surface of EC in response to heath stress, where phospho-annexin A2 seems to increase plasmin activity and therefore fibrinolysis (Gerke V 2002; Deora AB 2004).

Stress response and fibrinolysis are important events regulating vascular homeostasis after injury. Given the role of annexin A2 in these responses and its regulation by tyrosine-phosphorylation, further experiments focused on the possible role of annexin A2 in mediating ECPCM anti-inflammatory effects.

Real-time PCR showed that expression of the annexin A2 gene in HAEC is quite stable over time and is not significantly affected by treatment with collection medium or ECPCM, with or without IL-6 (Figure 33).

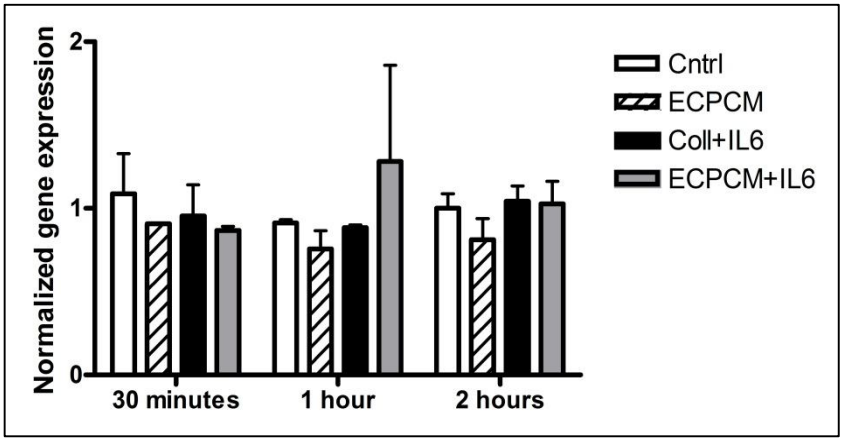

Figure 33: Gene expression of annexin A2 in HAEC. Cells were treated with or without 2.39 nM IL-6 in collection medium or ECPCM for 30 minutes and 1 or 2 hours. Cntrl = collection (control) medium only. Data = Mean ± SD; n (individual well) = 6 per group.

Western blot analysis of total and phosphorylated annexin A2 showed no differences between collection medium- and ECPCM-treated samples (Figure 34).

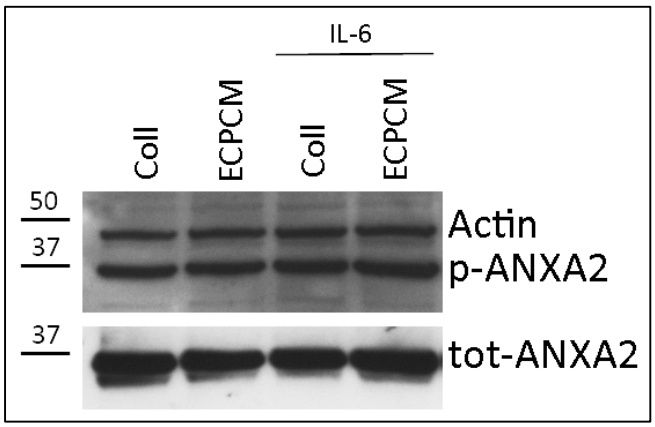

Figure 34: Protein levels of total and phosphorylated annexin A2 in HAEC. Cells were treated in collection medium (control) or ECPCM, with or without 2.39 nM IL-6 for 30 minutes. Western blot analysis was performed using anti-β-Actin and anti-total- and phospho-annexin A2. The image is representative of three independent experiments.

Based on these experiments annexin A2 and its phosphorylation were excluded as mediators of ECPCM anti-inflammatory activity in HAEC. The potential roles of annexins A1 and A5 in the anti-inflammatory activity of ECPCM will also be investigated in the future in order to thoroughly utilize the results from the mass spectrometry screening.

After this general analysis of phospho-tyrosines in EC, further experiments explored the specific pathways activated by TNFα and IL-6.

3.5.3 NF-kB pathway activation is necessary for TNFα-induced E-selectin and VCAM-1 expression in HAEC

NF-kB is a transcription factor known for its important role in modulating the immune and inflammatory response (Hayden MS 2008). As mentioned in the introduction, TNFα-induced gene expression is typically mediated by activation of NF-kB. Moreover, although the intracellular signalling cascade triggered by PF4 is still largely unknown, activation of NF-kB has been observed in HUVEC upon PF4

treatment (Yu G 2005). Based on this information, a possible association between the anti-inflammatory effects of ECPCM and the inhibition of NF-kB pathway was investigated. Activation of the pathway was assessed by looking at the protein levels of IkBα, the inhibitor that binds to NF-kB and retains it in the cytoplasm. IkBα needs to be phosphorylated and then degraded through the proteasome in order for the transcription factor to move into the nucleus to activate gene transcription (see introduction).

First, to verify that NF-kB is indeed necessary for TNFα-induced expression of E-selectin and VCAM-1 in HAEC, cells were treated with the cytokine in collection medium with or without BAY 1170-85, an irreversible inhibitor of IkBα phosphorylation and degradation, and therefore an inhibitor of NF-kB activation. The efficacy of BAY 1170-85 in blocking the NF-kB pathway was confirmed by western blot (figure 35).

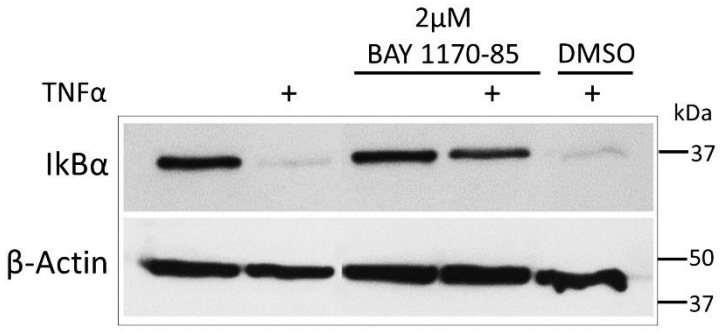

Figure 35: Inhibition of NF-kB activation by BAY 1170-85. HAEC were treated for 30 minutes with 0.1 nM TNFα in collection medium with or without 2 μM BAY 1170-85 or DMSO as vehicle control. Western blot analysis was performed with anti-IkBα and anti-β-Actin antibodies. The image is representative of three separate experiments.

Real-time PCR showed that inhibition of NF-kB activation during TNFα treatment blocked the induction of gene expression for E-selectin and VCAM-1 (figure 36). These data confirm the essential role of NF-kB in mediating the expression of the two adhesion molecules in HAEC upon TNFα treatment.

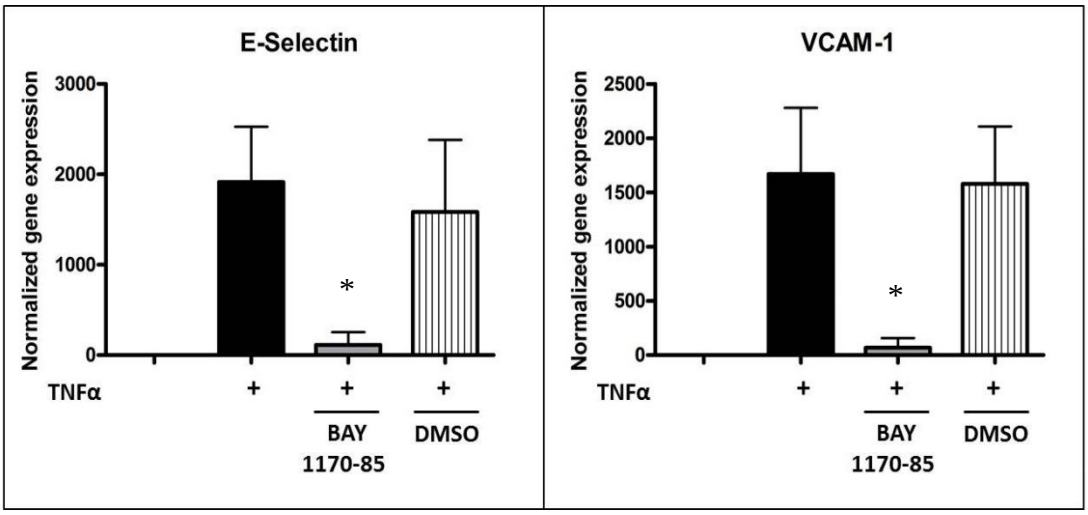

Figure 36: TNFα-induced gene expression for E-selectin and VCAM-1 is inhibited by BAY 1170-85. HAEC were treated for 2 hours with 0.1 nM TNFα in collection medium with or without 2 µM BAY 1170-85 or DMSO as vehicle control. *p-value <0.001 compared to treatment in collection medium + TNFα. Data = mean ± SD; n = 9 per group.

3.5.4 ECPCM does not affect IkBα degradation

In order to investigate whether ECPCM affects NF-kB activation, IkBα protein levels were analysed in HAEC treated with TNFα in collection medium or ECPCM. Treatment with TNFα in collection (control) medium caused the complete degradation of IkBα, indicating activation of NF-kB pathway as expected (figure 37). Importantly, cells treated with TNFα in ECPCM showed a decrease in IkBα levels comparable to that observed during treatment in collection medium (figure 37). These results show that ECPCM anti-inflammatory activity is not mediated by the inhibition of NF-kB pathway activation.

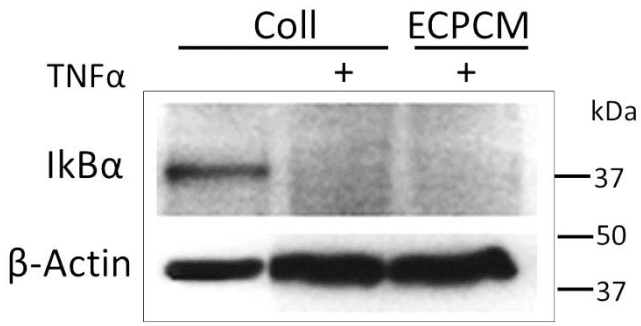

Figure 37: ECPCM does not affect NF-kB activation. Western blot of IkBα and β-Actin in HAEC treated for 30 minutes with collection medium or with 0.1 nM TNFα in collection medium or ECPCM. A representative image of three different experiments is shown.

3.5.5 ECPCM does not affect NF-kB translocation to the nucleus

An alternative mechanism for ECPCM to affect the NF-kB pathway would be to inhibit translocation of the activated transcription factor to the nucleus (Hayden MS 2008). To test this hypothesis, HAEC were treated for different times with TNFα in collection medium or ECPCM, then immunofluorescent staining was performed for p65 (RelA), which together with p50 makes up the activated NF-kB dimer for transcriptional activation (Hayden MS 2008). Figure 38 shows a clear accumulation of p65 in the nucleus after 30 minutes of treatment with TNFα in collection medium, with partial localization of the protein in the cytoplasm after 1 hour and a complete return of p65 to the cytosol after 2 hours of treatment. Interestingly, the staining pattern of p65 in HAEC treated with TNFα in ECPCM at various time points was very similar to that for TNFα in the collection medium control (figure 38). Quantification of p65 nuclear levels confirmed that there was no significant difference between ECPCM and collection medium treatment (figure 39). Based on these observations it can be concluded that ECPCM affects neither NF-kB activation nor its translocation into the nucleus.

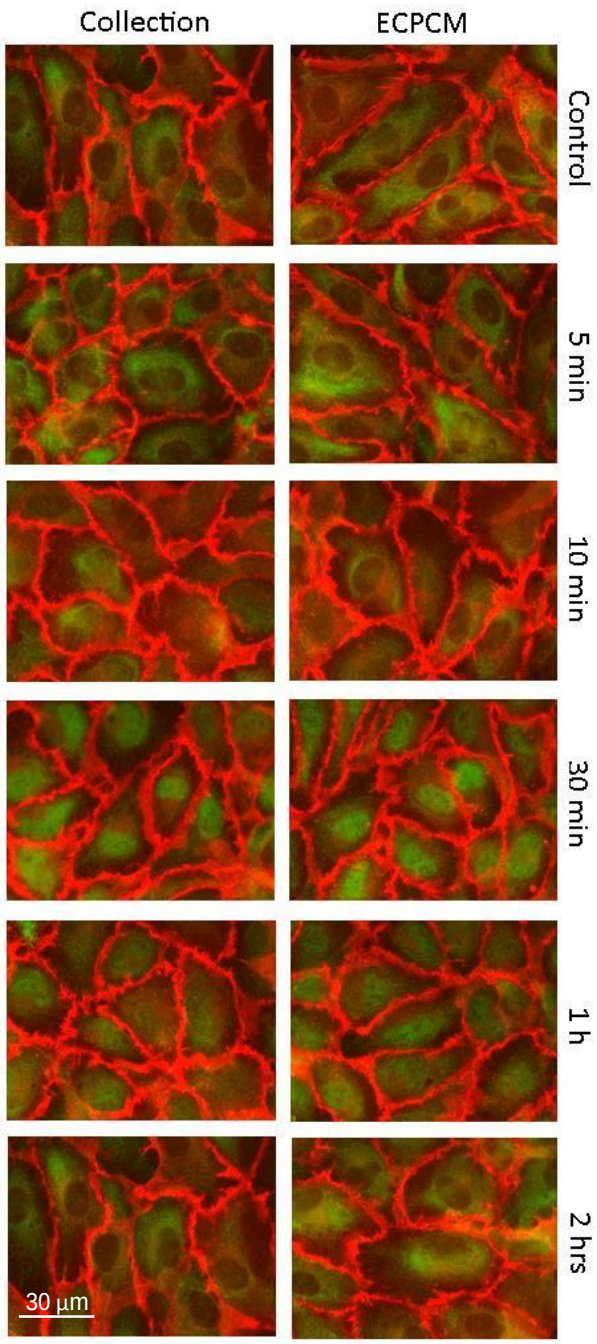

Figure 38: ECPCM does not affect p65 translocation to the nucleus. HAEC were treated with 0.1 nM TNFα in collection medium or ECPCM for up to 2 hours. Control cells were incubated in collection medium or ECPCM for 2 hours. After fixation in 4% paraformaldehyde and permeabilization with methanol, immunofluorescent staining was performed with anti-p65 (RelA) (green) and anti-PECAM-1 (red) antibodies. The image is representative of three separate experiments.

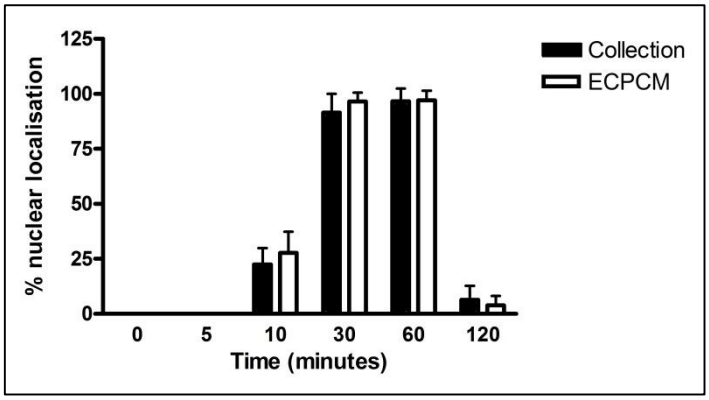

Figure 39: Quantification of p65 nuclear translocation in HAEC treated with 0.1 nM TNFα in collection medium or ECPCM for up to 2 hours. Data = Mean ± SD; n = 3 for each group.

3.5.6 IL-6 induces expression of E-selectin and VCAM-1 through activation of Stat3 and not NF-kB

IL-6 is known to stimulate gene transcription through activation of the Stat3 transcription factor (Heinrich PC 1998; Heinrich PC 2003). Although it is known that NF-kB is involved in gene expression of E-selectin and VCAM-1 (Montgomery KF 1991; Collins T 1995; Cook-Mills JM 2011), to date there are no reports of Stat3 being directly involved in the IL-6-induced transcription of these two genes. Nonetheless, in the *in vitro* assay described earlier IL-6 clearly induced both E-selectin and VCAM-1. In order to determine if in HAEC the induction of E-selectin- and VCAM-1 expression is indeed mediated by Stat3, experiments were performed using Stattic, a specific inhibitor of the Stat3 transcription factor. Stattic is a small molecule that selectively inhibits the activation of Stat3 by blocking its phosphorylation and dimerization (IC50 5.1±0.8 μM after 1 hour incubation at 37°C) (Schust J 2006). HAEC were treated with IL-6 in collection (control) medium in the presence or absence of 2 μM Stattic or DMSO as vehicle control. Stat3 activation was verified by western blot, and gene expression of E-selectin and VCAM-1 was analysed by real-time PCR.

As shown in figure 40, treatment with IL-6 induced Stat3 phosphorylation and therefore activation, whereas treatment in the presence of Stattic effectively blocked phosphorylation of the transcription factor. IL-6 did not activate the NF-kB pathway: IkBα levels were not affected by IL-6 treatment, which is in contrast with a dramatic reduction of IkBα upon TNFα treatment (positive control).

Real-time PCR data showed a marked decrease in IL-6-induced gene expression of E-selectin and VCAM-1 upon Stattic treatment (figure 41), demonstrating the essential role of Stat3 in the IL-6-induced transcription of both genes in HAEC.

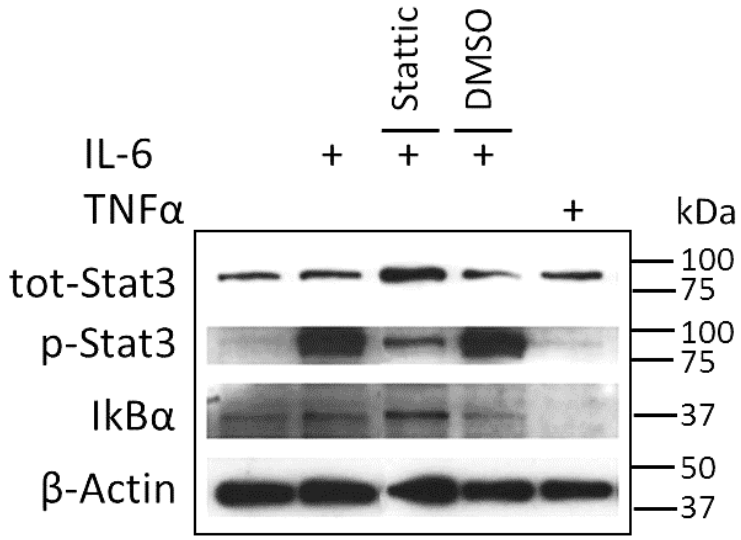

Figure 40: Effects of Stattic and IL-6 on the Stat3 and NF-kB pathways. After 1 hour pre-treatment with 2 µM Stattic or DMSO in collection medium, HAEC were treated for 30 minutes with 2.39 nM IL-6 in collection medium with or without Stattic or DMSO. Treatments in collection medium with IL-6 or 0.1 nM TNFα were also performed to verify activation of the Stat3 and NF-kB pathways, respectively. Western blot analysis was performed using antibodies specific for phosphorylated and total Stat3, IkBα and β-Actin. The image is representative of three separate experiments.

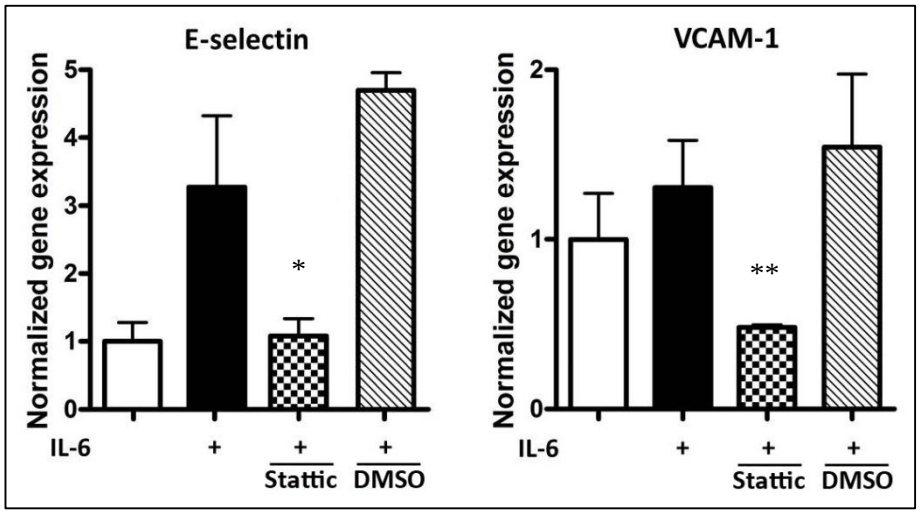

Figure 41: Stattic inhibits IL-6-induced E-selectin and VCAM-1 expression in HAEC. Real-time PCR was performed after 2 hours treatment in collection medium with 2.39 nM IL-6 with or without 2 µM Stattic or DMSO. P-value *< 0.01, **<0.001 compared to treatment in collection medium + IL-6. Data = mean ± SD; n = 6 for each group.

3.5.7 Stat3 activation is not affected by ECPCM

In order to study the effect of ECPCM on Stat3 activation in HAEC during IL-6 treatment, western blot analysis was conducted to visualize the total and the phosphorylated forms of the transcription factor. Figure 42 shows that IL-6 induced the phosphorylation of Stat3 in collection medium and ECPCM to similar levels, indicating that ECPCM does not affect the activation of Stat3.

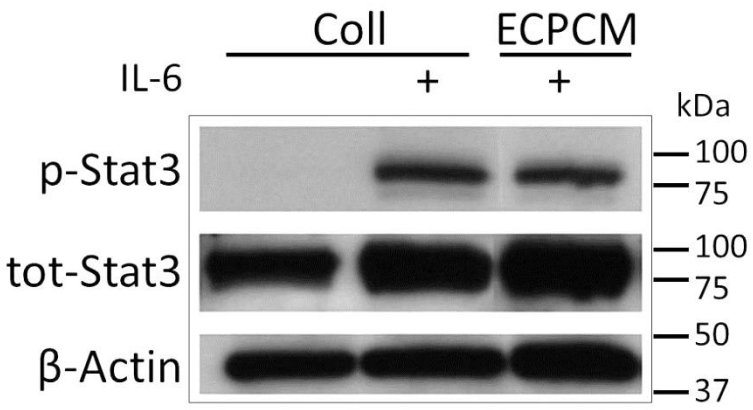

Figure 42: ECPCM does not affect Stat3 activation. HAEC were treated for 30 minutes with collection medium or with 2.39 nM IL-6 in collection medium or ECPCM. Western blot analysis was performed on β-Actin, total Stat3 and phosphorylated Stat3. The image is representative of three separate experiments.

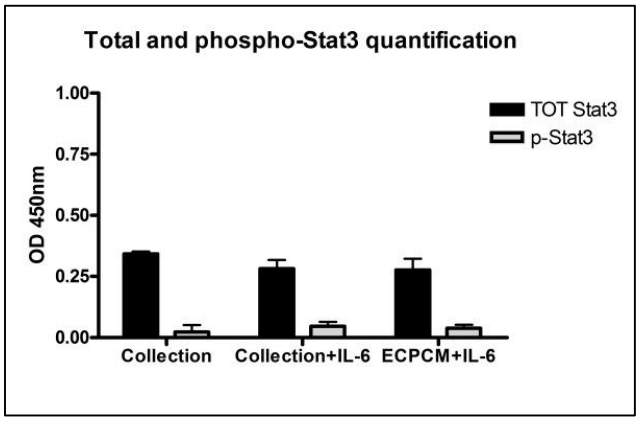

Figure 43: ELISA detection (expressed in absorbance at 450 nm) of total and phosphorylated Stat3 in HAEC treated for 30 minutes with 2.39 nM IL-6 in collection medium or ECPCM. Data = mean ± SD; n = 3 per group

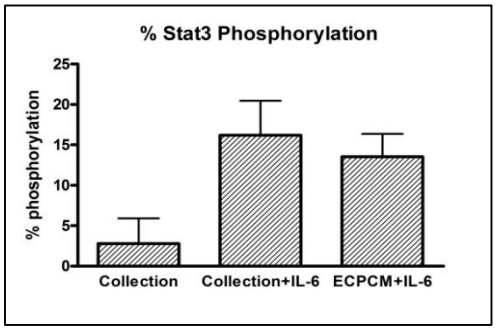

Figure 44: Percentage of phosphorylation of Stat3 as detected by ELISA. Data = mean ± SD; n = 3 per group.

To better quantify the differences in the phosphorylation and therefore activation of the transcription factor, an ELISA for total and phosphorylated Stat3 was performed on HAEC treated with IL-6 in collection medium or ECPCM. Phosphorylation levels for Stat3 were similar between the two treatment conditions (figures 43 and 44), confirming that activation of the Stat3 transcription factor by IL-6 is not affected by ECPCM.

3.5.8 Stat3 translocation to the nucleus is not affected by ECPCM

In order to determine if ECPCM prevents activated Stat3 from translocating into the nucleus and stimulating expression of E-selectin and VCAM-1, immunostaining for phosphorylated Stat3 was performed. HAEC were treated with IL-6 in either collection medium or ECPCM and fixed at different time points. As shown in figure 45, a similar pattern of staining was observed in cells treated with ECPCM and the collection medium control: activated Stat3 moved to the nucleus in the presence of collection medium or ECPCM; after only 5 minutes of IL-6 treatment an increase in nuclear phospho-Stat3 was observed, and even higher levels of the activated transcription factor in the nucleus were seen after 10 and 30 minutes. Phospho-Stat3 nuclear staining declined after 1 hour of treatment, although by 2 hours it still had not disappeared completely. Quantification of Stat3 nuclear localisation confirmed that there was no significant difference between ECPCM and collection medium treatment (figure 46). These data suggest that ECPCM does not affect activation or translocation of Stat3 transcription factor upon IL-6 treatment.

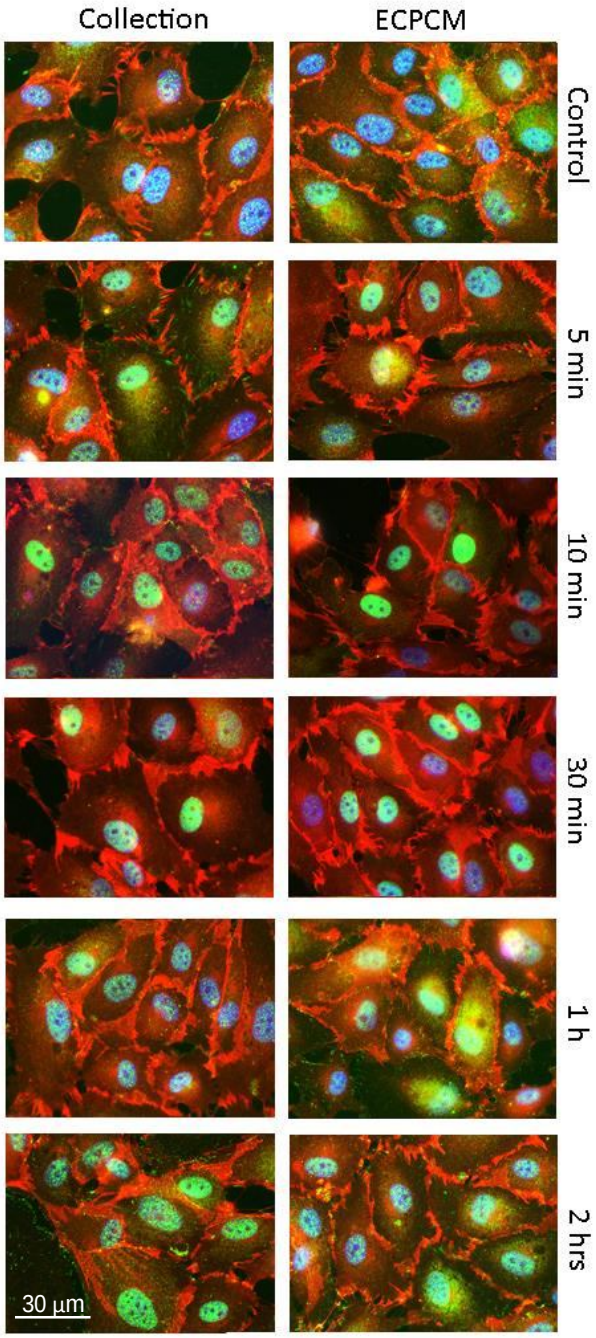

Figure 45: ECPCM does not affect translocation of activated Stat3 to the nucleus. HAEC were treated with 2.39 nM IL-6 in collection medium or ECPCM for up to 2 hours. Control cells were incubated in collection medium or ECPCM for 2 hours. After fixation in 4% paraformaldehyde and permeabilization in methanol, immunofluorescence staining was performed with anti-phosphoStat3 (green) and anti-PECAM-1 (red) antibodies followed by DAPI staining (blue). The image is representative of three separate experiments.

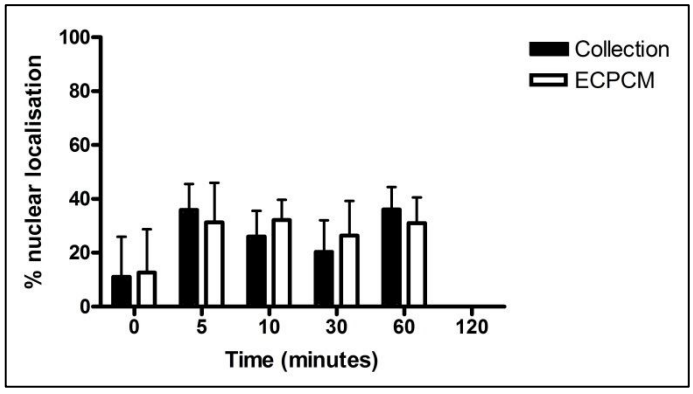

Figure 46: Quantification of phospho-Stat3 nuclear translocation in HAEC treated with 2.39 nM IL-6 in collection medium or ECPCM for up to 2 hours. Data = Mean ± SD; n = 3 for each group.

3.5.9 ECPCM does not affect stability of E-selectin or VCAM-1 mRNA

ECPCM affected neither activation nor translocation to the nucleus of NF-kB and Stat3, and yet it consistently inhibited TNFα- and IL-6-induced expression of E-selectin and VCAM-1 at both the mRNA and protein levels. Since RNA stability affects mRNA levels and ultimately protein levels, the ability of ECPCM to affect the stability of E-selectin- and VCAM-1 mRNA was investigated.

Gene expression was first induced in HAEC by treatment for 2 hours with TNFα or IL-6 in collection medium, then transcription was inhibited by addition of Actinomycin D, a polypeptide antibiotic that binds to DNA at the initiation of transcription complex and blocks RNA elongation and transcription by RNA polymerase (Sobell HM 1985). Next, cells were treated with IL-6 or TNFα in collection (control) medium or ECPCM in the presence of Actinomycin D for an additional 30 minutes, 1 hour, 2 hours and 4 hours. E-selectin and VCAM-1 mRNA levels were analysed at each time point by real-time PCR. The normal turnover of the mRNA for both E-selectin and VCAM-1 was also determined by treating HAEC for the same time with the cytokines in collection medium without Actinomycin D.

Figure 47-A shows that after the initial 2 hours of cytokine treatment, continued treatment with IL-6 in collection medium, but without Actinomycin D, induced a small rise in E-selectin mRNA levels for 1 additional hour, then the mRNA quickly decreased and reached basal levels at 4 hours. However, treatment in collection medium with IL-6 and Actinomycin D showed a steady and slow decrease in E-selectin mRNA levels from 30 minutes onwards. A similar trend in E-selectin mRNA reduction was observed during treatment with IL-6 and Actinomycin D in ECPCM. In HAEC treated with IL-6 but no Actinomycin D in collection medium, VCAM-1

mRNA levels rose for up to 2 hours past the initial induction and returned to initial levels after 4 hours (figure 47-B). In cells treated with IL-6 and Actinomycin D, mRNA levels of VCAM-1 at each time point were comparable between treatment in collection medium and ECPCM. In both cases the mRNA levels decreased regularly from 30 minutes of Actinomycin D addition, until reaching the initial levels after 4 hours (figure 47-B).

During TNFα treatment in collection medium, E-selectin mRNA levels increased for 1 hour after the initial induction and then decreased after 4 hours (figure 47-C). Treatment in collection medium with TNFα and Actinomycin D showed a regular but small reduction in E-selectin transcript levels. Treatment with ECPCM in the presence of TNFα and Actinomycin D revealed a similar trend, with a slow but steady decrease in mRNA expression from 1 hour onwards after the initial induction. At all time points examined the E-selectin mRNA levels were comparable between the two treatments (figure 47-C). For VCAM-1 expression, after the initial induction the transcript levels rose for 2 more hours in the presence of collection medium with TNFα and without Actinomycin D. In the presence of TNFα and Actinomycin D, VCAM-1 mRNA levels increased slightly for 30 minutes and then decreased steadily at each time point, and were comparable throughout the experiment upon treatment with collection medium or ECPCM (figure 47-D). As was observed with E-selectin, overall levels of VCAM-1 transcripts after 4 hours of TNFα treatment were still quite high compared to those observed upon IL-6 treatment. This is probably due to the fact that TNFα induces a much higher level of gene expression than does IL-6.

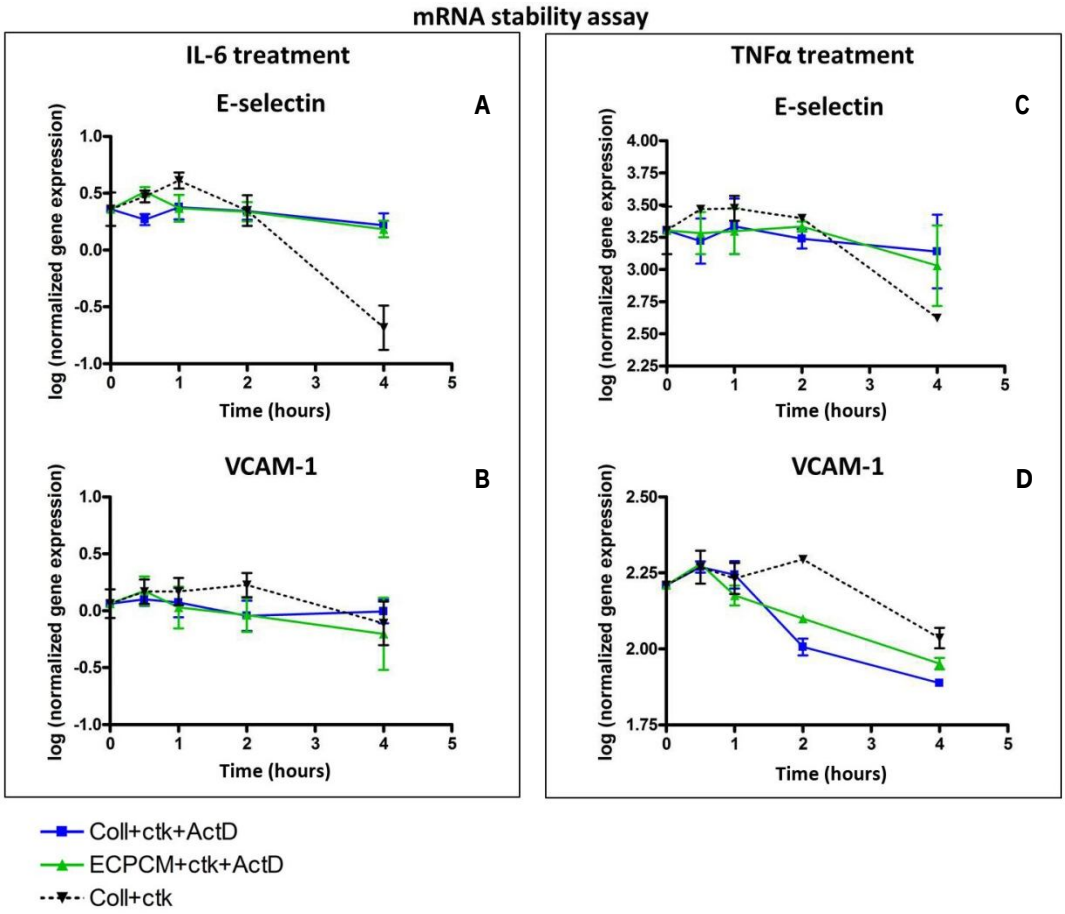

Figure 47: mRNA stability assay on E-selectin and VCAM-1 transcripts. HAEC were treated with the cytokine (ctk) (2.39 nM IL-6 or 0.1 nM TNFα) in collection medium for 2 hours. The media was then changed to collection medium + cytokine (dotted line), collection medium + cytokine + Actinomycin D (blue line) or ECPCM + cytokine + Actinomycin D (green line). Transcripts levels were assessed by real-time PCR after 30 minutes, 1 hour, 2 hours and 4 hours of treatment. Data = mean ± SD.

Although the kinetics of mRNA decrease were different during treatment with IL-6 and TNFα, and between E-selectin- and VCAM-1 genes, these results clearly show that ECPCM does not affect the mRNA stability of either gene. Therefore, it is highly unlikely that the anti-inflammatory effect of ECPCM involves the modulation of mRNA stability of the targeted genes.

3.5.10 ECPCM decreases TNFα-induced binding of p65 to the E-selectin and VCAM-1 promoters

Since the stability of E-selectin and VCAM-1 transcripts induced by IL-6 and TNFα was not affected by ECPCM, and the activation and nuclear translocation of NF-kB and Stat3 were also not inhibited by ECPCM, the effect on transcription was examined next as a potential mechanism for the anti-inflammatory effect of ECPCM. To this end, the promoter DNA binding activity of the transcription factors was analysed using a chromatin immuno-precipitation assay (ChIP). Because ChIP experiments require a large number of cells to give sufficient genomic DNA, which was not possible to obtain with primary cultures of HAEC, HUVEC were used for these experiments.

To validate HUVEC as a suitable cell type substitution for ChIP, IL-6 and TNFα treatment in collection medium or ECPCM was performed using the same timing and concentrations adopted for HAEC. Figure 48 shows that the anti-inflammatory activity of ECPCM on HUVEC is similar to that observed in HAEC. Indeed, ECPCM inhibited IL-6- and TNFα-induced expression of E-selectin by 85% and 35%, respectively, whereas expression of VCAM-1 was inhibited by ECPCM by 75% with IL-6 and 61% with TNFα treatment in HUVEC (figure 48).

To further validate the use of HUVEC, molecular signalling pathways were analysed in the cells during cytokine treatment. Western blot and immunofluorescent experiments were performed on p65 and Stat3 in HUVEC treated with TNFα or IL-6, in collection medium or ECPCM.

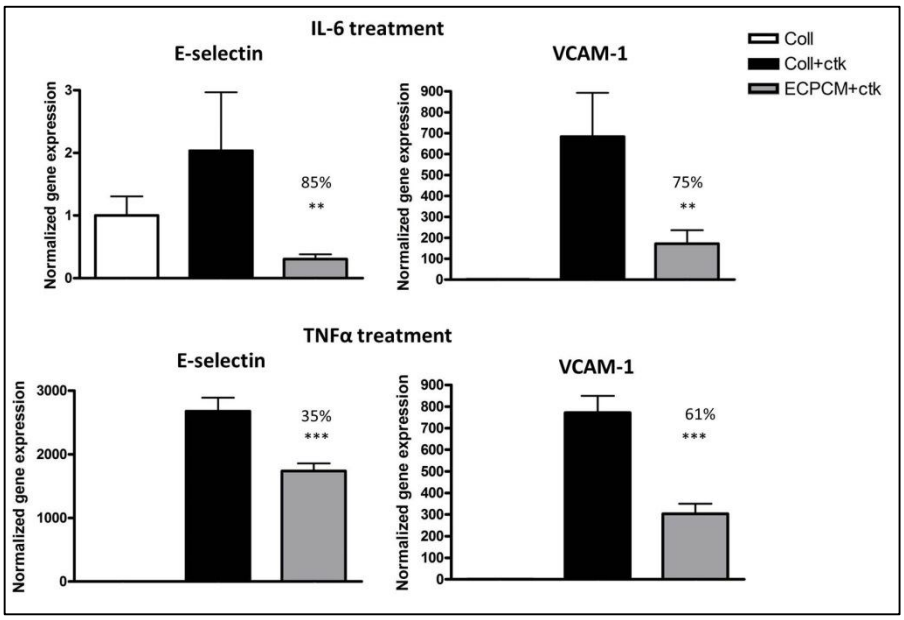

Figure 48: Inhibition of E-selectin- and VCAM-1 gene expression by ECPCM in HUVEC. Cells were treated with 2.39 nM IL-6 or 0.1 nM TNFα for 2 hours in collection medium or ECPCM. Percentage of gene expression inhibition and p-value compared to treatment with IL-6 or TNFα in collection medium are shown. p-value: *** <0.001; ** <0.01; * <0.05. Data = mean ± SD; n = 9 for each group.

As was observed with HAEC (see figures 37-40 and 42-46), treatment of HUVEC with TNFα in collection medium induced IkBα degradation (figure 49) and NF-kB pathway activation with p65 translocation to the nucleus (figures 50 and 51). Treatment in ECPCM did not affect the TNFα–dependent NF-kB activation and translocation (figures 50 and 51). Likewise, HUVEC treated with IL-6 showed activation and nuclear translocation of Stat3 in the presence of both collection medium and ECPCM (figures 49, 52 and 53). IkBα was not degraded during IL-6 treatment, confirming that in HUVEC, as in HAEC, IL-6-induced expression of E-selectin and VCAM-1 is Stat3- and not NF-kB-dependent.

These results demonstrate that IL-6 and TNFα activate the same molecular pathways in HAEC and HUVEC; they also show that ECPCM has comparable effects on the two cell lines. Therefore, HUVEC is a valid alternative to HAEC for ChIP experiments.

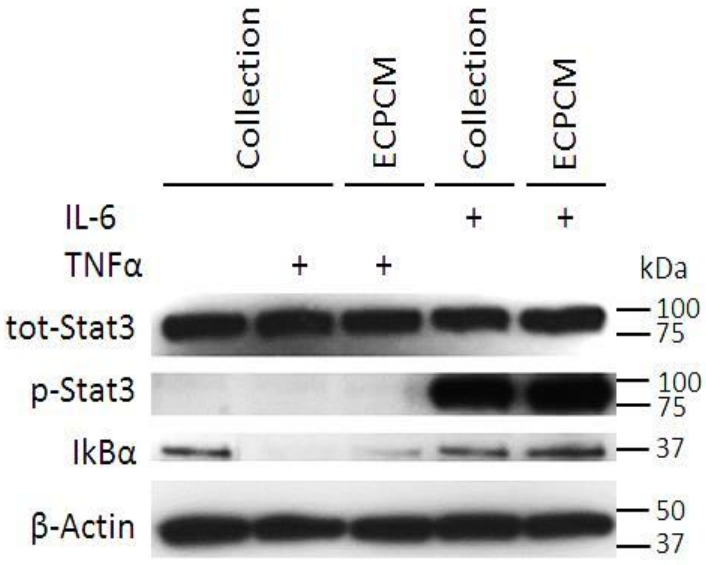

Figure 49: ECPCM does not affect activation of NF-kB or Stat3 in HUVEC. Cells were treated for 30 minutes with collection medium, 0.1 nM TNFα in collection medium or ECPCM and with 2.39 nM IL-6 in collection medium or ECPCM. Western blot analysis was performed on β-Actin, IkBα, total and phosphorylated Stat3. The image is representative of three separate experiments.

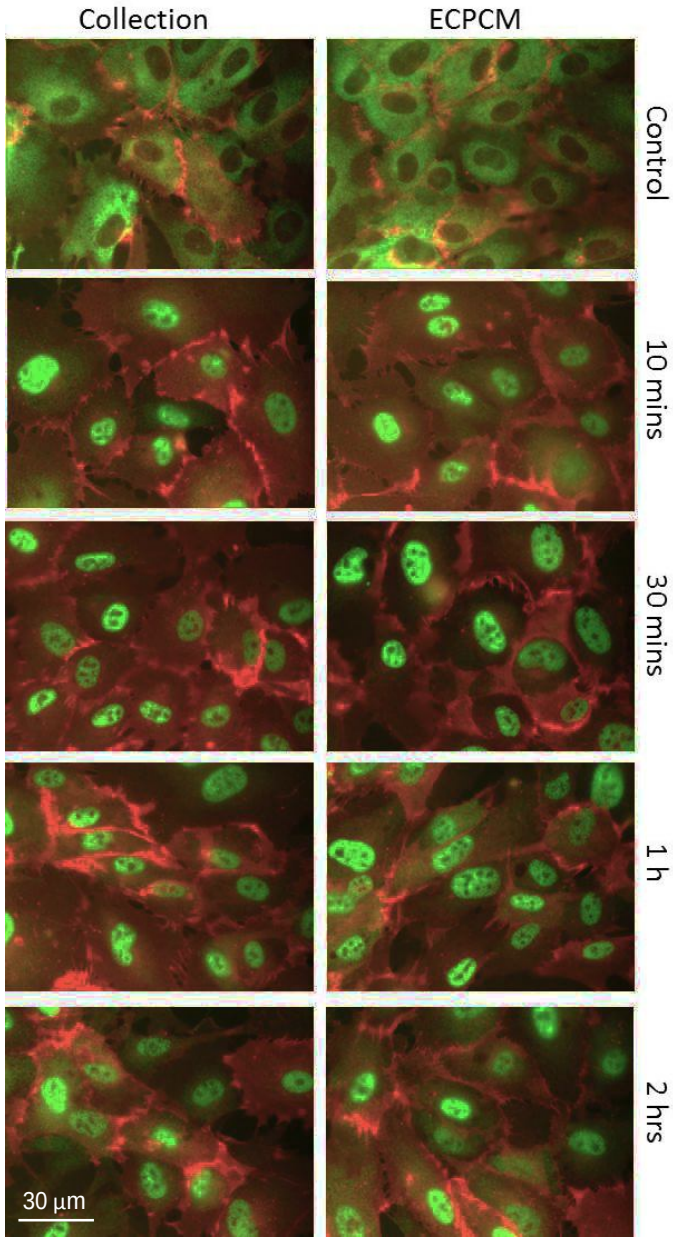

Figure 50: Translocation of activated p65 to the nucleus in HUVEC. Cells were treated with 0.1 nM TNFα in collection medium or ECPCM for 10 minutes, 30 minutes, 1 hour or 2 hours. Control cells were incubated in collection medium or ECPCM for 2 hours. After fixation in 4% paraformaldehyde and permeabilization with methanol, immuno-fluorescence staining was performed with anti-p65 (green) and anti-PECAM-1 (red) antibodies. The image is representative of three separate experiments.

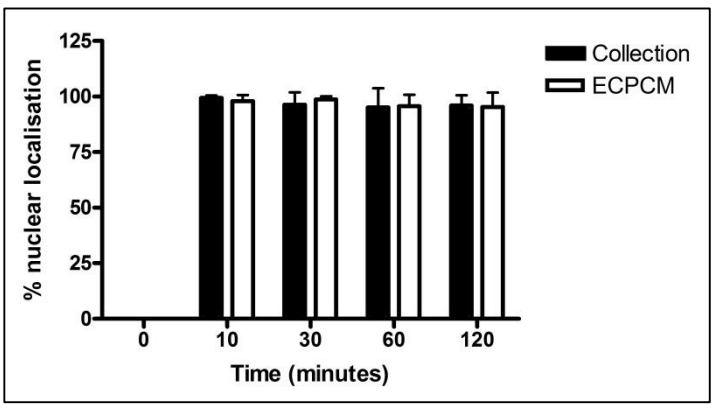

Figure 51: Quantification of p65 nuclear translocation in HUVEC treated with 0.1 nM TNFα in collection medium or ECPCM for up to 2 hours. Data = Mean ± SD; n = 3 for each group.

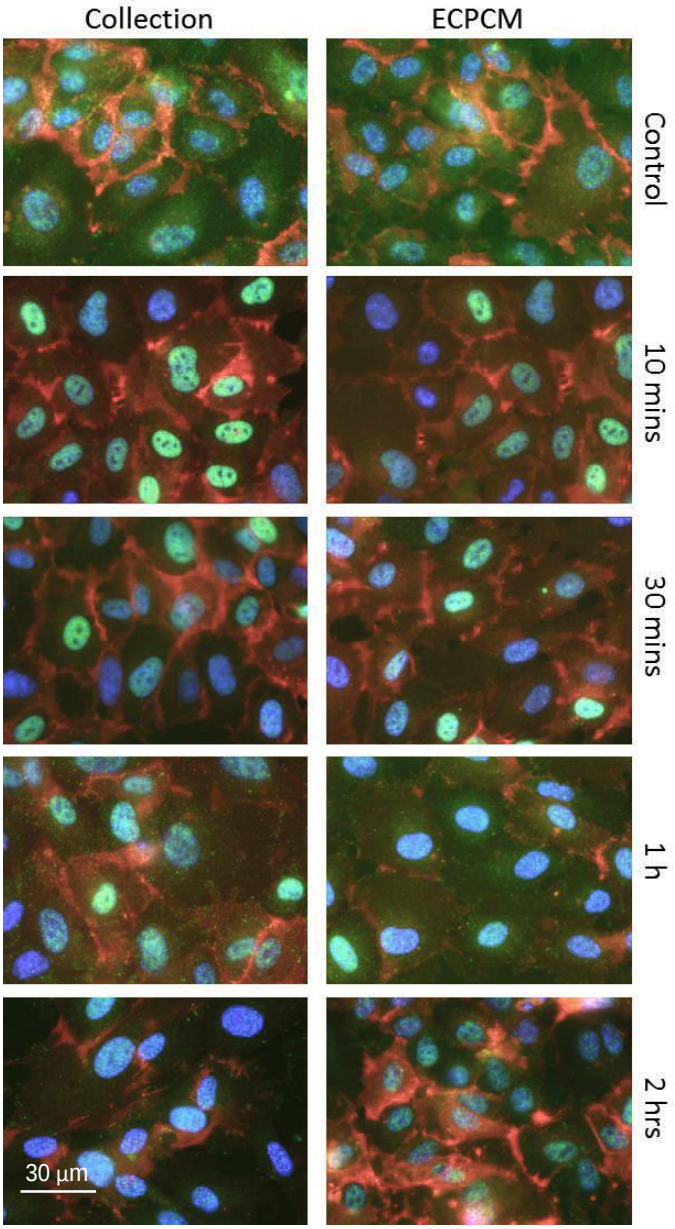

Figure 52: Stat3 activation and translocation to the nucleus in HUVEC. Cells were treated with 2.39 nM IL-6 in collection medium or ECPCM for 10, 30 minutes, 1 or 2 hours. Control cells were incubated in collection medium or ECPCM for 2 hours. After fixation in 4% paraformaldehyde and permeabilization with methanol, immuno-fluorescence staining was performed with anti-phospho-Stat3 (green) and anti-PECAM-1 (red) antibodies followed by DAPI staining (blue). The image is representative of three separate experiments.

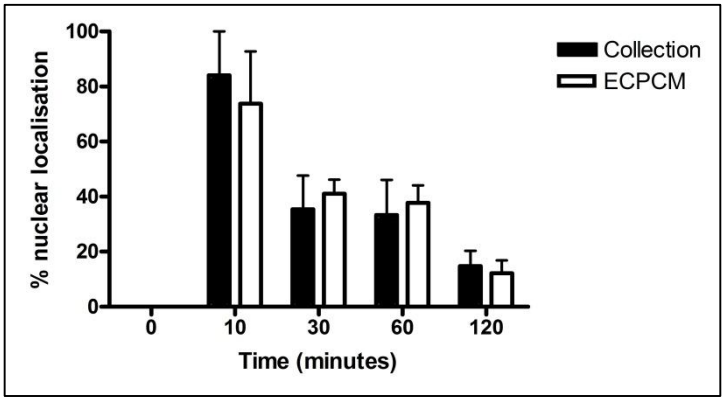

Figure 53: Quantification of phospho-Stat3 nuclear translocation in HUVEC treated with 2.39 nM IL-6 in collection medium or ECPCM for up to 2 hours. Data = Mean ± SD; n = 3 for each group.

For the ChIP experiment HUVEC were treated with or without TNFα in collection medium or ECPCM. After immunoprecipitation with an anti-p65 antibody, real-time PCR was used to determine the amount of DNA bound to p65 upon cytokine treatment. Isotype-matched IgG was used as a negative control for ChIP, and binding of p65 to its target DNA sequence was expressed as fold change over non-specific IgG binding. Primers and probes for real-time PCR were specifically designed to detect and amplify the regions recognized by p65 on E-selectin and VCAM-1 promoters (figure 54).

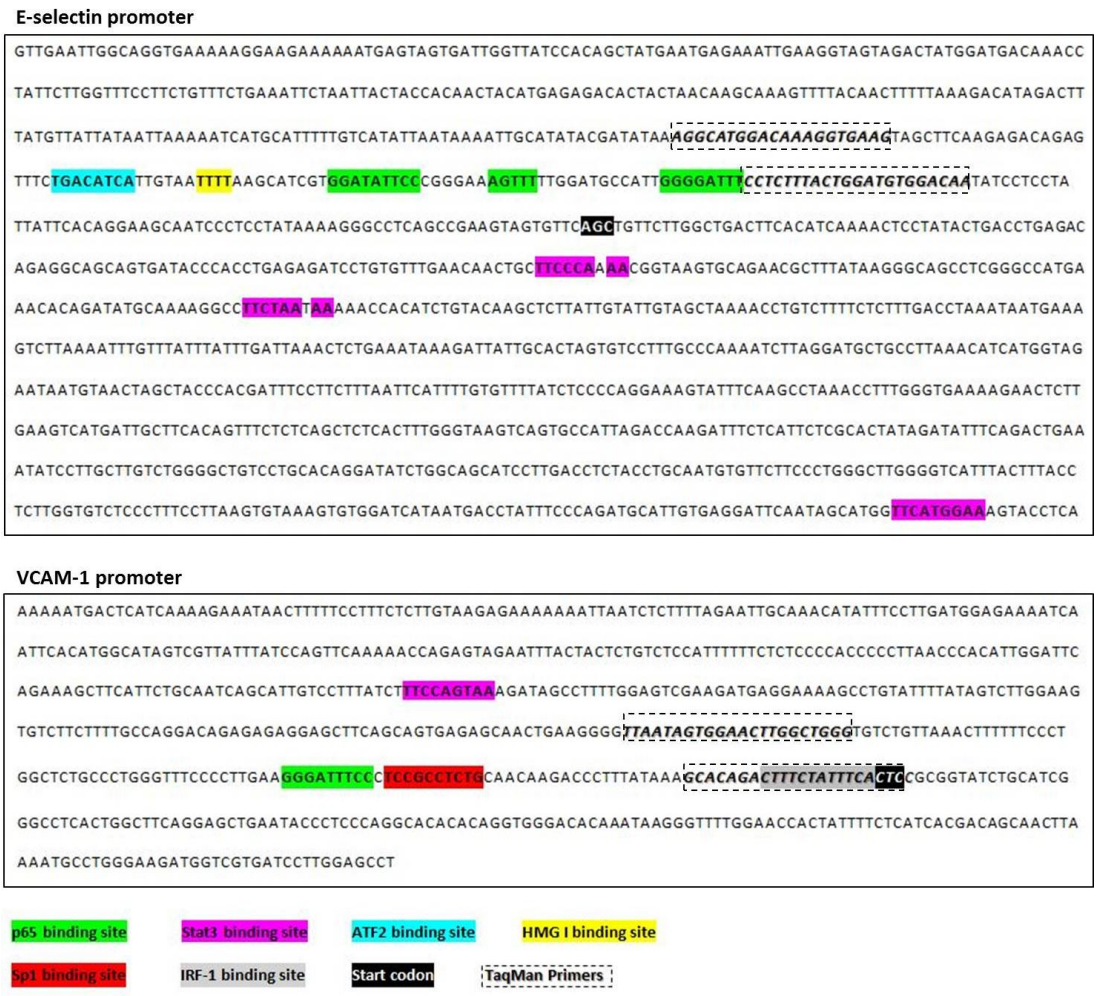

Figure 54: Nucleotide sequence of the E-selectin and VCAM-1 promoters. Binding sites for specific transcription factors are highlighted in different colours. The sequences between the TaqMan primers (dotted boxes) were amplified using real-time PCR.

As shown in figure 55, treatment of HUVEC with TNFα in collection (control) medium strongly induced p65 binding to E-selectin and VCAM-1 promoters. More importantly, ECPCM inhibited the binding of p65 to the promoters of both genes, down to levels comparable to the control treatment without TNFα. These data suggest ECPCM exerts its anti-inflammatory activity on TNFα mainly by inhibiting p65 binding to the promoters of E-selectin and VCAM-1 and thereby blocking their transcription.

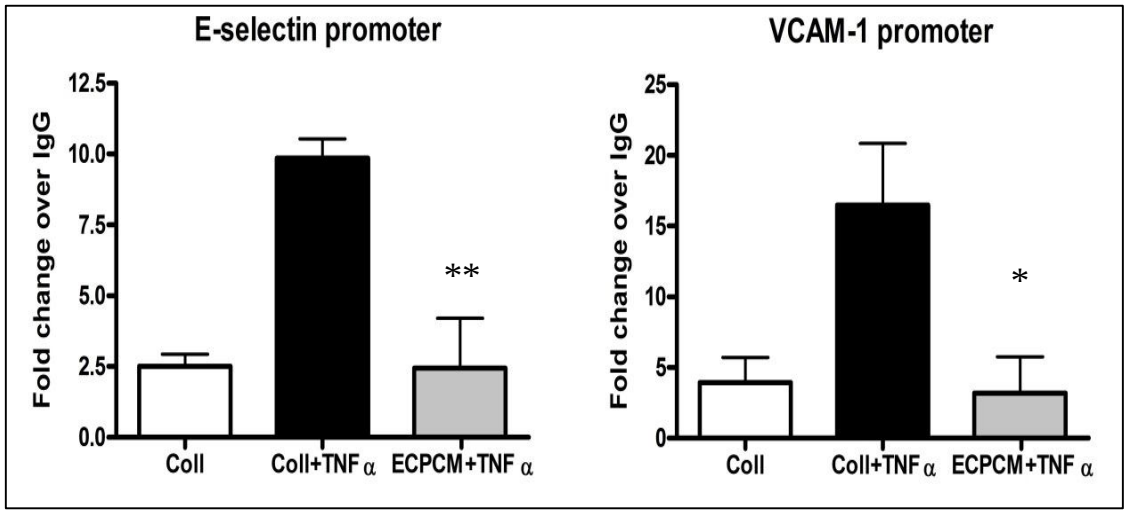

Figure 55: ECPCM significantly inhibits p65 binding to E-selectin and VCAM-1 promoters. HUVEC were treated for 1 hour with or without 0.1 nM TNFα in collection medium or ECPCM. The extracted chromatin was immunoprecipitated using anti-p65 antibody or isotype-matched IgG as control. The immunoprecipitated DNA was then quantified using real-time PCR. The level of p65 binding to the specific promoters was expressed as fold changes over the IgG control. p-value: *<0.01, **<0.001 comparing ECPCM + TNFα to collection medium + TNFα treatment. Data = mean ± SD; n = 3 for each group.

3.5.11 Molecular mechanisms underlying the anti-inflammatory effects of ECPCM: conclusions and discussion

In order to elucidate the molecular mechanism mediating ECPCM anti-inflammatory activity and the pathways affected by the formulation, analysis of protein tyrosine phosphorylation in HAEC was performed. Cells treated with ECPCM revealed a different pattern of tyrosine phosphorylation compared to collection medium treatment. Mass spectrometry analysis identified those differentially-phosphorylated proteins likely are annexins A1, A2 and A5. Further experiments analysing annexin A2 showed no changes in the level of gene expression or phosphorylation of the protein in EC upon ECPCM treatment, suggesting that annexin A2 is not affected by ECPCM and therefore is likely not involved in mediating its anti-inflammatory effects. Since the mass spectrometry analysis was performed only once, it is possible that the data obtained from that single experiment were not representative for changes in EC in the presence of ECPCM. Also, the mass spectrometry analysis provided a list of other proteins identified in the gel bands sent for investigation, and those proteins rather than annexin A2 might be the ones affected by ECPCM. Repeating the mass spectrometry analysis and exploring the role of other proteins identified in the list of potential candidates could provide interesting data on the molecular changes affecting EC response to inflammatory stimuli in the presence of ECPCM.

Experiments performed on HAEC and HUVEC confirmed that the canonical NF-kB pathway is activated in EC upon TNFα treatment. The cytokine induced degradation of IkBα, localization of p65 to the nucleus and strong gene expression of the inflammatory adhesion molecules E-selectin and VCAM-1. Use of a selective NF-kB

activation inhibitor, BAY 1170-85, abolished both IkBα degradation and cytokine-induced gene induction, proving the essential role of p65 in mediating the pro-inflammatory effects of TNFα in EC.

Stat3 is another pro-inflammatory transcription factor that is typically activated by IL-6. HAEC and HUVEC treated with IL-6 showed induction of Stat3 phosphorylation, accumulation of the activated Stat3 in the nucleus, and no reduction in IkBα levels. Induction of E-selectin and VCAM-1 gene expression was observed at the same time. Moreover, treatment of HAEC with IL-6 in the presence of a specific inhibitor for Stat3 phosphorylation prevented the cytokine-dependent gene induction. These results proved that Stat3, and not NF-kB, is mainly responsible for the pro-inflammatory gene expression induced by IL-6 in EC. Whereas involvement of Stat3 in ICAM-1 transcription has been reported (Wung BS 2005; Chen SC 2006), to my knowledge this is the first evidence of Stat3 being involved in gene expression of VCAM-1 and E-selectin.

Interesting, ECPCM did not prevent activation or nuclear translocation of p65 and Stat3 during treatment with TNFα and IL-6, respectively. Immunofluorescence analysis suggested that ECPCM did not affect the turnover or degradation of either activated transcription factor.

During TNFα treatment p65 activity is shut down by a negative feedback loop in which p65 promotes IkBα transcription (Sun SC 1993). Newly synthesised IkBα binds to NF-kB in the nucleus and moves the transcription factor to the cytoplasm (Sun SC 1993; Hayden MS 2008). Analysis of IkBα levels was not performed; however, since p65 levels and nuclear localisation were not affected by ECPCM over

a period of 2 hours compared to the control treatment, it is unlikely that ECPCM affects IkBα transcription or synthesis.

The mRNA stability of the cytokine-induced genes was also not affected by ECPCM. After inhibition of transcription, degradation of E-selectin and VCAM-1 mRNA was not enhanced by the presence of ECPCM. Based on the results of ECPCM digestion with proteinase K and RNase, miRNAs which are resistant to both enzymes were proposed as a possible mediator of ECPCM activity; these molecules are known to be involved in inhibition of adhesion molecule expression in EC (e.g. miR-126, -31 and -10a). The miRNAs act by inducing degradation of gene transcripts (Harris TA 2008; Fang Y 2010; Suarez Y 2010). More specifically, a recent publication suggests miRNAs mediate gene silencing by affecting protein translation first and mRNA deadenylation and decay second (Djuranovic S 2012). In the same study, inhibition of transcription with actinomycin D resulted in a substantial reduction in protein synthesis but no decline of mRNA levels at a single time point after induction. This scenario is not consistent with the ECPCM-mediated inhibition of E-selectin and VCAM-1 expression, which involved a decrease in levels of the genes transcripts first, at as early as 2 hours, and reduction of proteins levels later, after 5-6 hours. Based on these data miRNA molecules can be excluded as mediators of ECPCM activity.

An explanation of the mechanism of action of ECPCM was provided by the ChIP experiments, which showed that ECPCM reduced p65 binding to E-selectin and VCAM-1 promoters in HUVEC. Binding of p65 to DNA is controlled by ubiquitination and acetylation; four lysine residues (Lys-122, -123, -314 and -315) are targets for both poly-ubiquitination and acetylation (Li H 2012). Ubiquitination

of p65 promotes its release from chromatin by targeting the protein for proteasomal degradation (Li H 2012). If ubiquitination was involved in ECPCM activity, it would cause a decrease in detectable p65 levels in EC treated with TNFα in the presence of ECPCM. Since western and immunofluorescence analyses showed no reduction in p65 levels upon ECPCM treatment, this degradation mechanism can be excluded. Ubiquitination of p65 can also have non-degradative effects on the transcription factor, for example by preventing acetylation events on the same lysine acceptor sites. Interestingly, Lys-122 and -123 are the only residues contacting DNA in the minor grove (Chen FE 1998) and their acetylation reduces p65 binding to DNA (Kiernan R 2003). On the other hand, acetylation of Lys-310 enhances p65 transcriptional activity (Quivy V 2004; Schmitz ML 2004). It has been suggested that acetylated-p65 is more easily exported to the cytoplasm by IkBα (Kiernan R 2003). If that was the case, TNFα-induced p65 nuclear levels should decrease more quickly in EC treated in ECPCM compared to cells treated in collection medium. However, immunostaining data at different time points revealed no difference in the abundance and localization of p65 between ECPCM- and collection medium-treated EC. Nonetheless, it is still possible that ECPCM affects the DNA-binding ability of p65, for example by stimulating its acetylation on Lys-122 and -123.

Phosphorylation of other sites on p65 has been described. Phosphorylation on Ser-276, -311, -529, -536 and Thr-254 stimulates the transcriptional activity of the factor, either by increasing its stability and nuclear localization or by enhancing its interaction with DNA and co-factors; on the other hand, phosphorylation of Thr-435 and -505 inhibits p65 transcriptional activity (Campbell KJ 2004). The phosphorylation status of p65 in EC during treatment in ECPCM was not analysed, therefore it cannot be excluded that ECPCM is affecting this post-translational

modification. In the future it would be of interest to verify if p65 phospho-sites are involved in the reduction of p65 DNA binding in the presence of ECPCM. Similarly, it would be interesting to analyse the phosphorylated status of NF-kB transcriptional co-factors.

Alkylation on Cys-38 was shown to inhibit p65 binding to DNA (Garcıa-Pineres AJ 2001; Pande V 2009). This type of chemical modification is induced by sesquiterpene lactones and epoxyquinone A. Sesquiterpene lactones are the active molecules in many medicinal plants from the Asteraceae family that are used in traditional medicine to treat inflammation (Garcıa-Pineres AJ 2001). Epoxyquinone A is a synthetic derivative of the natural epoxyquinol A (Pande V 2009). Neither molecule is produced by human EC, but a similar chemical compound and/or the same alkylation modification could be responsible for the reduction in p65 DNA binding induced by ECPCM.

As mentioned in the introduction, p65 must interact with a number of co-factors in order to induce transcription of adhesion molecules. It is possible that the anti-inflammatory factor (or factors) in ECPCM reduces p65 binding to E-selectin and VCAM-1 promoters by disturbing the interaction between the NF-kB subunit and one or more of its many co-factors. Alternatively, ECPCM might contain a molecule that binds to the actively transcribed DNA, and thereby preventing the binding of transcription factors to their target sequences. This general mechanism of transcription inhibition would have particularly strong effects on the expression of genes highly induced by the local environment, such as the pro-inflammatory milieu simulated by EC *in vitro*.

Chapter 3.6: Therapeutic effects of ECPCM *in vivo*

3.6.1 A zymosan-induced peritonitis model of systemic inflammation was tested for study the anti-inflammatory activity of ECPCM activity *in vivo*

To further explore the anti-inflammatory effects of ECPCM *in vivo,* an animal model involving zymosan-induced peritonitis was developed. Zymosan is a protein-carbohydrate complex that is produced from the yeast cell wall and is able to activate an inflammatory reaction when injected into animals. It is a ligand for Toll-like receptor 2, which is part of the innate immune system and is involved in pathogen recognition and activation of non-specific immune reactions (Underhill DM 1999). Preliminary experiments were performed to determine the optimal dose of zymosan to induce detectable peritonitis. C57/BL6 female mice, 10-14 weeks old were used, and an intra-peritoneal (IP) injection of 0.5 mg of zymosan in 1 ml of collection medium was sufficient to induce a significant increase in the number of inflammatory cells in the peritoneum at 24 hours post-injection, as determined by peritoneal lavage (data not shown). To improve the assay, the time course of the zymosan treatment was also determined, with peritoneal lavage performed after 1, 2, 4, 8, 18 and 24 hours following IP injection of the compound. The data from the time course experiment showed that the number of inflammatory cells reached a maximum at 8 hours post-injection, then started to decrease slowly, though inflammatory cell numbers remained high even at 24 hours post-injection (Figure 56). Based on this experiment, the optimum conditions for the peritonitis assay were established as 0.5 mg zymogen and cell count by peritoneal lavage at 16-18 hours post injection. This time point was selected for technical convenience and because

ECPCM anti-inflammatory effect was expected to be maximal before or after the peak of immune cell response.

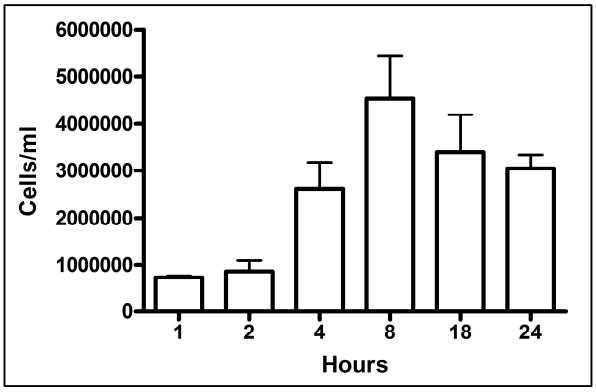

Figure 56: Time course of zymosan-induced peritonitis as assessed by peritoneal lavage. The graph shows the total number of inflammatory cells/ml in the peritoneal lavage recovered from animals at 1, 2, 4, 8, 18 and 24 hours after IP injection of 0.5 mg zymosan in collection (control) medium (n=3 per time point). Data = Mean ± SEM.

In order to test ECPCM activity in this model, animals were injected with 0.5 mg of zymosan resuspended in 1 ml of either collection medium or ECPCM. Injection of collection medium alone was used as a negative control for the assay. Peritoneal lavage and cell counting were performed at 16-18 hours post-injection. No significant difference in the inflammatory cell number/ml of peritoneal lavage was observed in animals treated with ECPCM compared to the collection medium-treated group (Figure 57).

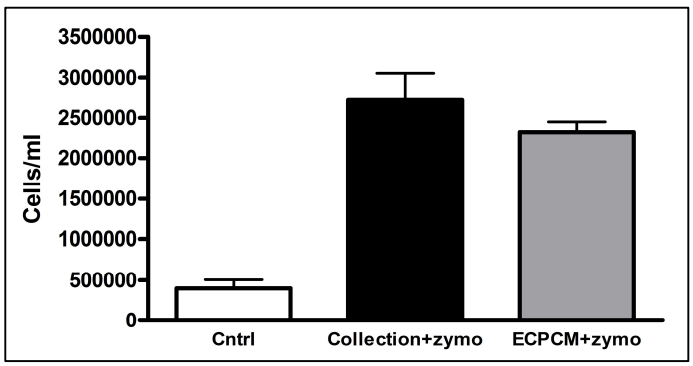

Figure 57: Number of inflammatory cells/ml in the peritoneal lavage recovered from animals 16-18 hours after injection with zymosan in collection medium or ECPCM (n=5 per treatment). The control animals (Cntrl) were injected with collection medium only. Data = Mean ± SEM.

These results suggest the ECPCM did not significantly suppress zymogen-induced peritonitis, likely because the dosage of zymosan at 0.5 mg was too high. However, no further study of this peritonitis model was performed because a vascular inflammation model was deemed to be more suitable for testing the anti-inflammatory activity of ECPCM.

3.6.2 A retinal leukostasis assay was tested for study of the anti-inflammatory potential of ECPCM *in vivo*

In order to evaluate the therapeutic activity of ECPCM *in vivo,* another animal model of vascular inflammation was developed: the retinal leukostasis assay. In this model inflammation is induced in the vasculature of the mouse retina through intra-vitreal (IVT) injection of a pro-inflammatory cytokine. After 24 hours cardiac perfusion with FITC-conjugated Concavalin A (Con-A) is performed. This allows staining of the blood vessels and leukocytes adhering to the luminal surface of the vasculature in areas of inflammation. The advantages of using this model to study vascular inflammation include the high vascularity of the retina, the accessibility of the tissue through IVT injections and the possibility to easily perform microscopic imaging of the retina. In pilot studies of the leukostasis assay, C57/BL6 mice received IVT injections of PBS, 2 pmol TNFα, and 1 or 2 pmol IL-6. After 24 hours the leukocytes adhering to the retinal vasculature were counted. As expected, treatment with pro-inflammatory cytokines induced an increase in leukocyte recruitment in the retinal vasculature (Figure 58).

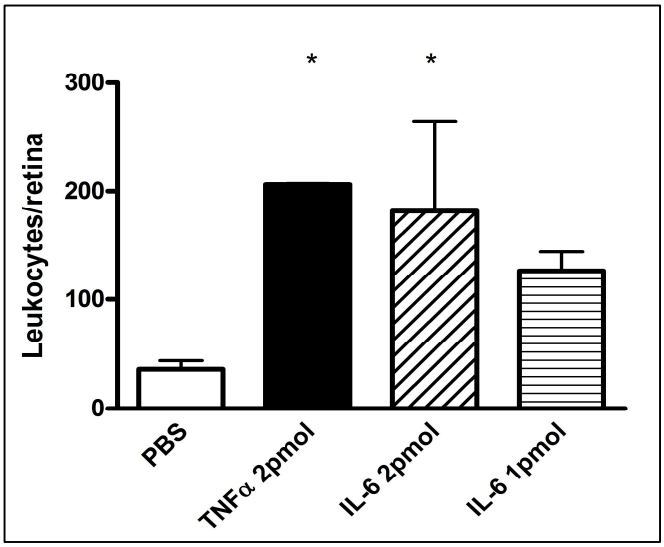

Figure 58: Induction of inflammation and leukocyte recruitment in the retinal vasculature. Mice received IVT injection of PBS, TNFα (2 pmol) or IL-6 (1 or 2 pmol); after 24 hours cardiac perfusion with FITC-ConA was performed, retinas were dissected and whole mounted and leukocyte number in the vasculature of each retina was determined using a fluorescent microscope (n=4 per treatment). *p-value <0.05 compared to the PBS control. Data = Mean ± SEM.

Further experiments were performed to study ECPCM effects. To this end, mice received IVT injections of 1 pmol IL-6, followed by IP injection of 1 ml collection medium (control) or ECPCM (treatment). After 24 hours the leukocytes in the retinal blood vessels were counted. Although the initial data were promising in that ECPCM treatment suppressed retinal leukostasis induced by IL-6, repeated experiments revealed that the assay was unreliable. In fact we discovered that due to an unanticipated technical issue, some of the retinas displayed insufficient and non-uniform perfusion. The incomplete staining of the vasculature made leukocyte counting virtually impossible (data not shown). Various technical modifications to the assay were tested in hopes of resolving the issue, but to date these have failed to improve the quality of retinal perfusion with FITC-ConA. Because of these technical problems, the retinal leukostasis assay was not used for further study of ECPCM anti-inflammatory activity *in vivo*.

3.6.3 ECPCM treatment suppresses choroidal neovascularization (CNV) development in a novel animal model of spontaneous CNV

Since the advantages of the retina for studying vascular inflammation remained, we turned to an alternate model of inflammatory vascular disease: CNV. The JR5558 mouse line is a novel genetic model of multi-focal, bi-lateral, spontaneous CNV (Nagai N 2011). These mice harbour a recessive homozygous mutation in an unknown gene (or genes) that leads to formation of sub-retinal neovascular tufts originating from the choriocapillaris post-natally between P10 and P15. From early (P15) to late (P30) development, CNV lesions are accompanied by macrophage infiltration. Depletion of circulating macrophages decreases lesion size, but not lesion number, confirming that macrophages have a significant role in driving the growth of existing CNV in this model (Espinosa-Heidmann DG 2003). CNV represents a non-specific wound repair response to an underlying disease, and all CNV lesions involve inflammation (Grossniklaus HE 2004). Since inflammation is a major component of CNV, JR5558 mice were used to assess the therapeutic anti-inflammatory effects of ECPCM *in vivo*.

CNV is examined and analysed by using fluorescein angiography (FA). The lesions appear as lacy, nodular or irregular hyperfluorescence in the early phase of angiography (Spencer WH 1996). In the mouse eye CNV is apparent as round-shaped areas of hyperfluorescence that increase in size over time. FA analysis was performed on JR5558 mice at P22 (day 0) to verify the presence of CNV and measure the baseline lesion area of each lesion in each retina. Animals were then treated for 7 days with daily IP injection of ECPCM or PBS. Lesion measurements

by FA were repeated at P29 (day 7), and eyes were collected for immunostaining analysis at P30 (day 8).

As shown in figure 59, treatment with ECPCM for 7 days significantly reduced the area of CNV lesions per retina compared to the control PBS treatment. Indeed, from P22 (day 0) to P29 (day 7) the CNV area/retina increased by ~6% in mice treated with PBS, whereas it decreased by ~30% in animals treated with ECPCM. No decrease in lesion number was detected (data not shown).

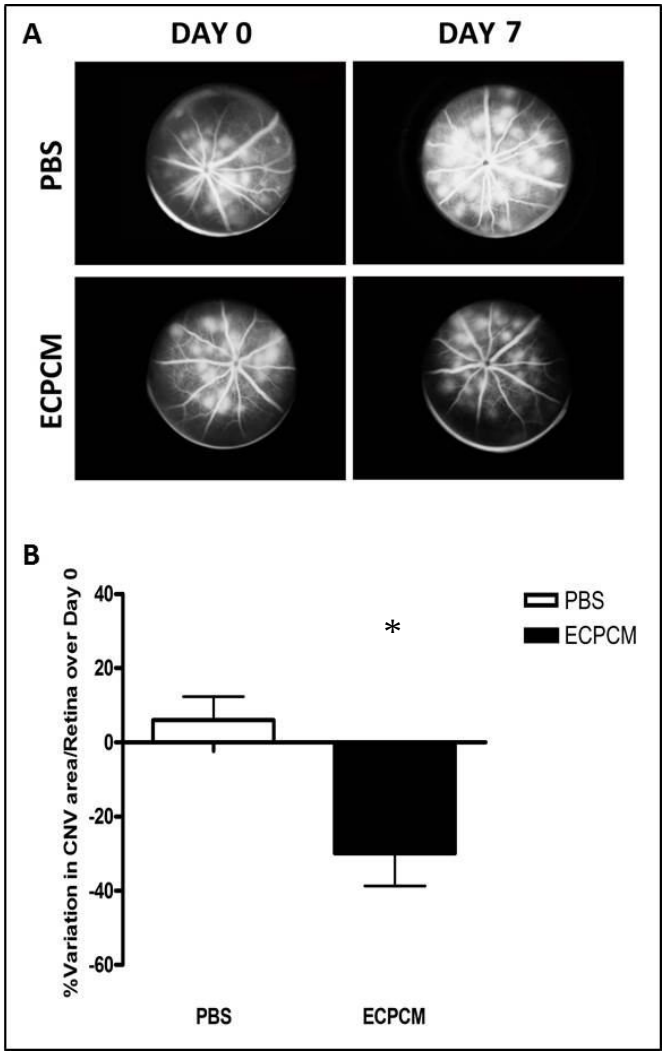

Figure 59: ECPCM reduces CNV area. JR5558 mice received daily intraperitoneal injection of 0.5 ml ECPCM or PBS from P22 (day 0) to P29 (day 7) (n=4 per treatment). CNV area/retina was determined at baseline (day 0) and after the treatment (day 7) using FA. **A**: Representative early phase FA images obtained from JR5558 before and after 7 days treatment with either PBS or ECPCM. **B**: Percentage of increase or decrease in CNV area/retina at day 7 compared to day 0 (+5.9% and -29.89% after PBS or ECPCM treatment respectively). *p-value <0.02 comparing ECPCM to PBS treatment. Data = mean ± SEM.

3.6.4 ECPCM reduces macrophage recruitment to CNV lesions in a spontaneous animal model of CNV

After the final FA, the eyecups of JR5558 animals were analysed by immunohistochemical staining for macrophage infiltration using F4/80 and PECAM-1 as markers of activated macrophages and EC, respectively. In animals treated with ECPCM, macrophage recruitment to the CNV lesions was significantly reduced compared to the recruitment observed in PBS-treated mice (figure 60).

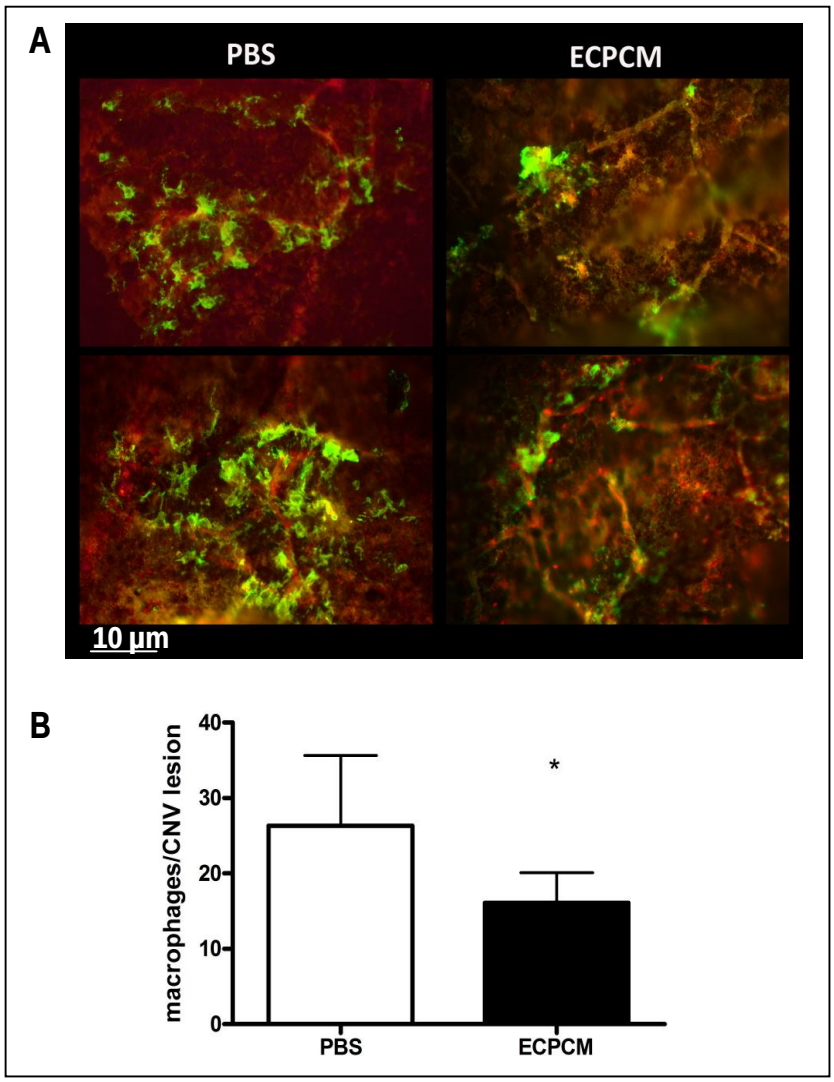

Figure 60: ECPCM reduces macrophage infiltration in spontaneous CNV lesions of JR5558 mice. **A**: Representative immunofluorescence images of the CNV. After 7 days of treatment with ECPCM or PBS, eyecups were collected and stained with anti-F4/80 antibodies to mark macrophages (green) and anti-PECAM-1 antibodies to mark endothelial cells (red). **B**: Quantification of macrophage recruitment at CNV lesions (n=9 per treatment) expressed as macrophage number around each lesion. *p-value <0.02. Data = mean ± SD.

An important feature of the CNV development and growth in JR5558 animals is inflammation. ECPCM reduced both the CNV area/retina and the macrophage recruitment to CNV, even when it was delivered at a distance from the diseased area by IP injection. These *in vivo* results confirm the presence of a potent soluble anti-inflammatory factor (or factors) in ECPCM and suggest its significant therapeutic potential.

3.6.5 *In vivo* therapeutic effects of ECPCM: conclusions and discussion

Establishing a suitable *in vivo* model to study the therapeutic effects of ECPCM was quite challenging. A major technical issue was the inability to concentrate the anti-inflammatory factor(s) in ECPCM, which meant a relatively large volume of ECPCM had to be injected into animals. The duration of treatment was another important aspect to consider in determining the *in vivo* effects of the formulation. Finally, there were challenges with animal models of inflammation chosen for the *in vivo* studies.

This project on ECPCM anti-inflammatory effects was inspired by preliminary data obtained from animal models of IH. Vascular inflammation is one of the important components in the pathogenesis of IH, which is a complex and multi-faceted disease accompanied by many other reactions (i.e. VSMC proliferation and migration) that are difficult to control in an experimental setting. For this reason experimental models of IH are not suitable for study of the anti-inflammatory effects of ECPCM.

The first model that was tested was the zymosan-induced peritonitis model. The failure of the ECPCM to reduce inflammatory cells recruitment might be explained by the molecular mechanism by which zymosan activates the inflammatory response.

Zymosan is a ligand for TLR2, a receptor involved in pathogen recognition and innate immunity that typically activates a non-specific immune reaction and numerous downstream inflammatory pathways (Underhill DM 1999). It is possible that effects of ECPCM on a single inflammatory pathway would be masked in the TLR2-dependent immune response involving multiple inflammatory pathways. Another explanation is that ECPCM might be effective in inhibiting the local activation of inflammatory cells and/or their adhesion to the vascular endothelium (activities suggested by the effects of ECPCM *in vitro*), rather than their recruitment to the peritoneal cavity. In any case, zymosan-induced peritonitis was not the ideal model for testing ECPCM *in vivo*.

In a second model, acute and robust vascular inflammation (leukostasis) was induced in the retina through intra-vitreal injection of pro-inflammatory cytokines such as IL-6 and TNFα. Recruitment and adhesion of leukocytes to the luminal surface of blood vessels in the retina was subsequently analysed by FITC-ConA perfusion. Technical challenges with the perfusion prevented this model from being adopted. More experiments are currently being carried out in the laboratory in order to resolve this issue, and it is possible that in the future the leukostasis model will provide more information about ECPCM anti-inflammatory activity *in vivo*.

The third model, an animal model of spontaneous CNV, was suitable for studying the anti-inflammatory activity of ECPCM. Since CNV is a major complication associated with AMD (Spencer WH 1996; Espinosa-Heidmann DG 2003), there is also great clinical relevance to a better understanding of this pathology. The JR5558 mice develop spontaneous CNV lesions, providing an inflammation-based model of eye disease. Moreover, the JR5558 mouse line represents a robust and reproducible

animal model for testing new therapeutics that target vascular inflammation as a means of suppressing CNV development.

JR5558 animals with established CNV lesions treated by intra-peritoneal injections of ECPCM for 7 days showed a significant decrease in CNV area/retina and a reduction in activated macrophage recruitment to the lesions. Since the growth of CNV lesions is supported by inflammatory events (see introduction), the reduction in the CNV area induced by ECPCM treatment is evidence of anti-inflammatory activity of ECPCM *in vivo*. The reduction in recruitment of macrophages to CNV lesions further demonstrates the *in vivo* anti-inflammatory effects of ECPCM. Importantly, these data support the idea of ECPCM containing soluble anti-inflammatory factors that, following delivery to the peritoneum, are able to reach the vessels in the eye and affect the local inflammatory reactions. Given the limitation on the volume that could be injected into the animals and the dilute concentration of the anti-inflammatory factor(s) in ECPCM, these results also provide strong evidence for great anti-inflammatory potency of the formulation.

Although the effects of ECPCM have promise with regards to treatment of CNV, it is possible that the anti-inflammatory effects observed with mouse CNV will not correlate with effects in human CNV. VEGF can mediate pro-inflammatory effects (Takahashi H 2005; Luo J 2011), and anti-VEGF treatments decrease leukocyte infiltration in experimental mouse models of CNV and ischemia/reperfusion liver injury, yet anti-VEGF treatment of human CNV has yielded surprising results (Tsuchihashi S 2006; Cao J 2010). Treatment of CNV with the anti-VEGF drug Bevacizumab in patients with AMD was paradoxically associated with an increase in the recruitment of leukocytes and especially macrophages to the CNV lesions, while having no effect on expression of E-selectin or ICAM-1 compared to untreated

controls (Tatar O 2008). It will be interesting to see if the promising therapeutic potential of ECPCM, which reduces both the CNV lesions' area and the infiltration of inflammatory cells in mice, will translate to effective CNV treatment in humans.

The experiments performed on the JR5558 mouse strain demonstrated how useful and powerful this animal model is. These mice with spontaneous CNV provide a model of inflammation in the eye, which can be easily analysed before, during and after treatment; they provide a localized and isolated site of inflammation, enabling the analysis of anti-inflammatory effects of systemic treatment with ECPCM. Moreover, each JR5558 animal provides data about both evolution of the CNV lesions and mediators of inflammatory reactions thanks to the compatible techniques of FA for CNV and immunohistological analysis for local inflammation. Gene expression analysis was not performed in this study, but it, too, is feasible.

In summary, the JR5558 mouse model of spontaneous CNV was used to demonstrate effects of ECPCM on vascular inflammation *in vivo*. ECPCM delivered by IP injection reduced CNV lesion area and recruitment of activated macrophages, findings with promising implications for treatment of AMD. In addition, the JR5558 mouse was further established as a promising new model for spontaneous CNV.

Chapter 4: Final discussion and future directions

4.1 Final discussion

EC are important producers of molecules that regulate vascular homeostasis and therefore the body's physiology and response to injury and disease. The factors released by the endothelium comprise a wide array of molecules, from proteins to lipids to gases. While the nature and mechanism of action for many of these molecules have been identified over the past three decades, other factors and/or their molecular effects remain to be understood. This thesis offers insight into the biochemical properties and mechanism of action for a potentially novel, soluble anti-inflammatory factor released by EC *in vitro*.

The work presented in this thesis was inspired by experiments performed using animal models of IH. In these experiments vessels were injured by angioplasty with or without stent placement, then a collagen matrix embedded with EC (EC/matrix) was placed around the damaged vessels. The animals treated with the EC/matrix showed less restenosis and reduced inflammation compared to control animals (Nathan A 1995; Nugent HM 1999; Nugent HM 2009; Nugent HM 2012). Since EC were placed at a distance from the injured luminal surface of the vessel, the data suggested that some soluble factors were being released from the EC/matrix, and that these factors were responsible for the positive effects on the vessels. Based on these observations, conditioned medium obtained from EC grown on a three-dimensional collagen matrix (ECPCM) was produced and analysed for its anti-inflammatory potential. Indeed, ECPCM was able to inhibit *in vitro* the cytokine-induced expression of pro-inflammatory adhesion molecules such as E-selectin and VCAM-1 in different types of EC (HAEC and HUVEC) in a dose-dependent manner. ECPCM

also reduced neovascularization and inflammatory responses in a mouse model of CNV.

Various experiments showed that previously identified anti-inflammatory molecules were not responsible for the beneficial effects of ECPCM; more precisely, TGF-β1, IL-10, cAMP, NO, PGI_2, resolvins and glucocorticoids were excluded as mediators of the anti-inflammatory activity. Instead, the data suggested ECPCM contains an as-yet unidentified anti-inflammatory molecule, or a mixture of molecules, which is resistant to both RNAse and proteinase K treatments. Moreover, the anti-inflammatory factor (or factors) was affected by treatments that involved inert materials such as agarose and cellulose.

The control exerted by ECPCM over EC activation by pro-inflammatory cytokines was mediated by the inhibition of transcription factors binding to pro-inflammatory gene promoters. In the presence of TNFα, ECPCM inhibited the binding of the transcription factor p65 to the promoters of E-selectin and VCAM-1. Given that ECPCM reduced not only TNFα inflammatory potential, but also IL-6- and PF4-induced gene expression, inhibition of the binding of transcription factors to their target sequences on pro-inflammatory genes might be the common molecular mechanism by which ECPCM exerts its anti-inflammatory activity.

It has been shown that the transcription factor Erg represses the expression of many NF-kB target genes in resting HUVEC by binding to the promoters of the genes, thereby preventing p65 binding (Dryden NH 2012). A similar mechanism of quiescence control could be provided by ECPCM in different ways: 1) ECPCM could potentiate the interaction between transcription regulators (such as Erg) already bound to the promoters of pro-inflammatory genes in quiescent EC; 2)

ECPCM might induce the rapid expression and/or the release from intracellular compartments of proteins that bind to the DNA and mask the promoters of pro-inflammatory genes; 3) ECPCM could contain a molecule that is able to quickly reach the nucleus of EC and bind to the DNA at sites recognized by pro-inflammatory transcription factors; 4) ECPCM could stimulate or inhibit post-translational modifications of transcription factors (for instance NF-kB) and/or their co-factors in such a way to prevent them from binding to the promoters; and 5) ECPCM could induce modifications on the promoter DNA that prevent recognition and binding by transcription factors. In every case the end result would be reduced access of activated p65 and/or other transcription factors and co-factors to the promoters of pro-inflammatory genes.

More experiments are needed to verify that ECPCM prevents transcription factor binding to pro-inflammatory promoters in the presence of IL-6 and PF4 as well as TNFα. Nevertheless, it is conceivable that the soluble factor (or factors) in ECPCM inhibits EC activation through maintenance of cell quiescence despite the presence of pro-inflammatory stimuli. The factor could mediate a general anti-inflammatory message throughout the blood vessel to promote homeostasis and avoid excessive or uncontrolled EC activation. Blockage of transcription factor binding to target sequences could serve as a gatekeeper function in EC responding to injury, in which accessibility to pro-inflammatory promoters is granted only when a certain stimulus level is sensed by EC. In this scenario, the endothelium would show a variable balance between anti- and pro-inflammatory factors recruited at the promoters of genes involved in inflammation. In normal physiologic conditions the balance would tilt towards quiescence due to the abundance of anti-inflammatory factors at the promoters, which counteract the smaller presence of pro-inflammatory factors and

act as a brake on gene expression. In the case of insult, the increase in concentration of pro-inflammatory factors would over-ride the anti-inflammatory factors and induce EC activation via binding to the promoters of inflammation-related genes. Given the dose-dependent anti-inflammatory effect of ECPCM, it is possible that quiescence-promoting/anti-inflammatory factors in ECPCM compete with the pro-inflammatory stimuli and allow EC activation only when inflammatory stimuli are sufficient and/or prolonged.

An endothelial-derived, anti-inflammatory molecule that does not inhibit the activation of a specific pro-inflammatory pathway in EC, but instead acts by controlling endothelial activation at the level of promoter recognition and binding, represents a new exciting finding in EC biology; importantly, it also offers new possibilities in drug discovery and development. Novel therapies could be established to inhibit the binding of pro-inflammatory transcription factors to their target promoters and/or to maintain the binding of repressors to the same promoters. A successful new treatment based on this concept could maintain or restore vascular homeostasis, providing unprecedented therapeutic impact in severe diseases in which endothelial inflammation is a major hallmark and trigger, including atherosclerosis, diabetes and metastatic cancer.

The data presented in this thesis show that conditioned medium obtained both from EC and from aortic SMC partly inhibited the TNFα- and IL-6-induced expression of E-selectin and VCAM-1 in HAEC. It has not been determined if SMCPCM also prevents p65 binding to the promoters of inflammatory genes in the presence of TNFα, but it is conceivable that this mechanism of inhibition is shared between the two conditioned medium. If this hypothesis is confirmed, it would suggest that cells

in the vascular system (at least in the aortic/arterial system) produce and release soluble, anti-inflammatory factors that act as general controllers of vessel homeostasis. These factors would represent a novel mechanism of cell communication among the layers and cell types in the blood vessels, in which quiescence is sustained at the level of gene promoter binding.

The *in vivo* experiments performed using an animal model of spontaneous CNV demonstrate the therapeutic potential of ECPCM. Indeed, ECPCM reduced the area of CNV and the recruitment of activated macrophages to the lesions, even when delivered at a great distance from the eyes, in the peritoneum. Since CNV growth is supported by local inflammation, this further supports the theory of ECPCM containing soluble anti-inflammatory factors. Additional experiments are required to elucidate the mechanism of action of ECPCM in this animal model. Notably, the data presented in this thesis suggest that the JR5558 mouse model of spontaneous CNV can be used not only as a model of neovascular eye diseases such as age-related macular degeneration, but also as a new vascular inflammation model which could provide insight into endothelial biology, immunology and drug discovery.

The goal of cell-based therapies is to provide active biological substances to modulate cell biology in those tissues where physiological function is lost due to disease. This is of extreme importance in vascular biology, since the paracrine function of the endothelium is essential in controlling vascular homeostasis. However, tissue engineering approaches have severe limitations with regards to cell availability and survival, delivery and host rejection. In terms of inflammation, ECPCM showed beneficial effects similar to those delivered by the cell-based therapy (EC/matrix), but without many of its limitations. In fact, ECPCM provided

effective soluble factor(s) at sufficient quantity to prevent EC activation in an inflammatory environment; for this reason it represents an exciting novel therapy that could be used in place of the EC/matrix. It also provides a new tool for study of EC biology and control of cellular quiescence, since it clearly contains factors necessary for endothelial homeostasis.

ECPCM offers exciting opportunities for personalised medicine. Production of ECPCM is a simple procedure, and personalised ECPCM could be made using EC isolated from the patient. Although endothelial control of vascular homeostasis is mediated by factors and molecular mechanisms shared among mammalian species, it cannot be excluded that each individual needs a different "mix" of factors released by the EC in order to maintain a healthy vascular system. In this respect, production of personalised ECPCM (with the potential to genetically enhance or modify its composition based on the needs of the patient) could provide the right type and amount of molecules required for proper vascular homeostasis in that specific patient. Personalized ECPCM might also be employed as a diagnostic tool. The formulation could provide important information regarding the status of the endothelium in that patient. Based on its composition, ECPCM could highlight imbalances in the paracrine function of EC and designate the most appropriate therapeutic intervention. This strategy would be of particular interest in the case of patients with inflammatory-based diseases (e.g. diabetes, cancer, atherosclerosis, hypertension, IH, transplant rejection) in which endothelial homeostatic control over the vasculature is lost. ECPCM produced with EC from these patients and used in *in vitro* and *in vivo* experiments could provide new insight into EC biology and its deregulation in pathology.

The findings presented in this thesis provide novel information about EC biology and confirm the prominence of EC control over the vascular system. They further suggest that paracrine activity of the endothelium is essential and as important as endothelial barrier function in regulating vascular homeostasis.

4.2 Future directions

The major advantage – and, at the same time, the major limitation – of the work presented in this thesis is the use of a cell-based assay to study the biological effects of ECPCM. Although this assay gave important insight into EC biology and EC response to pro- and anti-inflammatory stimuli, it severely limited the process of identification and isolation of the molecule (or molecules) responsible for the anti-inflammatory activity of ECPCM. Moreover, the lab lacked expertise in the biochemical processes necessary to isolate specific biological compounds. Nonetheless, useful information on the nature of the anti-inflammatory soluble factors was obtained. Future collaborations with laboratories specialized in analysis of complex solutions and identification and/or isolation of specific molecules will allow identification of the specific mediator of the ECPCM anti-inflammatory effects. Furthermore, although ChIP experiments provided a mechanism of action for ECPCM, these were preliminary findings; more in-depth studies are needed.

Further research is necessary to fully elucidate the molecular mechanisms of the anti-inflammatory activity of ECPCM. Additional experiments are required to understand how ECPCM affects p65 binding to the promoters of E-selectin and VCAM-1. For example, analysis of post-translational modifications known to affect p65 binding, such as acetylation, could give new insights into the mechanism. Since interactions between p65 and other co-factors might be affected by ECPCM, they should also be assessed. Moreover, it is possible that p65 binding to inflammatory gene promoters is prevented because other factors are already bound to the same sequences, thereby maintaining EC quiescence. One such candidate factor is Erg; analysis of Erg

binding to E-selectin and VCAM-1 promoters in the presence of ECPCM would be an appropriate starting point.

Another future experiment of interest would involve performing ChIP on Stat3 in EC treated with IL-6, in order to verify that the binding of this transcription factor to E-selectin and VCAM-1 promoters is affected by ECPCM. This experiment will demonstrate if inhibition of transcription factor binding to target sequences is a general mechanism of ECPCM-mediated anti-inflammatory activity or if other mechanisms are involved. ChIP data could also confirm the theory suggested in this thesis that Stat3, not p65, is essential in mediating IL-6-induced expression of E-selectin and VCAM-1. Expanding research into the role of Stat3 in inflammation and, more specifically, in the regulation of adhesion molecule expression in EC would allow for a greater understanding of this protein's role in vascular homeostasis.

Lastly, additional *in vivo* experiments are required to validate the therapeutic potential of ECPCM. Though the results presented in this thesis clearly suggest anti-inflammatory efficacy of ECPCM *in vivo*, the data were mainly qualitative. Quantification of macrophage recruitment is needed, together with analysis of the molecular pathways activated and/or repressed in the diseased eye (e.g. by western blot and immunofluorescent analysis of NF-kB and Stat3 pathways in the CNV lesions). Use of alternate animal models of inflammation, such as the leukostasis assay, would provide additional information about ECPCM efficacy and activity.

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