The role of sphingosine 1-phosphate in neutrophil trans-migration

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Abstract

Sphingosine 1-phosphate (S1P), a bioactive lipid mediator and ligand of 5 G-protein coupled receptors, is involved in many cellular processes including cell survival and proliferation, lymphocyte migration, and endothelial barrier function. As neutrophils are major mediators of inflammation, neutrophil trans-endothelial migration could be the target of therapeutic approaches to many inflammatory conditions. The aim of this project was to assess whether S1P can protect against inflammation by affecting neutrophil trans-endothelial migration, either by acting on neutrophils directly or indirectly through the endothelial cells.

The direct effects of S1P on isolated human neutrophils from healthy volunteers were assessed. It was shown that S1P signals in neutrophils mainly through the receptors S1PR1 and S1PR4 and it induces phosphorylation of ERK1/2. Moreover, S1P pre-treatment enhances IL-8 induced phosphorylation. However, in chemotaxis assays, S1P pre-treated neutrophils showed no altered migration towards IL-8 in comparison to untreated neutrophils. Additionally, in an *in vitro* flow-based adhesion assay, S1P pre-treatment did not have a significant effect on IL-8 induced neutrophil adhesion to VCAM-1 and ICAM-1.

Next, the effects of S1P on endothelial cells were measured. When HMEC-1 endothelial cell line and HUVEC primary endothelial cells were treated with S1P or S1P receptor agonists CYM5442 and CYM5541, the production of the chemokine IL-8 was induced. On the other hand, this treatment inhibited neutrophil trans-endothelial migration through HMEC-1 and HUVEC endothelial cells. This indicates S1P enhances endothelial barrier integrity, with a mechanism involving reduction of VE-cadherin phosphorylation. Finally, S1P treatment caused upregulation of the adhesion molecules VCAM-1 and ICAM-1, but inhibition of TNF-α induced upregulation, also shown as reduced neutrophil adhesion to endothelial cells under *in vitro* flow conditions.

To investigate the *in vivo* effects of S1P, two mouse models of cell recruitment were used, the peritoneum cell recruitment and the air pouch model. In the peritoneum cell recruitment model, S1P administration could successfully inhibit neutrophil recruitment at the peritoneum induced by IL-8.

In conclusion, functional assays indicated no direct effect of S1P on neutrophil migration, although S1P receptor signalling in neutrophils can activate MAPK/ERK

signalling pathways and enhance IL-8 signalling. However, S1P can affect neutrophil migration indirectly, either by inducing IL-8 and adhesion molecules expression by endothelial cells, or by enhancing endothelial barrier integrity leading to inhibition of trans-endothelial migration of neutrophils. The latter effect appears to be more pronounced *in vivo*.

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Table of contents

List of figures and tables

Chapter 1. Introduction

Chapter 2. Materials and Methods

Chapter 3. HL60 cell line as a neutrophil model

Chapter 4. Direct effects of S1P on neutrophils

Chapter 5. Effects of S1P on endothelial cells

Chapter 6. *In vivo* effects of S1P

Chapter 7. Discussion

Tables

Abbreviations

ADP	Adenosine diphosphate
AJ	Adherens junction
ANCA	Anti-neutrophil cytoplasmic antibody
APC	Allophycocyanin or Antigen-presenting cell
APS	Ammonium persulfate
BBB	Blood-brain barrier
BCA	Bicinchoninic Acid
BCR	B-cell receptor
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
C(x)	Complement component (x)
CASK	calcium/calmodulin-dependent serine protein kinase
CCL(x)	Chemokine (C-C motif) ligand (x)
CD(x)	Cluster of differentiation (x)
CD40L	CD40 ligand
Cdc42	Cell division control protein 42 homolog
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CGD	Chronic granulomatous disease
CNS	Central nervous system
CoA	Coenzyme A
CR(x)	Complement receptor (x)
CSK	c-SRC tyrosine kinase
CXCL(x)	Chemokine (C-X-C motif) ligand (x)
CXCR(x)	Chemokine (C-X-C motif) receptor (x)
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell

DiI-Ac-LDL	Dil-acetylated low density lipoprotein
DMEM	Dulbecco’s modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco’s Phosphate Buffered Saline
DPX	Di-n-butyl phthalate in Xylene
dsRNA	Double-stranded ribonucleic acid
EC_{50}	Half maximal effective concentration
Edg-(x)	Endothelial differentiation gene-(x)
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbant Assay
eNos	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
ET-1	Endothelin-1
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FcγR	Fcγ receptor
FDA	Food and Drug Administration
FITC	Fluorescein
fMLP	N-Formyl-Methionyl-Leucyl-Phenylalanine
FPR1	Formyl peptide receptor 1
FSC	Forward scatter
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCR1	Glycolytic genes transcriptional activator
gDNA	Genomic deoxyribonucleic acid

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
hCAP-18	Human cathelicidin-18
HDAC(1 or 2)	Histone deacetylase (1 or 2)
HDL	High-density lipoprotein
HMEC-1	Human microvascular endothelial cells-1
HOCl	Hypochlorous acid
HRP	Horseradish peroxidase
HSA	Human Serum Albumin
HUVEC	Human Umbilical Vein Endothelial Cells
i.p. / i.v.	Intraperitoneal / intravenous
ICAM-(1 or 2)	Intercellular adhesion molecule-1 or -2
IFN-(α or γ)	Interferon-(α or γ)
Ig(M/G/A/E)	Immunoglobulin (M/G/A/E)
IGF	Insulin-like Growth Factor
IL-(x)	Interleukin-(x)
IP-10	Interferon gamma-induced protein 10
IRI	Ischemia-reperfusion injury
ITAM	Immunoreceptor tyrosine-based activation motif
JAM	Junctional adhesion molecule
LDL	Low density lipoprotein
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
Mac-1	Macrophage-1 antigen
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase

MBL	Mannose-binding lectin
MCP-(1 or 3)	Monocyte chemotactic protein-(1 or 3)
MEK	Mitogen/Extracellular signal-regulated Kinase
MFI	Mean or median fluorescence intensity
MHC (class I/II)	Major histocompatibility complex (class I/II)
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NaCl	Sodium Chloride or saline
NADPH	Nicotinamide adenine dinucleotide phosphate
N-cadherin	Neuronal cadherin
NET	Neutrophil Extracellular Trap
NGAL	Neutrophil gelatinase-associated lipocalin
NK cells	Natural killer cells
NO	Nitric oxide
Nox2	NADPH oxidase 2
O_2^-	Superoxide
$ONOO^-$	Peroxynitrite
OPD	O-phenylendiamine
PAD4	Peptidylarginine deiminase 4
PAF	Platelet activating factor
PAI-I	Plasminogen activator inhibitor type I
PAMP	Pathogen –associated molecular pattern
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule-1
PerCP	Peridinin Chlorophyll

PGI_2	Prostaglandin I_2 (or prostacyclin)
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase or phosphatidylinositol 3-kinase
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear leukocyte
PMT	Photomultiplier tube
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
PVDF	Polyvinylidene fluoride
PYK2	Proline-rich tyrosine kinase 2
qPCR	Quantitative polymerase chain reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
Raf kinase	Rapidly Accelerated Fibrosarcoma kinase
Ras	Rat sarcoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Reverse transcription or reverse transcriptase
S1P	Sphingosine 1-phosphate
S1PR(x)	Sphingosine 1-phosphate receptor (x)
SDF-1	Stromal cell-derived factor-1
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
SphK(1 or 2)	Sphingosine kinase (1 or 2)
Src kinase	Sarcoma kinase
SSC	Side scatter

Syk	Spleen tyrosine kinase
TCR	T-cell receptor
TD	T cell-dependent
TEER	Trans-endothelial electrical resistance
TEM	Transmission Electron Microscopy
TEMED	N,N,N,N –tetramethylethylenediamine
TF	Tissue factor
Tfh	T follicular helper cell
TFPI	Tissue factor pathway inhibitor
TGF-β	Transforming growth factor-β
Th1	T helper type 1 cell
Th17	T helper type 17 cell
Th2	T helper type 2 cell
THI	2-acetyl-4-tetrahydroxybutylimidazole
TI	T cell-independent
TJ	Tight junction
TLR (-1 to -10)	Toll-like receptor (1 to 10)
TNF-α	Tumour-necrosis factor-α
t-PA	Tissue plasminogen activator
Treg	T regulatory cell
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule-1
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VE-PTP	Vascular endothelial protein tyrosine phosphatase
VLA-4	Very Late Antigen-4
vWF	von Willebrand factor
ZO- (1 or 2)	Zonula occludens- (1 or 2)

CHAPTER 1

Introduction

Chapter 1. Introduction

1.1 Immune system

The immune system is a system of biological functions, cells and molecules that protects the host organism from parasitic infection and other diseases. In order to act effectively, it must recognize foreign microorganisms, from bacteria and viruses to parasitic worms, and distinguish them from healthy host tissue. When the immune system "overacts", allergy, autoimmunity and inflammatory conditions can develop. Allergy is a hypersensitivity of the immune system induced by common antigens that are often innocuous, leading to adverse immune reactions when subsequent exposure to the same substance occurs (Kay, 2000). In autoimmune diseases, the hyperactive immune system attacks healthy self-tissue, as if it was foreign, causing damage to it. Such conditions include rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, etc (Grammatikos and Tsokos, 2012). In inflammatory diseases, persistent inflammation occurs even without infection or injury (Ferrero-Miliani *et al.*, 2007). An example of such a condition is ischemia-reperfusion injury that can occur after transplantation or myocardial infarction (Carden and Granger, 2000). Inflammation is the rapid response of the immune system to initiate host defense against infection or injury and help the healing process to occur. When it is not controlled, tissue damage can occur.

There are two types of immune response, innate and adaptive immunity. In innate immunity, the response is non-specific, it starts rapidly after infection and forms no memory of the pathogen. In adaptive immunity, which is initiated by innate immunity, a pathogen-specific response is instigated, and memory is formed against future infection by the same pathogen. (Medzhitov and Janeway, 2000)

1.1.1 Innate immunity

The first line of defence in innate immunity begins with mechanical, chemical and biological barriers. Epithelial cells of the skin or mucosal surfaces form a physical barrier pathogens have to penetrate. Even if the epithelium is disturbed through wounding, rapid repair occurs. Mucus as well as tears and saliva trap and remove infectious agents, with the help of cilia movement at the respiratory tract or peristalsis at the gastrointestinal tract. Enzymes and other antimicrobial proteins and peptides such as defensins coat epithelial barriers and enhance anti-microbial function. Non-pathogenic

bacteria, called the microbiota, normally occupy epithelial surfaces and provide further defence against pathogenic microorganisms by competing for nutrients and surface attachment or even by producing antimicrobial agents themselves (Gallo *et al.*, 2002; Koczulla and Bals, 2003).

Another major non-cellular component of innate immune response is the complement system. It contains more than 30 different proteins that normally exist in inactive forms, but are activated during infection by proteolysis. There are three different pathways of complement activation, all of which result in the killing of the pathogen either directly by cell lysis, or indirectly by inducing phagocytosis (through opsonisation of the pathogen) and promoting inflammation. The lectin pathway is activated by the pattern recognition receptors MBL and ficolins that bind to carbohydrate structures on the surface of the pathogen. The classical pathway is activated by C1 which binds to pathogen directly or to antibodies bound to the surface of pathogens. Finally, the alternative pathway is activated by spontaneous hydrolysis of C3 which binds to microbial surfaces with the help of properdin (Cooper, 1985; Jack *et al.*, 2001; Arlaud *et al.*, 2002; Dodds, 2002). Several complement regulatory proteins exist to make sure complement will not act on healthy host cells and tissue, such as factors I and H (Kirschfink, 1997; Zipfel *et al.*, 1999; Pangburn, 2000). Complement proteins are highly conserved with homologs existing not only in other vertebrates but also in many invertebrates' species (Smith *et al.*, 1999; Nakao *et al.*, 2006).

The next stage of the innate immune response involves several types of phagocytic cells and the initiation of an inflammatory response. There are three major classes of phagocytes: macrophages and monocytes, granulocytes, and dendritic cells. Macrophages mature from blood monocytes and reside normally in tissue throughout the body where they play a surveillance role and are usually the first cells to encounter the pathogen. Granulocytes refer to neutrophils, basophils and eosinophils, all characterized by the presence of granules; however neutrophils are the most abundant and play the biggest role in removal of infectious agents. Neutrophils or polymorphonuclear leucocytes (PMNs) are the most abundant leukocytes in the blood but they only migrate to tissue after recruitment by inflammatory signals. Dendritic cells reside in tissue, but unlike neutrophils and macrophages, their major role is not to kill the pathogens but to act as a bridge between innate and adaptive responses, by antigen presentation and cytokine production (Ezekowitz and Hoffmann, 1996; Fearon and Locksley, 1996).

Macrophages and neutrophils mainly kill microbes by phagocytosis which is the recognition, internalization and degradation of the pathogen. These cells express a wide range of receptors on their surface including pattern recognition receptors (PRR), such as C-type-lectin-like receptors, that can recognize pathogen components or complement proteins leading to binding and internalization of microbes and their derivatives (Feizi, 2000; Linehan *et al.*, 2000). The pathogen inside the phagosome is eventually destroyed, with mechanisms including acidification, action of enzymes and antimicrobial peptides, reactive nitrogen species and reactive oxygen species (ROS) produced by a process known as oxidative (or respiratory) burst. All these substances, contained in lysosomes in macrophages or primary and secondary granules in neutrophils that merge with the phagosome, can also be secreted to the extracellular space to battle microbes directly at the site of infection, but will thus damage host cells too (Aderem and Underhill, 1999; Dahlgren and Karlsson, 1999; Chertov *et al.*, 2000).

Within hours of infection or wounding, an inflammatory response is initiated. This includes the release of pro-inflammatory cytokines and chemokines by macrophages and other immune as well as stressed cells which then leads to the recruitment of more effector cells from the blood, especially neutrophils, and influx of plasma proteins to the tissue. Blood vessel endothelial cells are also activated to express chemokines and cell-adhesion molecules aiding in leukocyte extravasation. Inflammation, moreover, induces coagulation and repair of the injured tissue (Svanborg *et al.*, 1999; van der Poll, 2001). Toll-like receptors (TLRs) on the surface (or sometimes intracellularly on endosome membranes) of macrophages, dendritic cells and other immune or epithelial cells recognize pathogen components such as lipopolysaccharide (LPS) and signal the production of pro-inflammatory molecules. There are 10 different expressed TLRs in humans; each one recognising a different pathogen –associated molecular pattern (PAMP), e.g. TLR-4 recognises LPS (component of Gram-negative bacteria outer membrane), TLR-5 recognises flagellin (component of bacterial flagella), TLR-3 recognises dsRNA (genetic material or intermediate of viruses), etc (Kopp and Medzhitov, 1999; Barton and Medzhitov, 2002).

Another important cell type of innate immunity, are natural-killer cells (NK cells). These are cytotoxic cells that can distinguish virus infected or tumour cells from normal host cells by recognising alterations to MHC class I molecule expression. This leads to release of cytotoxic granules to the target cell that eventually causes apoptotic death, and production of interferon-γ (IFN-γ) (Biron *et al.*, 1999; Borrego *et al.*, 2002). There

are also some innate-like lymphocytes that can be considered a part of innate immunity. Intraepithelial γδ T cells have receptors of limited diversity and recognize molecules expressed by cells only when infected instead of pathogen particles presented on MHC molecules, like other lymphocytes (Jouen-Beades *et al.*, 1997; Boismenu and Havran, 1998). Similarly, the B-1 subset of B cells that are present in the peritoneum, bind general bacterial polysaccharide components, can produce antibodies of IgM class without the help of T cells, and no memory is generated; making them more like innate cells instead of the conventional antibody-producing B cells of adaptive immunity (Bos *et al.*, 2000).

1.1.2 Adaptive immunity

Adaptive immunity is activated much later and only if innate immunity has not dealt with the pathogen completely. Innate immunity initiates adaptive immunity through cytokine and other molecule production and by antigen presentation, performed by specialized antigen-presenting cells (APCs) such as dendritic cells (DCs). The main cell families of adaptive immunity are the APCs, T lymphocytes, and B lymphocytes (Germain, 1994).

Immature DCs reside in every tissue and can take up antigen by phagocytosis or macropinocytosis. The antigen is then combined with major histocompatibility complex (MHC) molecules, and is presented on the cell surface. MHC class I molecules are expressed in all cells, not only antigen-presenting cells, and they form complexes with antigens on the cytoplasm, to present on the cell surface, especially in the event of a viral infection (Williams *et al.*, 2002). MHC class II molecules are mainly expressed by immune cells that can perform antigen presentantion, primarily DCs but also macrophages and B cells; they bind antigen peptide components from ingested pathogen such as bacteria and parasites in endosomal compartments (Villadangos, 2001). Immature DCs are activated by signals through their PRRs such as TLRs, or by cytokine receptors and are transported to peripheral lymphoid organs, such as the lymph nodes and spleen, where they gradually mature into specialized activators of naïve T cells, expressing more MHC and co-stimulatory molecules with little to no phagocytic abilities (Clark *et al.*, 2000; Guermonprez *et al.*, 2002). Macrophages and B cells can also present antigen to T cells, but mainly as a way to acquire help from effector T cells, in order to increase their effectiveness in killing the ingested pathogens or to stimulate antibody production respectively (Lanzavecchia, 1990; Underhill *et al.*, 1999).

Naïve T cells are produced in the thymus and constantly circulate the lymphatic system going through peripheral lymphoid organs and scanning for specific antigens. When they come across antigen as a complex with MHC molecules on the surface of an APC, they bind it with their antigen specific T-cell receptor (TCR) (Itano and Jenkins, 2003). In order to become activated and start proliferating though, they also need co-stimulatory signals such as the binding of B7 molecules (B7-1 or CD80 and B7-2 or CD86) on the APC surface to CD28 on the T cell surface (Gonzalo *et al.*, 2001; Bour-Jordan and Blueston, 2002). Activation causes the expression of the cytokine IL-2 and IL-2 receptor α subunit (CD25), providing an additional proliferation and differentiation signal (Appleman *et al.*, 2000). T cells eventually differentiate into effector T cells, acting immediately when they encounter antigen without the need of co-stimulation anymore (Gudmundsdottir *et al.*, 1999). There are two major T cell subtypes, CD4 and CD8 T cells.

Activated CD4 T cells are effector cells that provide help to other types of cells, and bind to MHC class II - antigen complexes. There are at least five currently distinguishable CD4 T cell subsets: Th1, Th2, Th17, T follicular helper cells (Tfh) and regulatory T cells (Treg). Th1, Th2 and Th17 are distinguished by the different array of cytokines they produce. Th1 or type 1 cells mainly produce IFN-γ and their chief purpose is to activate macrophages infected with intravesicular pathogens, stimulating their microbicidal mechanisms to destroy the pathogen.Th2 or type 2 cells primarily produce the cytokines IL-4, IL-5 and IL-13, and help control parasite infections by stimulating eoshinophils and mast cells, and inducing IgE antibody production. Pathophysiological Th2 responses are related to allergies (Dong and Flavell, 2001). Th17 cells secrete cytokines of the IL-17 family, helping to recruit neutrophils directly or indirectly by activating epithelial and other cells to produce chemokines (Weaver *et al.*, 2006). Tfh cells, unlike the previous subtypes that can function at the site of infection, only reside on lymphoid follicles and their sole role is to activate B cells to produce antibody. Tfh cells can produce either type 1 or type 2 cytokines, leading to production of IgG or IgE antibody isotypes by B cells respectively (Ma *et al.*, 2012). Treg are not one distinct T cell subset; they can either be produced while still in the thymus, or differentiated by naïve T cells, known as induced regulatory T cells. Most Treg express the transcription factor FoxP3, secrete the anti-inflammatory cytokines IL-10 and TGF-β and their role is to inhibit T cell responses, preventing autoimmunity (Suvas and Rouse, 2006).

Activated CD8 T cells are all cytotoxic cells, whose purpose is to recognize, by binding to MHC class I - antigen complexes, and kill the target cell, which is usually infected with viruses or other intracellular pathogens. However, CD8 T cells also produce cytokines, especially IFN-γ which has virus fighting properties, and there are cases where CD8 T cells express type 1 or type 2 cytokines (Mosmann *et al.*, 1997; Amel-Kashipaz *et al.*, 2001; Barry and Bleackley, 2002).

Naïve B lymphocytes or B cells are produced in the bone marrow, then circulate the peripheral lymphoid organs and reside in lymphoid follicles (Hardy and Hayakawa, 2001). There they can encounter specific antigen that enters through the lymph or blood, or is retained on the surface of specialized macrophages and follicular DC (Batista *et al.*, 2007). Antigen is recognized by the specific B-cell receptor (BCR), which is surface immunoglobulin, and is then internalized and can be degraded and presented as a complex with surface MHC class II molecules (Noorchashm *et al.*, 1999). Specific helper T cells, like Tfh can then recognize it and stimulate the B cell through binding of molecules such as the CD40 ligand (CD40L) of the T cell to the CD40 on the B cell surface, or production of cytokines such as IL-4 (Schultze *et al.*, 1999). Naïve B cells will then proliferate and eventually differentiate into specialized antibody secreting cells, known as plasma cells (Maseda *et al.*, 2014).

Antibody responses can be classified into two categories based on the requirement for T cell help. These categories are T cell-dependent (TD) and T cell-independent (TI) antigens. B cells acquire, process and present antigen in complex with MHC class II, primarily in order to employ T cell help in the form of CD40-CD40L interactions (Schultze *et al.*, 1999). However TI antigens can either crosslink multiple BCRs and provide sufficient activation in the absence of T cell help or can activate B cells polyclonally and induce antibody secretion (Obukhanych and Nussenzweig, 2006). Proliferating stimulated B cells form germinal centers in lymphoid follicles, where they undergo procedures known as class switching and somatic hypermutation. Class switching allows them to produce antibody of a specific isotype that is most effective for the type of infection. IgM isotype is produced by naïve or newly stimulated B cells. Mature B cells that undergo class switching will then produce mostly immunoglobulin of the IgG isotype, but also IgA and IgE, especially in the event of a parasitic infection. Somatic hypermutation alters the variable regions of the immunoglobulin genes leading to the production of higher affinity antibodies. The mutated cells that have the higher

affinity are selected and undergo differentiation into plasma cells or memory B cells (Wagner *et al.*, 1996).

Memory is another function that largely distinguishes the adaptive immune response from the innate one. During the maturation of both T cells and B cells, some memory cells are produced. These cells remain even after the resolution of infection and are ready to act in the event of a second or subsequent infection with the same pathogen. Memory B cells are long lived cells, that divide very slowly or not at all, and express BCR but do not secrete antibody (Franz *et al.*, 2011). During a second encounter with their specific antigen, they will be the first to respond, proliferating and differentiating into plasma cells, which produce high affinity antibody, mostly of IgG isotype, since they have already undergone somatic hypermutation and class switching (Cumano and Rajewsky, 1986; Franz *et al.*, 2011). Similarly, memory T cells are long lived cells that persist after the resolution of infection. They resemble more effector cells than naïve T cells, and in the event of a re-infection, they quickly mature into effector T cells, rapidly producing cytokines, and providing help or killing target cells depending on their CD4 or CD8 nature (Bradley *et al.*, 1992; Rogers *et al.*, 2000; Kaech *et al.*, 2002a). The presence of memory cells assures that a secondary or subsequent immune response will be more rapid and more effective than a primary immune response, with usually no appearance of disease symptoms. This is the principle onto which vaccinations are based, providing protection from serious diseases, without having to be exposed to the disease on the first place. This is accomplished by injection of immunogenic antigens of the pathogen that do not cause disease, but lead to an immune response that generates memory (Rogers *et al.*, 2000; Kaech *et al.*, 2002b; Kamphorst *et al.*, 2015).

1.2 Neutrophils

Neutrophils are one of the main cells of the innate immune system, and are amongst the first cells that are recruited to the infected or injured tissue. They are also called polymorphonuclear leukocytes (PMN), due to their lobular nucleus, and are the most abundant leukocytes in human peripheral blood. Their numbers increase even more during infection and inflammation (Kolaczkowska and Kubes, 2013). Neutrophils are short lived in the circulation, with a half-life of around 8 hours, before they translocate to the liver, spleen and bone marrow to undergo apoptosis and be phagocytosed by macrophages (Dancey *et al.*, 1976; Hong *et al.*, 2012; Casanova-Acebes *et al.*, 2013). A more recent study however, argues that circulating neutrophil lifespan is longer than 5 days (Pillay *et al.*, 2010), though others disagree, claiming that the labelling process is also marking immature neutrophils in the bone marrow (Li *et al.*, 2011; Tofts *et al.*, 2011). Neutrophil activation by chemokines or bacterial products during inflammation can increase their lifespan by several fold, ensuring their presence at the inflammation site (Colotta *et al.*, 1992; Summers *et al.*, 2010).

The reason for the short lifespan of neutrophils might be their highly destructive nature that can damage host tissue together with pathogens. Indeed, when neutrophils accumulate to affected tissue, they release reactive oxygen species (ROS), proteolytic enzymes and other microbicidal factors that do not discriminate between host and microbe. These molecules are stored in special granules inside the neutrophil, which is the reason why neutrophils belong to the granulocyte family of cells (Spitznagel, 1990; Elsbach, 1998; Lehrer and Ganz, 1999). There are four distinct granule types, with the two most well-known being primary and secondary granules, each containing a diverse set of molecules. Often, they contain an inactive form of an antimicrobial factor that needs the action of a molecule or enzyme contained in a different granule or in the cytoplasm to become activated. Amongst the molecules neutrophils store in these granules are lysozyme, elastase, myeloperoxidase, gelatinase, etc (Faurschou and Borregaard, 2003). Another important anti-pathogenic ability of neutrophils is phagocytosis, during which the pathogen or cell debris is internalised by the cell membrane into the phagosome, which later fuses with the antimicrobial granules that eventually kill the pathogen (Underhill and Ozinsky, 2002). Finally, neutrophils can undergo NETosis, which is an active form of cell death that releases neutrophil uncondensed chromatin mixed with anti-microbial molecules. These structures are

termed Neutrophil Extracellular Traps (NETs), and can detain pathogens and eventually kill them (Fuchs *et al.*, 2007).

1.2.1 Neutrophil activation

Neutrophils become activated when they interact with the activated endothelium through selectins and rolling, which causes the expression of other adhesion molecules such as integrins β2 for firmer adhesion and eventually trans-endothelial migration. Various kinase families are responsible for this activation event, including Src family, Syk, phosphoinositide 3-kinase (PI3K), and p38 mitogen-activated protein kinase (Mueller *et al.*, 2010; Yago *et al.*, 2010). Oxidative or respiratory burst is also initiated causing the release of ROS, such as hydrogen peroxide (H_2O_2). One of the enzymes that contribute in this event is the NADPH oxidase, which has to be assembled on neutrophil secondary granule membrane, before it is activated (Jesaitis *et al.*, 1990). Neutrophils follow chemotactic gradients to lead them to the infected tissue, by chemokines such as CXCL8 (or IL-8) produced by the host, but also chemoattractants of pathogen origin, such as the peptide N-Formyl-Methionyl-Leucyl-Phenylalanine (fMLP). These molecules usually signal through G protein-coupled receptors (GPCRs), such as CXCR1 and CXCR2 for IL-8 and FPR1 for fMLP. The signalling pathway stimulated is the MAPK/ERK pathway, leading to further neutrophil activation and assembly of the oxidative burst complexes. Often, several molecules act together to activate neutrophils, such as lipopolysaccharide (LPS) and fMLP, which together highly induce the oxidative burst mechanism (Guthrie *et al.*, 1984; El-Benna *et al.*, 2008). LPS contributes to the assembly of the NADPH oxidase on neutrophil membranes, then fMLP induces enzyme activation by phosphorylation of its components (El Benna *et al.*, 2002; El-Benna *et al.*, 2008). Different concentrations of chemokines can have different effects on neutrophils. An important example is IL-8, which at low concentrations causes induction of integrin β2 expression, at slightly higher concentrations activates the oxidative burst, and at the highest concentrations induces neutrophil degranulation (Ley, 2002). The final stages of neutrophil activation lead to the full execution of neutrophil antimicrobial functions of phagocytosis, degranulation and NETosis. This usually occurs when the neutrophil has reached a site where no discernible gradients exist and the chemoattractant is at the highest concentration. On the other hand, ligand desensitization may occur, when the binding of the chemoattractant leads to internalization of its receptor making the neutrophil unresponsive to repeated stimulation with this particular ligand (Didsbury *et al.*, 1991; Claing *et al.*, 2002).

1.2.2 Neutrophil – endothelial cell interactions

The initial interaction of endothelial cells with neutrophils is mediated by the selectin family of adhesion molecules. Endothelial cells constitutively produce P-selectin, which they store intracellularly into Wiebel-Palade bodies. After activation they move P-selectin to the cell surface where it can interact with carbohydrate-based ligands, such as P-selectin glycoprotein ligand-1 (PSGL-1) and sialyl-Lewis^x on the neutrophil surface. They also produce E-selectin later after activation, which acts similarly. Neutrophils, on the other hand, express L-selectin, which interacts with the endothelial cell surface through similar ligands, but might also be a ligand for P-selectin (Kubes *et al.*, 1995). The selectin interactions in either cell cause neutrophil localisation close to the site of inflammation and "rolling" to the surface of the endothelium through loose adhesion (Chamoun *et al.*, 2000).

Next, neutrophils are activated and upregulate another family of adhesion molecules, the β_2-integrins, especially the CD11b/CD18 (Mac-1, α_M/β_2) and the CD11a/CD18 (LFA-1, α_1/β_2) complexes. The integrins are constitutively expressed by neutrophils, but are upregulated and become clustered after activation (Constantin *et al.*, 2000; Liu *et al.*, 2002). CD11b/CD18 in particular is relocated to the cell surface from intracellular stores (Jones *et al.*, 1988). Integrins acquire an activated conformation through rolling and chemokine signalling, with increased affinity for their ligands (Constantin *et al.*, 2000; Hogg *et al.*, 2011). They interact with a different set of molecules, members of the immunoglobulin superfamily, which are expressed on the surface of endothelial cells after activation, such as ICAM-1, VCAM-1, and PECAM-1. ICAM-1 is the counterligand for CD11b/CD18, and their interaction allows a firm adhesion of neutrophils to the endothelium. Neutrophil myeloperoxidase (MPO) has been shown to bind to Mac-1 integrin and may facilitate firm adhesion and electrostatically driven neutrophil recruitment, as was also shown for elastase and proteinase 3 (Cai and Wright, 1996; Johansson *et al.*, 1997; David *et al.*, 2003; Lau *et al.*, 2005; Klinke *et al.*, 2011). Another indication of neutrophils' potentially destructive nature is the finding that neutrophil adhesion to the coronary endothelium can cause damage to the endothelium, leading to endothelial dysfunction (Jordan *et al.*, 1999).

Firm adhesion to the endothelium initiates a neutrophil crawling along the endothelial surface, mediated by integrin-ICAM-1 interactions, until an appropriate site for egress is identified, eventually leading to the trans-endothelial migration of neutrophils into the

injured tissue (Phillipson *et al.*, 2006). PECAM-1 (CD31) is localised at the intraendothelial cell junctions and is thought to mediate this process (Muller *et al.*, 1993; Schenkel *et al.*, 2004). Although neutrophils preferentially migrate through the intercellular endothelial junctions (paracellular migration), and especially at the sites where three cells meet, they can also migrate through endothelial cells themselves (transcellular migration) by creating intracellular pores (Burns *et al.*, 1997a; Feng *et al.*, 1998; Carman and Springer, 2004; Phillipson *et al.*, 2008). The crossing of the endothelium, or diapedesis, is completed with the penetration of the basement membrane with the help of metalloproteases and other proteolytic enzymes, and passing through gaps between the pericytes (Huber and Weiss, 1989; Mandeville *et al.*, 1997). Once in the tissue, chemotactic gradients lead them to the exact site of inflammation.

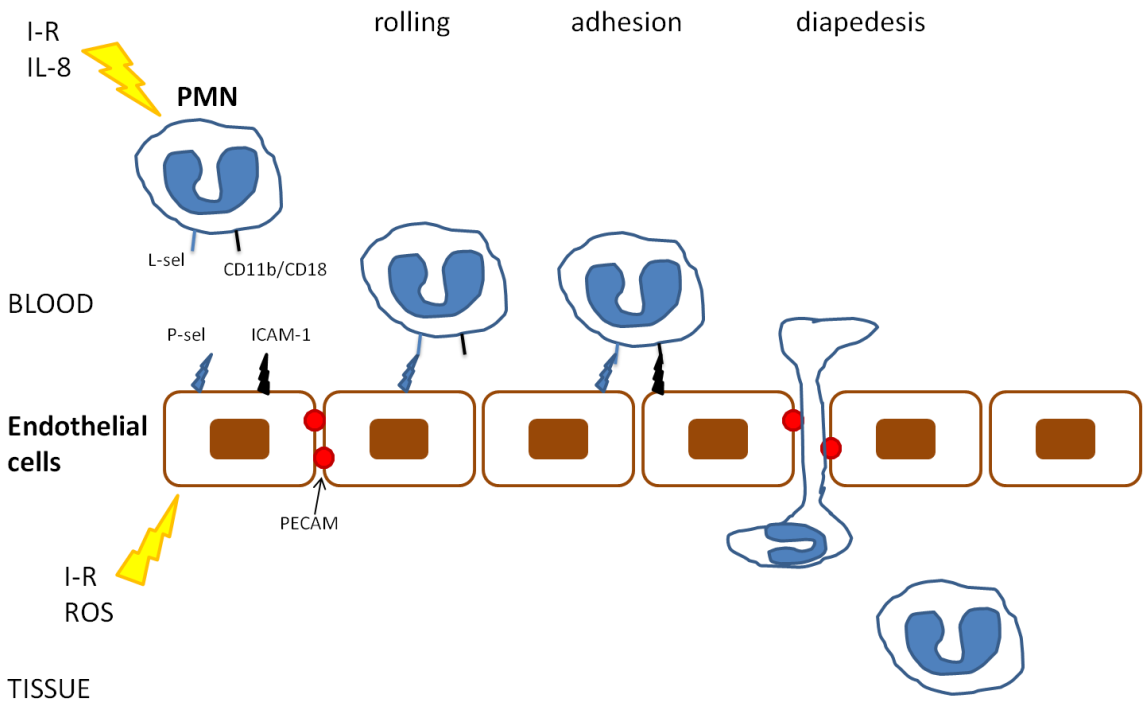


Figure 1.1. Neutrophil-endothelial cell interactions. Transendothelial migration of neutrophils starts with rolling, mediate by selectins, followed by firm adhesion mediated by integrins and ICAM-1, and finally diapedesis. PMN: neutrophil, I-R: ischemia-reperfusion, L-sel: L-selectin, P-sel: P-selectin, ROS: reactive oxygen species

1.2.3 Neutrophil degranulation

Neutrophil granules start forming during the early stages of maturation from myeloblasts to the mature polymorphonuclear leukocytes. There are four distinct types of granules. Primary granules (also known as azurophilic granules or peroxidase-positive granules) are the first to form; they are the largest granules with diameters of approximately 0.3μm. The main component of primary granules is MPO, an important enzyme for respiratory burst, but they also contain defensins, lysozyme, bactericidal/permeability-increasing protein, and serine proteases such as elastase, proteinase 3, and cathepsin G (Nusse and Lindau, 1988; Faurschou and Borregaard, 2003; Lacy, 2005). The next granules to form are the secondary granules (also known as specific granules), which are smaller in size, 0.1μm diameter, do not contain MPO and their main component is lactoferrin. They also contain other anti-microbial compounds and enzymes including lysozyme, NGAL, hCAP-18 and collagenase (Faurschou and Borregaard, 2003; Lacy, 2005). The tertiary granules (or gelatinase granules) are formed last during neutrophil maturation and are even smaller than secondary granules. As their name suggests they mainly contain gelatinase, as well as other metalloproteases and do not contain many microbicidal products (Kjeldsen *et al.*, 1992; Kjeldsen *et al.*, 1993). Finally, there is another type of granule called the secretory vesicles, which are endocytic in origin so they mainly contain plasma-derived proteins such as albumin, but their membranes are rich in important neutrophil activation proteins such as β2 integrins, complement receptors, etc (Faurschou *et al.*, 2002; Borregaard *et al.*, 2007).

Mobilization of these granules after inflammatory signalling is progressive, with secretory vesicles being the first to mobilize, followed by gelatinase granules, then secondary granules, with primary granules being the most difficult to mobilize. This differential mobilization appears to be mediated by calcium signalling (Borregaard *et al.*, 1992; Kjeldsen *et al.*, 1992; Sengelov *et al.*, 1993; Borregaard *et al.*, 1994). Each granule type is mobilized during different stages of neutrophil activation. After initial neutrophil rolling and activation, the secretory vesicles are mobilized to ensure firm adhesion, by transfer of β2 integrins to the cell membrane and continuous activation (Borregaard *et al.*, 1994; Faurschou *et al.*, 2002). During neutrophil diapedesis, gelatinase granules are thought to degranulate, so the metalloproteases they contain can assist neutrophils in traversing the basement membrane (Singer *et al.*, 1989; Delclaux *et al.*, 1996). When the neutrophils have finally reached the inflamed tissue, full activation occurs, with mobilization of primary and secondary granules, allowing initiation of the

oxidative burst and phagocytosis. These granules can either fuse with the plasma membrane to release their contents into the tissue or with the phagosomes to allow degradation of ingested microorganisms. Secondary granules' membrane contains flavocytochrome b558, an essential component of the NADPH oxidase complex, an important oxidative burst enzyme which can be assembled after secondary granule mobilization (Jesaitis *et al.*, 1990).

1.2.4 Neutrophil respiratory burst

Respiratory or oxidative burst in neutrophils is the process that produces ROS and other reactive species to be released in the tissue to contribute in the killing of microbes. The NADPH oxidase is assembled on neutrophil membranes and reduces molecular oxygen to superoxide anion (O_2^-) (Babior, 1984; Hampton *et al.*, 1998). NADPH oxidase is a protein complex that consists of both cytosolic and membrane - bound components. The membrane proteins p22phox and gp91phox form the cytochrome b558 complex that exists on secondary granule membranes and is transferred on the plasma membrane or the phagosome membrane. When neutrophils are activated, the cytosolic proteins p40phox, p47phox, and p67phox migrate to the membrane, after phosphorylation of p47phox, to associate with the cytochrome b558 and form the active enzyme (DeLeo and Quinn, 1996; Hampton *et al.*, 1998; Babior, 1999; Clark, 1999; Winterbourn, 2008). Downstream of superoxide many other ROS are generated. Superoxide can dismutate to form hydrogen peroxide (H_2O_2) (Nathan, 1987). Alternatively, it can react with nitric oxide to produce peroxynitrite ($ONOO^-$) (Beckman and Koppenol, 1996; Bogdan *et al.*, 2000). Patients with chronic granulomatous disease (CGD) have deficient NADPH oxidase, leading to failed oxidative burst responses, and inability to kill ingested pathogens. CGD patients are thus susceptible to recurrent bacterial and fungal infections that can even be life-threatening, but also autoinflammatory conditions, since NADPH oxidase can have regulatory functions (Gallin, 1991; Segal and Abo, 1993; Segal *et al.*, 2000; Seger, 2010).

MPO is another important enzyme of the respiratory burst machinery that reacts with hydrogen peroxide to produce a vast array of reactive species, such as hypochlorous acid (HOCl) (Klebanoff, 1968; Klebanoff, 2005). MPO is a heme protein that can be found inside the primary granules of neutrophils and has a wide range of substrates that can produce different oxidant byproducts. Among them, aminoacids can be chlorinated to produce chloramines, tyrosine peroxide and reactive aldehydes can be generated, and

serum proteins and lipoproteins can be oxidated (Winterbourn *et al.*, 1990; Leeuwenburgh *et al.*, 1997; Hazen *et al.*, 1998; Heinecke, 1999). MPO can act in the phagosome, creating a microbicidal environment to destroy ingested microbes, or it can be released in the extracellular space (Winterbourn *et al.*, 2006). Apart from their antimicrobial action the released ROS can also modify host molecules. This might lead to stress signalling from affected cells to progress inflammatory responses by generation of pro-inflammatory cytokines and recruitment of more immune cells. Moreover, various proteases, phosphatases and other enzymes can be regulated through oxidation of cysteine residues (Johnson and Travis, 1979; Nathan, 2003; Shao *et al.*, 2005).

1.2.5 Neutrophil phagocytosis

Phagocytosis is the process of active internalization of a particle or microbe to be eventually destroyed inside the phagocyte. It is the main mechanism utilized for pathogen or cell debris removal. Phagocytosis is mediated by FcγR or complement receptors, or by PRRs that recognize PAMPs. The mechanism of action of PAMP-mediated direct phagocytosis is poorly understood. On the other hand, neutrophils can bind to IgG molecules by FcγR receptors or complement proteins by complement receptors to indirectly ingest opsonized pathogens (Underhill and Ozinsky, 2002).

FcγRIIA (CD32) and FcγRIIIB (CD16) are the two transmembrane Fc receptors on neutrophils that play a role in phagocytosis. Multiple FcγRIIA receptors bind to IgG immune complexes, and this aggregation leads to engulfment of the opsonized pathogen by pseudopod extensions. FCγRII binding to IgG-antigen complexes causes phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on its cytoplasmic tail by Src tyrosine kinases. This allows docking of Syk kinase which subsequently activates downstream signalling pathways that involve PI3K and Rho proteins (Swanson and Baer, 1995; Greenberg *et al.*, 1996; Massol *et al.*, 1998). Neutrophils from Syk deficient mice cannot successfully ingest IgG-opsonized particles (Crowley *et al.*, 1997; Kiefer *et al.*, 1998). RhoA appears to be involved in F-actin mediated formation of pseudopods for engulfment of the complex, with Cdc42 further required for extension of the pseudopods over the edge of the complex (Hackam *et al.*, 1997; Caron and Hall, 1998; Massol *et al.*, 1998). Finally, Rac1 and PI3K activate membrane fusion and closure of the phagosome (Cox *et al.*, 1997; Massol *et al.*, 1998). Specifically, PI3K mediates the myosin-induced contraction of the pseudopods to close the phagosome (Swanson *et al.*, 1999; Cox *et al.*, 2002). FcγRIIIB involvement in

phagocytosis is not thoroughly investigated. It appears that FcγRIIIB is a co-receptor, which after binding immune complexes, can recruit FcγRIIA to lipid raft membrane domains, allowing clustering of ITAMs for enhanced signalling (Chuang *et al.*, 2000).

Phagocytosis through complement receptors CR1 (CD35) and CR3 (or CD11b/CD18 integrin) is performed by a different mechanism than that through Fcγ receptors. It does not involve active formation of pseudopods, but rather "sinking" of complement-coated particles into the cell after binding of C3bi molecules by CR3 in cell protrusions. Like FcγRIIIB, CR1 appears to be more of a co-receptor, merely assisting in the phagocytosis process rather than mediating it. Binding of CR1 and CR3 to C3b particles is not enough to promote phagocytosis; further activation by phorbol-12-myristate-13-acetate (PMA) or fMLP and contact with fibronectin or laminin is required, to phosphorylate CR1 which can then signal to stimulate CR3 binding capacity (Brown, 1986; Wright and Meyer, 1986). CR3 - mediated phagocytosis requires RhoA signalling, but unlike FcγR - mediated phagocytosis it does not require Cdc42 or Rac1 (Caron and Hall, 1998). Moreover, CR3-mediated phagocytosis is independent of inositol phosphates production or rise of free calcium cations (Ca^{2+}), which are required in FcγR-mediated phagocytosis (Fallman *et al.*, 1989). Oxidative burst activation also appears to be unconnected with complement receptor-mediated phagocytosis, unlike FcγR-mediated (Wright and Silverstein, 1983; Yamamoto and Johnston, 1984).

The two phagocytosis mechanisms can also work co-operatively. Neutrophils from CR3 deficient patients have defective IgG-dependent phagocytosis as well as complement-dependent phagocytosis, indicating a cross-talk between the complement and Fcγ receptors (Dana *et al.*, 1984). This is further supported by findings of lectin-carbohydrate interactions between CR3 and FcγRIIIB (Todd and Petty, 1997). Moreover, C3b-coated targets can be also IgG-opsonized, activating both CR3 and Fcγ receptor-mediated phagocytosis at the same time (Ehlenberger and Nussenzweig, 1977). The engulfment of the opsonized targets through phagocytosis stimulates signalling pathways for activation of degranulation and respiratory burst inside the phagosome, by fusion of neutrophil granules with phagosome membranes, leading to the creation of a microbicidal environment to support killing of the ingested pathogen.

1.2.6 Neutrophil Extracellular Traps

NETosis is an active form of programmed cell death that releases neutrophil de-condensed chromatin and granule anti-microbial molecules in the extracellular

environment. The formed NETs can trap pathogens and kill them with the contribution of histones, granule proteins and cytoplasmic anti-microbial molecules. The mechanism that causes NET formation and NETosis is still under investigation. It appears to require various respiratory burst mediators such as the NADPH oxidase and MPO, indicating the involvement of ROS in NET formation signalling (Brinkmann *et al.*, 2004; Fuchs *et al.*, 2007).

NET formation can be triggered by various pathogens, such as bacteria, fungi, viruses and protozoa, but also chemical compounds like PMA and host inflammatory stimuli such as IL-8, ROS, TNF-α and activated platelets. Often various factors cooperate to stimulate NET formation (Brinkmann *et al.*, 2004). NADPH oxidase isoform Nox2 and the ROS it generates, appear to be vital for NET formation. This is supported by evidence of lack of NET formation on CGD patients, who have deficient NADPH oxidase activity. In these patients, treatment with H_2O_2 leads to restored NET formation function, indicating the oxidative abilities of NADPH oxidase to be important (Fuchs *et al.*, 2007; Nishinaka *et al.*, 2011). ROS activate Raf-MEK-ERK signalling pathway that mediates NET formation in neutrophils (Hakkim *et al.*, 2011; Keshari *et al.*, 2012).

After NET formation has been initiated, chromatin decondensation in the cell nucleus occurs by a mechanism involving neutrophil elastase and MPO which leave the azurophilic granules and infiltrate the nucleus. Elastase partially degrades histones that keep the DNA packed, leading to chromatin decondensation, whereas MPO causes further DNA relaxation with a mechanism independent of its enzymatic activity (Papayannopoulos *et al.*, 2010; Metzler *et al.*, 2011). Histone hypercitrullination also appears to play a role in chromatin decondensation and NET formation. Citrullination is the conversion of histone arginine residues to citrulline, mediated by the enzyme peptidylarginine deiminase 4 (PAD4) which can be found in neutrophil nucleus (Wang *et al.*, 2009; Li *et al.*, 2010; Leshner *et al.*, 2012; Neeli and Radic, 2013). Neutrophils from PAD4-knockout mice cannot hypercitrullinate histones, and are unable to form NETs (Li *et al.*, 2010).

The next step in NET formation is nuclear envelope disintegration to release the decondensed chromatin in the cytoplasm, where it mixes with anti-microbial factors from neutrophil granules, which have also disintegrated. Finally, the plasma membrane bursts, releasing the formed NET to the extracellular space (Brinkmann and Zychlinsky, 2012). This process leads to the neutrophil's death via NETosis, 2-4 hours after initial

activation. However, another mechanism for rapid NET release has been described that does not lead to cell death and is independent of NADPH oxidase ROS formation. Stimulation with *Staphylococcus aureus* or *Streptococcus pyogenes* leads to the release of vesicles containing decondensed chromatin by neutrophils which then combines with granule molecules that have also been released in the extracellular space. The whole process only takes 5-60 minutes and leaves behind a still intact cytoplast that can crawl and phagocytose microbes (Pilsczek *et al.*, 2010; Yipp *et al.*, 2012). Taking into consideration that neutrophils are terminally differentiated cells with low transcriptional activity, loss of the nucleus would not have a huge impact to their functions, allowing for multiple anti-microbial mechanisms to act at the same time by rapid NET release, while still performing phagocytosis.

NETs can effectively kill microbes by trapping them and exposing them to a high concentration of microbicidal factors. However, NETs have been implicated in various host diseases, including autoimmune and autoinflammatory conditions, thrombosis and cancer. NETs expose self molecules extracellularly, which can lead to the production of autoantibodies against chromatin and neutrophil components, such as anti-neutrophil cytoplasmic antibodies (ANCAs), antibodies against histones and DNA, which are characteristic in the diverse autoimmune disease systemic lupus erythematosus (SLE) (Hakkim *et al.*, 2010; Villanueva *et al.*, 2011). NETs have been also found in the airways of cystic fibrosis patients, a condition characterized by high sputum viscosity that leads to sputum accumulation and chronic airway inflammation (Marcos *et al.*, 2010; Papayannopoulos *et al.*, 2011). Neutrophil elastase and MPO as well as extracellular DNA had been found in the past to be factors of the disease, making the discovery of NETs' involvement even more compelling (Vogelmeier and Doring, 1996; Ratjen, 2008). Moreover, NETs can bind platelets, von Willebrand factor and other coagulation factors to form scaffolds for thrombus, in deep vein thrombosis (Si-Tahar *et al.*, 1997; Fuchs *et al.*, 2010; Brill *et al.*, 2012; von Bruhl *et al.*, 2012). NETs have been discovered in pediatric Ewing sarcoma (ES) tumours, where they might play a role in tumour cell killing or they might enhance tumour cell proliferation with the release of elastase which has been shown to promote tumour growth (Houghton, 2010; Berger-Achituv *et al.*, 2013).

1.3 Ischemia-reperfusion injury

An inflammatory condition where neutrophils play a large role to is ischemia-reperfusion injury (IRI). Ischemia is the restriction of blood supply to an area of tissue. Reperfusion is the subsequent return of the blood supply to the ischemic tissue. The term ischemia-reperfusion injury (IRI) is used to describe the phenomenon of damage and inflammation caused during ischemia and deteriorated during the reperfusion process. Ischemia reperfusion injury can occur after myocardial infarction, other tissue infarcts or during organ transplantation. While ischemia interrupts the aerobic metabolism, causing tissue necrosis due to hypoxia, and oxidative damage through accumulation of anaerobic metabolites and free radicals, the vital procedure of reperfusion can lead to the initiation of a cascade of events, causing extra damage due to oxidative stress, inflammation and leukocyte-mediated cell injury. Ischemia causes the accumulation of intermediate metabolites, resulting in an overwhelming generation of reactive oxygen species (ROS), after aerobic metabolism is restored with the return of oxygen during reperfusion. Severe oxidative damage is caused to the cells, which, as a response, produce several cytokines and other pro-inflammatory molecules. These molecules activate the vascular endothelium, causing endothelial cells to express adhesion molecules and release cytokines and chemokines such as interleukin-1 (IL-1) and IL-8. As a result, leukocytes, especially neutrophils, are attracted to the site of injury and pass through the endothelium. The neutrophils then cause even more damage to the afflicted area, by releasing oxygen free radicals and proteolytic enzymes, such as elastase, which attack the tissue. This causes the secretion of even more inflammatory molecules by the injured tissue, the endothelium and the neutrophils, resulting in a vicious cycle that can eventually affect multiple organs. (Boyle *et al.*, 1996; Jordan *et al.*, 1999).

1.4 Endothelial cells

Endothelial cells line the interior of all blood and lymphatic vessels forming a monolayer called the endothelium. The endothelium is of mesodermal origin and it is characterized by a thin layer of squamous cells, approximately 1 - 6 x 10^{13} in total, weighing approximately 1kg and covering a surface area of 1 - 7 m^2 in an adult human (Augustin *et al.*, 1994). The endothelium acts as a partly permeable barrier between the blood and tissue. However, it is a dynamic layer that has many active functions apart from physically acting as a barrier. Endothelial cells have a variety of important autocrine, paracrine and endocrine actions and can influence the behaviour of other cells and blood elements, especially smooth muscle cells that line the vessel, circulating leukocytes and platelets. Actions where the endothelium is involved include regulation of vascular tone, coagulation and thrombolysis, platelet activation and aggregation, inflammation and immune modulation, vascular permeability, vascular smooth muscle cell proliferation and angiogenesis (Herrmann and Lerman, 2001).

Endothelial cells can control vascular tone and regulate the blood flow and blood pressure by releasing vasodilator or vasoconstrictor substances. Vasodilator substances include nitric oxide (NO) and prostacyclin (PGI_2), which are released in response to chemical stimuli such as thrombin, arachidonic acid, ADP and bradykinin, or physiological stimuli such as changes on blood shear stress and flow (Pohl *et al.*, 1986; Vanhoutte, 2003). Both of these substances can cause smooth muscle cell relaxation leading to vasodilation, but can also inhibit platelet aggregation (Radomski *et al.*, 1987; Mendelsohn *et al.*, 1990; McGuire *et al.*, 2001; Vane and Corin, 2003). NO can additionally inhibit platelet and leukocyte adhesion to the endothelium and smooth muscle cell migration and proliferation, providing it with anti-atherosclerotic effects (Garg and Hassid, 1989; Mendelsohn *et al.*, 1990; Kubes *et al.*, 1991; Marks *et al.*, 1995).

On the other hand, vasoconstricting substances such as endothelin-1 (ET-1), prostaglandins H2 and platelet activating factor (PAF), are released in response to hypoxia, shear stress, adrenaline and ischemia (Boulanger and Luscher, 1990; Cines *et al.*, 1998). ET-1 in particular, signals through two G-protein coupled receptors ET_A and ET_B, expressed by smooth muscle cells and endothelial cells respectively, with conflicting effects (Boulanger and Luscher, 1990; Herrmann and Lerman, 2001). Activation of ET_A on smooth muscle cells leads to potent vasoconstriction through an

increase in intracellular calcium concentration, mediated by phospholipase C (Simonson and Dunn, 1990; Pernow *et al.*, 1996; Kiely *et al.*, 1997). Autocrine activation of ET_B on endothelial cells however gives vasodilation signals, mediated by phospholipase A_2 which stimulates the production of other endothelial factors such as PGI_2 and NO (Molnar and Hertelendy, 1995; Giardina *et al.*, 2001). This mechanism might be in place to avoid excessive vasoconstriction.

The endothelium surface under normal conditions has anticoagulant, anti-thrombotic properties that allow uninterrupted blood flow. Perturbation of the endothelium however, by chemical or physical factors leads to cell activation and transformation to a pro-thrombotic surface (Bombeli *et al.*, 1997). Molecules such as heparan sulfate, and glycosaminoglycans (GAGs), or dermatan sulfate in the subendothelium, prevent platelet adhesion and promote thrombin inhibitors like antithrombin III, or heparin cofactor II, enhancing an anti-thrombotic environment (Marcum and Rosenberg, 1984; Tollefsen and Pestka, 1985). Thrombin is a serine protease, which is involved in platelet activation, fibrin production, factor V, factor XIII, and fibrinogen activation and endothelial cell pro-coagulant induction through thrombin receptor (Francis *et al.*, 1983; Kanthou *et al.*, 1995; Di Cera, 2008) Thrombomodulin is another molecule that binds to thrombin, inhibiting its pro-coagulant activities but also catalysing the activation of the anticoagulant protein C by thrombin (Esmon, 1993; Esmon, 1995). Protein S is also produced by endothelial cells, and is a co-factor for activated protein C (Fair *et al.*, 1986).

The main step in the transformation of the endothelium from anti-thrombotic to pro-thrombotic is the induction of tissue factor (TF) expression. Stimulants such as thrombin, shear stress, cytokines, hypoxia and endotoxins can induce the production of TF by endothelial cells. TF acts by enhancing factor VIIa-dependent activation of factors X and IX (Drake *et al.*, 1989; Nemerson, 1995; Rapaport and Rao, 1995). Endothelial cells physiologically express tissue factor pathway inhibitor (TFPI) which binds to factors VIIa and Xa and inhibits activity of the tissue factor coagulation pathway; this can be depleted in pro-thrombotic conditions (Broze, 1995; Jesty *et al.*, 1996). Another important pro-coagulant molecule expressed by endothelial cells is von Willebrand factor (vWF), which is mainly stored intracellularly in specialized organelles called Weibel-Palade bodies, but can be released on the cell surface after activation. Once there, vWF interacts with the platelet glycoprotein receptor complex, initiating platelet adhesion and aggregation, and promoting thrombosis (Wagner and

Bonfanti, 1991). Endothelial cells also produce tissue plasminogen activator (t-PA) which generates plasmin from plasminogen to induce fibrinolysis, maintaining blood fluidity (Chang *et al.*, 1997; Levin *et al.*, 1997). Anexin II, which is expressed on endothelial cell surface, is a receptor for t-PA which enhances its activity (Barnathan *et al.*, 1988; Hajjar *et al.*, 1994; Ishii *et al.*, 2001a). In contrast, plasminogen activator inhibitor type I (PAI-I), which is produced by endothelial cells after activation by inflammatory stimuli, is the main inhibitor of t-PA activity and hence fibrinolysis (Levin and Santell, 1987).

Endothelial cells play a major role in inflammatory and immune reactions, by regulating leukocyte trafficking into tissue and permeability to macromolecules. Activated endothelial cells express PAF, which stimulates platelet adhesion to endothelium through a mechanism involving interactions of the platelet glycoprotein IIb/IIIa with fibrinogen and endothelial vitronectin receptors. Adherent platelets then act together with P-selectin to induce neutrophil adherence to the endothelium. CD154 (or CD40L) on activated platelets binds to CD40 on endothelial cells stimulating endothelial chemokine production and leukocyte adhesion molecule expression to induce leukocyte adherence (Barry *et al.*, 1997; Thienel *et al.*, 1999). Leukocytes extravasation occurs in at least four distinct phases, starting with loose adhesion and rolling, mediated by selectin molecules P-selectin and E-selectin in endothelial cells and L-selectin in leukocytes. Next, activation and firm adhesion occurs, mediated by β integrins such as LFA-1 and Mac-1 in PMN and VLA-4 (integrin α_4/β_1) in lymphocytes that bind immunoglobulin family molecules in endothelial cells ICAM-1, ICAM-2 and VCAM-1 (Albelda and Buck, 1990; Carlos and Harlan, 1994; Cines *et al.*, 1998). ICAM-2 is constitutively expressed by endothelial cells whereas ICAM-1 and VCAM-1 need to be upregulated by inflammatory signalling. Finally, leukocyte diapedesis or trans-endothelial migration occurs. Activated endothelial cells produce IL-8 which promotes PMN chemotaxis and degranulation (Muller and Weigl, 1992; Springer, 1995; Butcher and Picker, 1996; Burns *et al.*, 1997b; Huo *et al.*, 2000).

Morphological changes occur during inflammation that lead to increased vascular permeability and leakage. Endothelial cell contraction and injury result in leakage of serum and interstitial fluid. On the other hand, endothelial permeability increases indirectly by vasodilation mediated by NO and PGI_2. In general, endothelial barrier integrity and vascular permeability are regulated by various factors, such as the intercellular junctions, cell surface binding proteins, the electrostatic charge of

endothelial monolayers, and the basement membrane. Most important of these factors appear to be the endothelial cell junctions that mediate cell attachment (Dardik *et al.*, 1999; Toborek and Kaiser, 1999).

1.4.1 Endothelial cell junctions

Endothelial cells can form three types of junctions with adjacent cells: gap junctions, tight junctions (TJs) and adherens junctions (AJs) (Rubin, 1992; Anderson *et al.*, 1993; Beyer, 1993; Gumbiner, 1993; Schmelz and Franke, 1993). Gap junctions are transmembrane ion channels clustered together to allow transfer of ions and small molecules between the cells. Gap junctions are usually intercalated with tight junctions *in vivo*, as shown by their frequencies that correlate (Beyer, 1993).

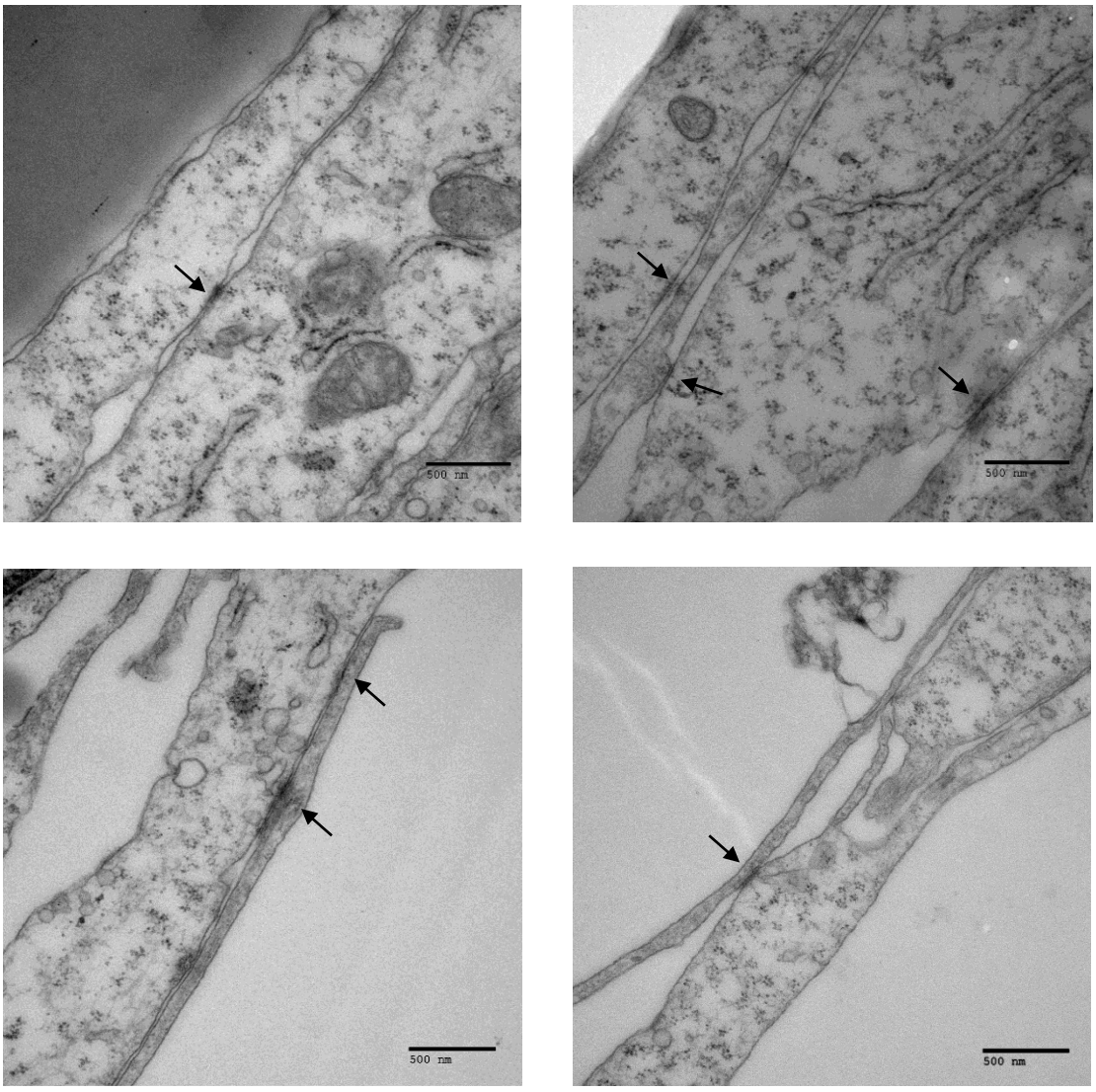


Figure 1.2. Electron micrographs of endothelial cell junctions. Transmission electron microscopy images of endothelial cells forming junctions with each other (arrows). (See Chapter 5 for description of experiment)

TJs (or zonula occludens) usually form closest to the luminar surface of the cells, and allow very close contact between adjacent cells. They consist of transmembrane adhesion proteins that interact with their partners on the adjacent cell, as well as intracellular signalling molecules that interact with the cytoskeleton (Farquhar and Palade, 1963; Staehelin, 1974; Milton and Knutson, 1990). Transmembrane proteins that mediate adhesion in TJs include occludin, claudins 1, 5 and 12 and junctional adhesion molecules (JAMs) (Furuse *et al.*, 1993; Furuse *et al.*, 1998; Martin-Padura *et al.*, 1998; Aurrand-Lions *et al.*, 2001; Muller, 2003). Among the intracellular components of TJs are zonula occludens- 1 and 2 (ZO-1 and ZO-2), cingulin and calcium/calmodulin-dependent serine protein kinase (CASK) (Stevenson *et al.*, 1986; Anderson and Van Itallie, 1995; Cereijido *et al.*, 2000; Anderson, 2001). These interact with the actin cytoskeleton to help stabilize the junction and perhaps regulate the opening and closing of the junction. Moreover, they have signalling functions or they can act as scaffolds for other junction proteins (Fanning and Anderson, 1999; Stevens *et al.*, 2000; Dudek and Garcia, 2001; Matter and Balda, 2003). The number of tight junctions in endothelial cells *in vivo* can be varied, depending on the vascular bed and the requirements for vascular permeability. Whereas endothelial cells of the post-capillary venules and especially the high endothelial venules of the lymphoid system where constant lymphocyte extravasation occurs form little to no tight junctions, cells of large arteries and especially the endothelium in the brain microvasculature have a very high frequency of organised tight junctions to strictly control permeability (Simionescu and Simionescu, 1991; Wolburg and Lippoldt, 2002; Nitta *et al.*, 2003).

Adherens junctions (or zonula adherens) are contacts of the cellular membrane through transendothelial cadherin molecules. Cadherins are a family of single-chain glycoproteins with a highly conserved cytoplasmic region and an extracellular domain consisting of calcium-binding motifs. Cell-cell adhesion mediated by cadherins is homophilic and calcium-dependent (Takeichi, 1991; Kemler, 1993). The main endothelial cadherin that exists only in endothelial cells is VE-cadherin, which mediates endothelial cell-cell adhesion. Endothelial cells also express neuronal cadherin (N-Cadherin) which is present in other types of cells such as neural cells and mediates adhesion of endothelial cells with pericytes or smooth muscle cells (Lampugnani *et al.*, 1992; Gerhardt *et al.*, 2000; Bazzoni and Dejana, 2004; Paik *et al.*, 2004). Other cadherins such as P-cadherin and T-cadherin can be also expressed in different types of endothelial cells (Ivanov *et al.*, 2001). Inside the cell, cadherins interact with many

intracellular proteins and the actin cytoskeleton through their cytoplasmic tails (Geiger and Ayalon, 1992; Tsukita *et al.*, 1992). VE-cadherin associates with the intracellular junction proteins β-catenin, p120 and plakoglobin. Subsequently, β-catenin and plakoglobin bind to α-catenin which can interact with several actin binding molecules such as α-actinin, ajuba, and ZO-1 (Weis and Nelson, 2006). VE-cadherin with its associated junction molecules appears to be able to influence the actin cytoskeleton and can be influenced by it. VE-cadherin is very important in regulating endothelial barrier integrity and vascular permeability, and molecules that increase permeability like VEGF, thrombin and histamine target VE-cadherin (Rabiet *et al.*, 1994; Rabiet *et al.*, 1996; Esser *et al.*, 1998).

Endothelium mechanisms that modulate vascular permeability affect AJ organization by directly targeting VE-cadherin and other junctional molecules either by phosphorylation, internalization or cleavage. As a general rule, tyrosine phosphorylation of VE-cadherin and other AJ components appears to increase endothelial permeability and impair barrier function. This is supported by the fact that endothelial permeability increasing molecules such as VEGF, TNF-a, histamine and PAF induce tyrosine phosphorylation of VE-cadherin as well as β-catenin, plakoglobin and p120 (Esser *et al.*, 1998; Andriopoulou *et al.*, 1999; Shasby *et al.*, 2002; Hudry-Clergeon *et al.*, 2005; Angelini *et al.*, 2006). Several tyrosine kinases and phosphatases have been implicated in this mechanism, including SRC kinase, c-SRC tyrosine kinase (CSK), proline-rich tyrosine kinase 2 (PYK2), and vascular endothelial protein tyrosine phosphatase (VE-PTP) (Nawroth *et al.*, 2002; Baumeister *et al.*, 2005; Weis and Cheresh, 2005).

VE-cadherin can be phosphorylated at various different tyrosine and serine sites; different studies report different residues to be important for endothelial barrier integrity (Baumeister *et al.*, 2005; Potter *et al.*, 2005; Gavard and Gutkind, 2006; Allingham *et al.*, 2007; Wallez *et al.*, 2007; Turowski *et al.*, 2008). For example, Wallez et al. found only tyrosine 685 phosphorylation of VE-cadherin by SRC after VEGF stimulation (Wallez *et al.*, 2007). In another study, neutrophil adhesion to the endothelium via ICAM-1 caused phosphorylation of tyrosines 658 and 731of VE-cadherin by SRC and PYK2 (Allingham *et al.*, 2007), whereas a different work showed activated lymphocyte adhesion to ICAM-1induced the phosphorylation of tyrosines 645, 731 and 733, and was mediated by Rho GTPases, but not by SRC (Turowski *et al.*, 2008). Further work is needed to understand the conflicting data. Other AJ molecules such as β-catenin, plakoglobin and p120 can also be phosphorylated by similar mechanisms, although the

resulting effect on junction stability is still unknown (Lampugnani *et al.*, 1997; Esser *et al.*, 1998).

Phosphorylation at a serine residue, at Ser665, activates another mechanism of increased permeability, that of VE-cadherin internalization. VE-cadherin phosphorylation at Ser665 induces the recruitment of β-arrestin, which promotes VE-cadherin internalization in a clathrin-dependent manner (Gavard and Gutkind, 2006). Interestingly, VE-cadherin internalization appears to be modulated by the binding of p120, which could be a signal for retention of VE-cadherin to the cell membrane (Xiao *et al.*, 2005). Moreover, the VE-cadherin extracellular domain can be easily digested by various enzymes, such as metalloproteases, elastase, cathepsin G or trypsin, providing another way for increased permeability and induced leukocyte diapedesis. Leukocytes and especially neutrophils can release most of these enzymes, which would allow their extravasation by VE-cadherin cleavage (Lampugnani *et al.*, 1992; Herren *et al.*, 1998; Xiao *et al.*, 2003; Luplertlop *et al.*, 2006).

1.5 Sphingosine 1-phosphate

Sphingosine 1-phosphate (S1P) is a bioactive lipid mediator, metabolite of membrane sphingolipids. It was first identified in the early 1990s as a novel lipid involved in cell proliferation and signal transduction (Ghosh *et al.*, 1990; Zhang *et al.*, 1991). Since then, S1P has been implicated in several different biological functions both intracellularly and extracellularly. The identification of at least five G-protein coupled receptors (GPCRs) involved in S1P signalling, termed S1PR1-5, and their wide expression by different cell types, led to a better understanding of the complex signalling pathways S1P can mediate, and its potential role in regulation of several pathophysiological processes.

1.5.1 S1P metabolism

S1P is a 379Da member of the lysophospholipid family. It is the direct metabolite of sphingosine through the action of two sphingosine kinases SphK1 and SphK2. The main metabolic pathway starts with the hydrolysis of sphingomyelin, a membrane sphingolipid, into a member of the ceramide family by the enzyme sphingomyelinase and the subsequent production of sphingosine by ceramidase (figure 1.3). Ceramides can also be produced *de novo* in the endoplasmic reticulum (ER) from serine and palmitoyl CoA, through multiple intermediates. Ceramides are a family of molecules comprised of sphingosine and a fatty acid, and can also be salvaged from sphingosine by the ceramide synthase enzyme family. S1P production is regulated by various S1P-specific and general lipid phosphatases, as well as S1P lyase, which irreversibly degrades S1P into phosphoethanolamine and hexadecanal (Swan *et al.*, 2010). The balance between intracellular S1P and its metabolite ceramide, can determine cellular fate. Ceramides promote apoptosis, whilst S1P suppresses cell death and promotes cell survival (Cuvillier *et al.*, 1996; Alvarez *et al.*, 2007; Kihara *et al.*, 2007; Hannun and Obeid, 2008). This creates an S1P-ceramide "rheostat" inside the cells. S1P lyase expression in tissue is higher than it is in erythrocytes and platelets, the main "suppliers" of S1P in blood (Yatomi *et al.*, 1997; Hanel *et al.*, 2007). This causes a tissue-blood gradient of S1P, which is important in many S1P mediated responses, including the lymphocyte egress from lymphoid organs (Schwab *et al.*, 2005; Pappu *et al.*, 2007).

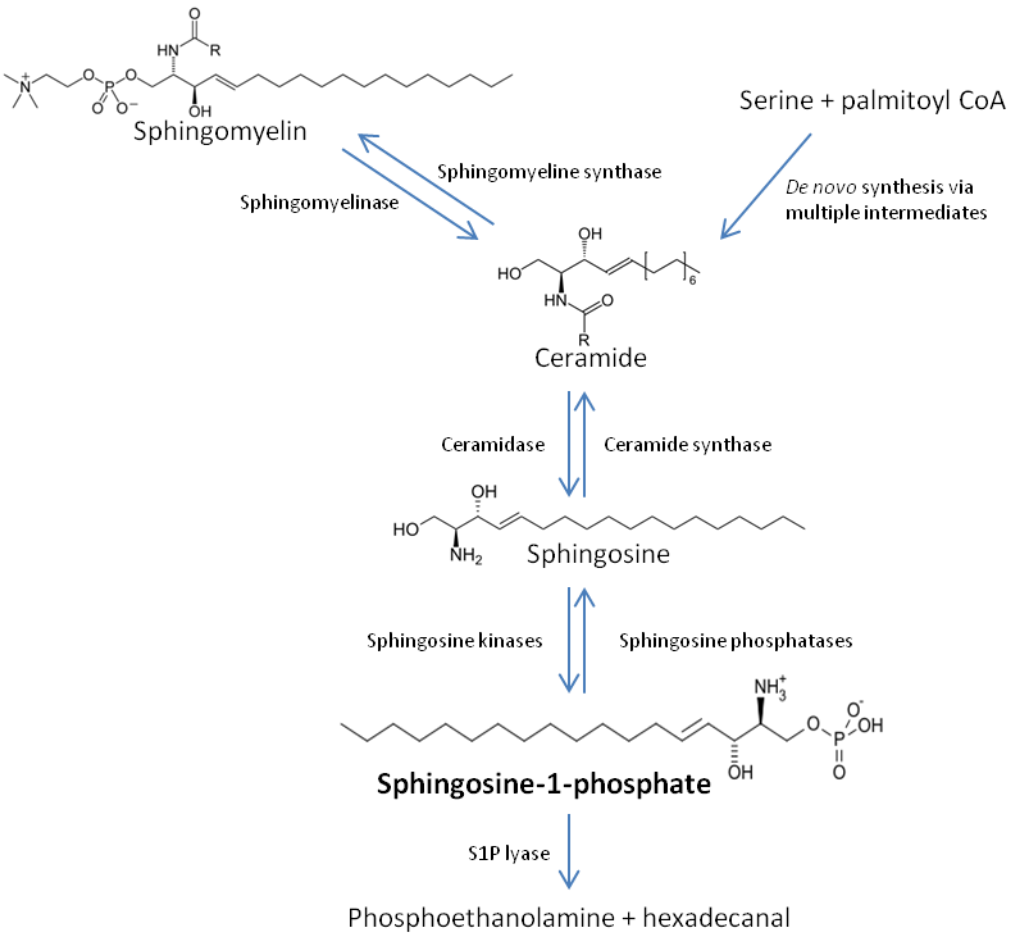


Figure 1.3. Sphingosine 1- phosphate metabolism. Ceramides, produced either *de novo* or from sphingomyelin, are hydrolysed into sphingosine which is then phosphorylated to become S1P. S1P can be irreversibly degraded by S1P lyase to phosphoethanolamine and hexadecanal.

1.5.2 S1P receptor signalling

Sphingosine 1-phosphate is produced inside cells; it can, however, also be found extracellularly, in a variety of different tissues. It is abundant in the blood, at concentrations of 0.4-1.5μM, where it is mainly secreted by erythrocytes and platelets (Yatomi *et al.*, 1997; Hanel *et al.*, 2007). Blood S1P can be found separately, but mainly it exists in complexes with high-density lipoprotein (HDL) (~60%). Many of the cardioprotective effects of HDL are hypothesized to involve S1P (Tamama *et al.*, 2005). Before 1996, S1P was thought to act mainly intracellularly as a second messenger. The identification, though, of several G-protein-coupled receptors that bind S1P led to the initiation of many studies on extracellular S1P signalling through those receptors. In 2001, Tamama and colleagues showed that S1P mediated regulation of DNA synthesis and migration of rat aortic smooth muscle cells does not involve intracellular S1P, but

extracellular S1P signalling through the Edg-5 receptor, now known as S1PR2 (Tamama *et al.*, 2001). There are five receptors that have been currently identified. These can be coupled with different G proteins. Assuming that each receptor coupling with a G protein has a slightly different function, one can recognize the complexity of S1P receptor signalling.

1.5.2.1 S1PR1

S1PR1 (formerly Edg-1) is widely expressed by cells of the immune system, brain, heart, lung, kidney, spleen and vasculature. It is coupled with G_i only, as pertussis toxin sensitivity suggests, so it acts through the ERK, phosphatidyl inositol 3-kinase (PI3K)/Akt, Ras/MAP, Rac and eNos pathways (Siehler and Manning, 2002). *S1PR1* knockout in mice is lethal, mainly due to severe vascular disruption (Liu *et al.*, 2000). Experiments with partial inactivation suggest that S1PR1 plays key roles in angiogenesis, neurogenesis, immune cell trafficking, endothelial barrier integrity and regulation of vascular tone (Matloubian *et al.*, 2004; Singleton *et al.*, 2005; Sanna *et al.*, 2006; Takuwa *et al.*, 2008). S1PR1 seems to be important for lymphocyte egress from the thymus, as well as T cell migration through the lymph nodes and other lymphoid organs (Allende *et al.*, 2004; Matloubian *et al.*, 2004). It seems that upon S1P ligation, S1PR1 is internalized and can be degraded. In micromolar saturating concentrations of S1P, as it is in blood and lymph, this effect is more persistent. In the thymus and other tissue however, with nanomolar concentrations of S1P, there is a mechanism that recycles S1PR1 on the cell surface, resulting in dynamic receptor stabilization. There are cases however where lymphocyte egress from lymphoid organs is inhibited, either for accumulation of cells before their release, or in immunosuppressive situations. In those cases, there seems to be a crosslinking of S1PR1 with CD69, a T cell activation antigen, causing greater levels of internalization and degradation (Shiow *et al.*, 2006; Bankovich *et al.*, 2010) and disrupting the balance. Data from our group suggest that, 24h following T cell activation, CD69 expression increases, causing a disruption to S1PR1 signalling. After 3 days of activation though, subsequent T cell mitosis causes a decrease in the surface levels of CD69, allowing the re-acquisition of S1PR1 responsiveness and the egress of mature effector T cells from the lymph nodes (Swan *et al.*, 2012). Recent evidence suggests that S1PR1 is also involved in immature B cell egress from the bone marrow into the blood (Allende *et al.*, 2010; Pereira *et al.*, 2010).

1.5.2.2 S1PR2

S1PR2 (formerly Edg-5), unlike S1PR1, couples with several different types of G protein, including G_i, G_q and $G_{12/13}$. This means that it signals through all the pathways S1PR1 does, with the addition of phospholipase C (PLC) and Rho pathways. *S1PR2* knockout mice do not have an apparent phenotype, but there are reports of epileptic seizure cases (MacLennan *et al.*, 2001) as well as deafness (Herr *et al.*, 2007; Kono *et al.*, 2007). This S1P receptor is also widely expressed. S1PR2 has been suggested to regulate macrophage recruitment and inflammatory cytokine production (Wang *et al.*, 2010; Skoura *et al.*, 2011). S1PR2 signalling is proposed to promote smooth muscle cell differentiation through RhoA activation (Medlin *et al.*, 2010). S1PR2 is also induced in microvascular endothelial cells and skin mast cells by various inflammatory factors (Du *et al.*, 2012; Wang *et al.*, 2012).

1.5.2.3 S1PR3

Like the previous receptors, S1PR3 (formerly Edg-3) is ubiquitously expressed by immune cells, as well as cells in the heart, lung, spleen, kidney, intestine, diaphragm and cartilage. Both vascular endothelial cells and smooth muscle cells can express it and S1PR3 can mediate vasoconstriction or vasodilation depending on the vascular bed and the G protein signalling pathway which is activated. S1PR3 couples with G_i, G_q and $G_{12/13}$ leading to activation of distinct pathways with contradictory effects. It can activate Akt and eNos through G_i or Rho through $G_{12/13}$ as well as PLC and Ca^{2+} mobilization through G_q. The cardioprotective effects of HDL are suggested to involve S1PR3 signalling (Theilmeier *et al.*, 2006). On the other hand, S1PR3 can cause endothelial barrier disruption, in contrast to S1PR1 which helps maintain barrier integrity (Singleton *et al.*, 2006). Studies suggest S1PR3 can promote inflammatory recruitment of monocytes and macrophages (Keul *et al.*, 2011). *S1PR3* knockout mice do not seem to have an obvious phenotype, although they lack several S1P effects on cardiovascular system (Ishii *et al.*, 2001b).

1.5.2.4 S1PR4

S1PR4 (formerly Edg-6) expression, unlike the previous receptors, is mainly restricted to cells of the immune system, especially lymphocytes and hematopoietic cells. A *S1PR4* knockout mouse model has only recently been investigated (Schulze *et al.*, 2011). It seems that S1PR4 deficiency doesn't affect lymphoid organ structure and

lymphocyte numbers in blood, but dendritic cell (DC) function is severely altered, leading to reduced Th17 T cell differentiation (Schulze *et al.*, 2011). S1PR4 mainly couples with G_i and $G_{12/13}$, and it acts through ERK, PLC and Rho signalling pathways. S1PR4 is upregulated during megakaryocyte development and plays a role in their differentiation into pro-platelets (Golfier *et al.*, 2010). There is also evidence to suggest S1PR4 signalling can inhibit T cell proliferation and cytokine secretion (Wang *et al.*, 2005).

1.5.2.5 S1PR5

Like S1PR4, S1PR5 (formerly Edg-8) expression is more restricted, mainly observed in cells of the central nervous system (CNS). It can similarly couple to G_i and $G_{12/13}$, acting through ERK, Akt, or Rho signalling pathways. S1PR5 signalling plays a dual role: it can promote the survival of mature oligodendrocytes, through Akt-dependent pathway, or lead to process retraction of pre-oligodendrocytes, through Rho activation (Jaillard *et al.*, 2005). Moreover, S1PR5 is expressed in natural killer (NK) cells, where it acts, similarly to S1PR1 on lymphocytes, to regulate cell trafficking of mature cells through the bone marrow and from blood to the lymph nodes (Jenne *et al.*, 2009; Mayol *et al.*, 2011). *S1PR5* knockout in mice leads to absence of observed oligodendrocyte retraction (Jaillard *et al.*, 2005) and deficient NK cell trafficking (Mayol *et al.*, 2011).

1.5.3 S1P as a second messenger

S1P is involved in many cellular processes through its GPCR signalling; however there are studies demonstrating that S1P is acting at an intracellular level, too (Strub *et al.*, 2010). As was mentioned before, intracellular S1P plays a role in maintaining the balance of cell survival signal to apoptotic signals, creating a cell "rheostat" between S1P and its precursor ceramide. Important evidence that S1P can act intracellularly as a second messenger came from yeast (*Saccharomyces cerevisiaei*) and plant (*Arabidopsis thaliana*) cells. Although yeast cells do not express any S1P receptors, they can be affected by S1P during heat-shock responses (Mao *et al.*, 1999; Skrzypek *et al.*, 1999). Similarly, *Arabidopsis* has only one GPCR-like protein, termed GCR1 which does not bind S1P; however S1P regulates stomata closure during droughts (Ng *et al.*, 2001).

In mammals, the sphingosine kinases have been found to localize in different cell compartments, being responsible for the accumulation of S1P in those compartments to give intracellular signals. In mitochondria, for instance, S1P was recently found to

interact with prohibitin 2, a conserved protein that maintains mitochondria assembly and function (Strub *et al.*, 2011). According to the same study, SphK2 is the major producer of S1P in mitochondria, and the knockout of its gene can cause disruption of mitochondrial respiration and cytochrome-c oxidase function. SphK2 is also present in the nucleus of many cells, and has been implicated to cause cell cycle arrest (Igarashi *et al.*, 2003). Furthermore, it causes S1P accumulation in the nucleus (Hait *et al.*, 2009). It seems that nuclear S1P is affiliated with histone deacetylases HDAC1 and HDAC2, inhibiting their activity, thus having an indirect effect in epigenetic regulation of gene expression (Hait *et al.*, 2009). In the endoplasmic reticulum (ER), SphK2 has been identified to translocate during stress, and promote apoptosis. It seems that S1P has specific targets in the ER that cause apoptosis, probably through calcium mobilization signals (Ghosh *et al.*, 1994).

1.5.4 S1P signalling on immune cell trafficking

As has been mentioned above, S1P has important roles in various immune cells' trafficking through different S1P receptor signalling and S1P gradients. More thoroughly investigated is the regulation of lymphocyte trafficking through the lymph nodes by S1PR1 signalling (Matloubian *et al.*, 2004; Cyster, 2005). Naïve T cells follow S1P gradients to move from peripheral lymphoid organs to the lymph and blood. However, when activated, they express CD69 on their surface, causing internalization of S1PR1 and lymphocyte arrest in the lymph nodes (Shiow *et al.*, 2006). Three days later CD69 expression decreases sufficiently, allowing normal S1PR1 expression and egress from the lymph nodes (Swan *et al.*, 2012). FTY720-phosphate (FTY720-P), an S1P structural analog, which can activate S1PR1, 3, 4 and 5, binds antagonistically to S1PR1 causing internalization of the receptor as does S1P, but instead of recycling it back to the cell surface, it promotes its ubiquitination and degradation at the proteasome (Cohen and Chun, 2011). This has a direct effect on lymphocyte trafficking through the lymph nodes, which relies on S1PR1 signalling, leading to reduced lymphocytes in the blood. FTY720, a pro-drug, which is phosphorylated *in vivo* by SphK2 into FTY720-P, has been approved by FDA to be used as a drug against multiple sclerosis (MS).

The effects of S1P on neutrophil trafficking have not been as thoroughly investigated as the effects on lymphocyte trafficking. Neutrophils mainly express S1PR1, S1PR4 and S1PR5, although in pneumonia S1PR3 expression seems to be induced (Rahaman *et al.*, 2006). There are studies that argue S1P pre-treatment has a negative effect on neutrophil

chemotaxis towards the chemokine CXCL8 (IL-8) or the potent chemoattractant fMLP (Kawa *et al.*, 1997; Rahaman *et al.*, 2006). S1P pre-treatment might also inhibit trans-endothelial migration of neutrophils, without affecting their adhesion in the endothelium (Kawa *et al.*, 1997). However, different S1P concentrations in blood and tissue, and the activation state in which the neutrophils are, could have various effects on neutrophil trafficking *in vivo*. So, we need to take everything into consideration, if we want to assess the effect S1P has on neutrophil trafficking during inflammatory conditions. S1P effects on neutrophil migration towards IL-8 might be the result of S1PRs crosslinking with the IL-8 receptors in neutrophils, CXCR1 and CXCR2. Indeed, there is evidence suggesting S1PR4 and S1PR3 form heterodimers with CXCR1 in neutrophils (Rahaman *et al.*, 2006).

On the other hand, there are several studies showing S1P can increase IL-8 expression and/or secretion by several diverse types of cells, including human bronchial epithelial cells (Cummings *et al.*, 2002), alveolar epithelial cells (Milara *et al.*, 2009), airway smooth muscle cells (Rahman *et al.*, 2014), gingival epithelial cells (Eskan *et al.*, 2008), umbilical vein endothelial cells (HUVEC) (Lin *et al.*, 2006), immature dendritic cells (Oz-Arslan *et al.*, 2006), retinal pigment epithelial cells (Qiao *et al.*, 2012), and ovarian cancer cells (Schwartz *et al.*, 2001). This would cause an indirect positive effect on neutrophil trafficking.

Another indication that S1P plays a role in neutrophil trafficking is a study on S1P lyase deficiency, which impairs neutrophil migration from blood to tissue in knockout mice (Allende *et al.*, 2011). Little is known on the mechanism in which S1P lyase acts, although S1PR4 deficiency seems to alleviate the phenomenon, implicating S1P receptor signalling in neutrophil trafficking. These findings suggest S1P lyase and S1PRs in neutrophils as new therapeutic targets against ischemia/reperfusion injury and inflammatory conditions in general. Consistent with these results, another study showed that inhibition of S1P lyase can have a protective effect on the heart after ischemia/reperfusion injury, and this is alleviated when pre-treated with an S1PR1 and S1PR3 antagonist (Bandhuvula *et al.*, 2011). Inhibition was achieved with an FDA approved food additive, 2-acetyl-4-tetrahydroxybutylimidazole (THI), providing a possible new drug perspective (Schwab *et al.*, 2005; Bandhuvula *et al.*, 2011). Another S1P lyase inhibitor, LX2931, a synthetic analogue of THI, has been shown to cause peripheral lymphopenia when administered in mice, providing a potential treatment for autoimmune diseases and prevention of graft rejection after transplantation (Bagdanoff

et al., 2010). This molecule is currently under Phase II clinical trials in rheumatoid arthritis patients.

1.5.5 Cardiovascular effects of S1P

S1P can have direct effects on the cardiovascular system. Vascular endothelial cells mainly express S1PR1 and S1PR3, only a few types express S1PR2 (Panetti, 2002; Waeber *et al.*, 2004). S1PR1 and S1PR3 activation on these cells, has been shown to enhance their chemotactic migration, probably through direct phosphorylation of S1PR1 by Akt (Lee *et al.*, 2001), in a PI3K and Rac1 dependent signalling pathway (Kimura *et al.*, 2000; Gonzalez *et al.*, 2006). Moreover, it stimulates endothelial cell proliferation through an ERK pathway (Kimura *et al.*, 2000; Tamama and Okajima, 2002). S1PR2 activation however, inhibits endothelial cell migration, morphogenesis and angiogenesis (Sanchez *et al.*, 2005; Skoura *et al.*, 2007), most likely through Rho-dependent inhibition of Rac signalling pathway, as Inoki *et al* showed in mouse cells with the use of S1PR1 and S1PR3 specific antagonists (Inoki *et al.*, 2006). Apart from endothelial cells, S1PR2 has an inhibitory effect on vascular smooth muscle cell migration and proliferation as well. These cells express S1PR2 and S1PR3 mainly, and it seems that their activation has similar contradictory effects as in endothelial cells, through the activation of equivalent pathways (Okamoto *et al.*, 2000; Ryu *et al.*, 2002; Tamama and Okajima, 2002; Sugimoto *et al.*, 2003; Waeber *et al.*, 2004).

Regarding permeability of the vascular endothelium and endothelial barrier integrity, S1P receptors can have different effects. S1PR1 activation enhances endothelial barrier integrity by stimulation of cellular adhesion and upregulation of adhesion molecules (McVerry and Garcia, 2004; Singleton *et al.*, 2005). On the other hand, S1PR2 and S1PR3 have been shown to have barrier-disrupting effects *in vitro*, and vascular permeability increasing effects *in vivo* (Singleton *et al.*, 2006; Sanchez *et al.*, 2007). All the effects S1P can have on vascular endothelium and smooth muscle cells suggest, that activation of S1PR2, but not S1PR1 and S1PR3, signalling, perhaps with the use of S1PR2 specific agonists, could be used therapeutically to inhibit angiogenesis and disrupt vasculature, suppressing tumour growth and progression. On the other hand, in several other situations, including transplantation and ischemia/reperfusion injury, S1PR1 activation and inhibition of S1PR2, could have positive effects in promoting angiogenesis and barrier function for recovery of the transplant or injured tissue respectively.

1.5.6 S1P in ischemia-reperfusion injury and other conditions

In a mouse model of myocardial ischemia-reperfusion injury (IRI), S1P and its carrier HDL can help protect myocardial tissue and decrease the infarct size (Theilmeier *et al.*, 2006). It seems they reduce cardiomyocyte apoptosis and neutrophil recruitment to the ischemic tissue and they may decrease leukocyte adhesion to the endothelium. This effect appears to be S1PR3 mediated, since in *S1PR3* knockout mice it is alleviated (Theilmeier *et al.*, 2006). Fortunately for therapeutic potential, this cardioprotective effect of S1P can be produced not only by administering it before ischemia (pre-conditioning), but also during reperfusion (post-conditioning). Ischemia activates SphK1 which is then translocated to the plasma membrane (Jin *et al.*, 2004). This leads to an increase of intracellular S1P, helping to promote cardiomyocyte survival against apoptosis, induced by ceramide (Bielawska *et al.*, 1997). *SphK1* knockout mice cannot be pre-conditioned against ischemia reperfusion injury (Jin *et al.*, 2007), whereas *SphK1* gene induction in the heart protects it from IRI (Duan *et al.*, 2007). Interestingly, a recent study shows SphK2 may also play a role, since its knockout reduces the cardioprotective effects of pre-conditioning (Vessey *et al.*, 2011). Furthermore, administration of S1P or sphingosine during reperfusion results in better recovery and attenuation of damage to cardiomyocytes (Vessey *et al.*, 2008). Like with pre-conditioning, SphK1 deficiency also affects post-conditioning of mouse hearts after ischemia-reperfusion (Jin *et al.*, 2008).

S1P does not protect the heart only, from IRI. During intestinal ischemia-reperfusion, multiple organs can be damaged, including the lungs. S1P treatment of mice during intestinal IR seems to have a protective effect on lung injury, probably due to suppression of iNOS-induced NO generation (Ding *et al.*, 2012). Ischemia-reperfusion injury after rat lung transplantation was attenuated when recipient animals were treated with S1P before reperfusion. The treatment resulted in decreased pro-inflammatory molecule production and signalling, as well as reduced neutrophil infiltration and pulmonary oedema(Okazaki *et al.*, 2007). In renal ischemia reperfusion injury, SphK1 seems to be important, since its deficiency increased the damage in kidney tissue, whereas the lentiviral overexpression of *SphK1* gene protected from injury (Park *et al.*, 2011). Another study suggests that apoptotic renal cells, after IRI, release S1P, which recruits macrophages through S1PR3 activation and might contribute in kidney regeneration and restoration of renal epithelium (Sola *et al.*, 2011). On the other hand,

SphK2 is negatively implicated in hepatic IRI, its inhibition helping protect hepatocytes and restoring mitochondrial function (Shi *et al.*, 2012).

Studies are implicating S1P signalling or SphKs to several kinds of cancer. The first indication that S1P signalling might be involved in cancer came with the evidence that *SphK1* overexpression in NIH3T3 fibroblasts transforms them into cancer-like cells, in the sense that they proliferate in serum free conditions, they have increased growth *in vitro*, and form fibrosarcomas *in vivo* (Xia *et al.*, 2000). Although there is no proof that *SphK1* is an oncogene, cancer cell lines overexpress it and depend on it for growth and survival (Vadas *et al.*, 2008). Even more, in various human cancers, such as stomach (Li *et al.*, 2009), lung (Johnson *et al.*, 2005), brain (Van Brocklyn *et al.*, 2005) and breast cancer (Ruckhaberle *et al.*, 2008), increased levels of SphK1 mRNA and/or protein have been identified. There are several studies implicating the intracellular S1P-ceramide rheostat to cancer cell survival or apoptosis and resistance to chemotherapy or irradiation *in vitro* (Pyne and Pyne, 2010). Studies with SphK1 inhibition in pancreatic (Guillermet-Guibert *et al.*, 2009), prostate cancers (Pchejetski *et al.*, 2005; Akao *et al.*, 2006) and leukaemia (Baran *et al.*, 2007), show increased ceramide/S1P ratio and induction of apoptosis. On the other hand, S1P receptor signalling plays conflicting roles in cancer cell migration and metastasis. Generally, it seems that S1PR1 and S1PR3 activation stimulates cancer cell migration, whereas S1PR2 inhibits cancer cell motility (Yamamura *et al.*, 2000; Arikawa *et al.*, 2003; Fisher *et al.*, 2006; Yamashita *et al.*, 2006; Malchinkhuu *et al.*, 2008).

The pro-drug FTY720, whose phosphorylated form can inhibit lymphocyte S1PR1 dependent egress from the lymph nodes, causing lymphopenia, is already used in MS. It works as an immunosuppressant by stopping lymphocytes migrating into the brain, but it may also have direct effects on the CNS through neuroprotection. FTY720 can pass the blood-brain barrier (BBB) and it could be phosphorylated by local SphKs, to act through S1PR1 and S1PR3 receptors that are mainly expressed in CNS. In MS lesions, astrocytes upregulate those two receptors, and it has been shown that FTY720-P treatment *in vitro* inhibits astrocyte production of inflammatory cytokines (Van Doorn *et al.*, 2010). Another study confirms the importance of S1PR3 signalling on activated astrocytes, which upregulate this S1P receptor and SphK1 expression, promoting the secretion of the potentially neuroprotective cytokine CXCL1 (Fischer *et al.*, 2011).

FTY720 is investigated for other autoimmune conditions and for transplantation. Unfortunately, Phase II and III clinical trials for the prevention of kidney graft rejection did not show an advantage over standard therapies (Tedesco-Silva *et al.*, 2005; Mulgaonkar *et al.*, 2006; Salvadori *et al.*, 2006; Tedesco-Silva *et al.*, 2006; Tedesco-Silva *et al.*, 2007). Moreover, FTY720 can have some adverse cardiac effects, such as bradycardia, due to the activation of other S1P receptors, especially S1PR3. However, there are other S1PR1 antagonists that could be considered instead, including KRP-203 (Shimizu *et al.*, 2005), AUY954 (Pan *et al.*, 2006), SEW2871 (Sanna *et al.*, 2004), etc. KRP-203 in particular has been shown to prolong rat skin and heart allograft survival, and attenuate chronic rejection, without causing bradycardia (Shimizu *et al.*, 2005), especially when combined with other immunomodulators (Takahashi *et al.*, 2005; Suzuki *et al.*, 2006). Moreover, it seems to have positive effects in mouse and rat models of autoimmune conditions, such as chronic colitis (Song *et al.*, 2008), autoimmune myocarditis (Ogawa *et al.*, 2007) and autoimmune kidney disease (Wenderfer *et al.*, 2008). KRP-203 is currently undergoing Phase II clinical trials for cutaneous lupus erythematosus.

1.6 Project aims

The overall aim of this project was to investigate the potential role sphingosine 1-phosphate has on neutrophils trans-endothelial migration. This role could be direct or indirect, by having effects on neutrophils with direct signalling or by affecting endothelial cells and through them neutrophils. Neutrophils are major effectors in many inflammatory conditions including ischemia-reperfusion injury. They migrate from blood to the inflamed tissue after pro-inflammatory signalling by chemokines such as CXCL8 and the effect of adhesion molecules that allow their adhesion and subsequent extravasation from the endothelial layer. There are indications that S1P might have effects on neutrophils but it has not been explored thoroughly. Studies show S1P can be involved in ischemia-reperfusion injury, but it is not yet clear if the effects are only on an intracellular protective level or if neutrophil migration is also affected. Moreover, the S1P receptors involved have to be investigated to determine which receptor stimulation or inhibition could have positive effects for future therapeutic solutions.

In order to examine all these, the current study investigated:

- Which S1P receptors are expressed by neutrophils and which signalling pathways are activated
- Whether S1P can modulate neutrophil migration and adhesion directly
- What effects S1P has on endothelial cell chemokine and adhesion molecule expression
- Whether S1P affects endothelial barrier integrity and neutrophil adhesion to endothelium indirectly
- The S1P receptors responsible for any effects observed
- When using *in vivo* models of neutrophil migration which effect was apparent, if any

CHAPTER 2

Materials and Methods

Chapter 2. Materials and Methods

2.1 Tissue culture

All tissue culture work was performed under sterile conditions inside Class II Microbiological Safety Cabinets. Proper protective equipment was worn and safety procedures followed according to Newcastle University Safety Policy. Relevant COSHH and BIOCOSHH forms were read and signed before the use of any chemical or biological reagents.

All cultured cells were maintained at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. They were cultured in polystyrene 75cm^2 or 25cm^2 tissue culture flasks with vented cap, or grown in polystyrene tissue culture plates with 6, 12 or 24 wells (Greiner Bio-One GmbH, Austria).

2.1.1 Cell counting

When there was a need to count cells, this was done using a haemocytometer. A small volume of cell suspension, 10-20µl, was pipetted in the haemocytometer chamber, under the coverslip. The cells inside the 5x5 grid were counted under an inverted microscope at 200x magnification. This measurement was then multiplied with 10^4 to give an estimate of number of cells per ml of cell suspension. To measure absolute cell number, this was then multiplied with the volume of available cell suspension.

If viable cell counts were required, trypan blue dye was used to exclude dead cells. Trypan blue was mixed 1:1 with cell suspension before being added in the haemocytometer as before. Only cells that were not dyed blue were measured. Calculations were made as before, accounting for the 1:2 dilution step because of dye addition.

2.1.2 Cryopreservation

All cell lines used and some primary cells were recovered from frozen aliquots stored in liquid nitrogen. To culture them, the cryovial was rapidly thawed in a 37 ^{o}C waterbath, and then the contents were transferred in a tube with prewarmed culture media, appropriate for the cell line. Cells were washed by centrifugation at 500 x g for 5 minutes, removal of supernatant and resuspension at appropriate cell density in prewarmed media, before being transferred to culture flasks or plates.

When there was a need for renewal of frozen stock of specific cells, an appropriate aliquot of cells was washed in Phosphate Buffer Saline (PBS; Sigma-Aldrich, USA), resuspended in ice cold cryopreservation medium, consisting of 90% Foetal Bovine Serum (FBS; Lonza, Switzerland) and 10% Dimethyl Sulfoxide (DMSO; Sigma-Aldrich, USA) and transferred in cryopreservation vials. These were then stored at -80 ^{o}C in a Nalgene Mr. Frosty Cryo 1°C Freezing Container (Thermo Scientific), for a controlled cooling rate. For long term storage they were moved in a liquid nitrogen tank.

2.1.3 Dissociation of adherent cells for subculturing

All adherent cell lines or primary cells were detached from culture vessels for subculturing using the following method, unless mentioned otherwise. For dissociation of cells 0.25% Trypsin-EDTA solution (2.5g porcine trypsin and 0.2g EDTA, Sigma-Aldrich, USA) was used. Firstly, media was removed from flask and cells were washed with an excess amount of PBS, to remove any traces of serum that contains trypsin inhibitors. Trypsin-EDTA was then added and incubated for about 5-10 minutes at 37 ^{o}C until all cells could be seen to detach under an inverted microscope. Complete media was then added to inactivate trypsin, and cells were collected into a universal tube. Cells were pelleted by centrifugation at 400 x g for 5 minutes, supernatant was removed, and pellet was resuspended in complete media in desired concentration, before being transferred to new culture vessels with proper amounts of complete media.

2.1.4 Cell lines

2.1.4.1 HL60

The HL60 cell line (ATCC® CCL-240™) was derived from peripheral blood leukocytes of a 36-year old human female with acute myeloblastic leukaemia (Collins *et al.*, 1977). The cells grow in suspension with doubling times from 20 - 45 hours. Morphologically they consist of large, blast-like cells with large rounded nuclei containing 2-4 distinct nucleoli, and a basophilic cytoplasm with azurophilic granules. HL60 cells can be induced to differentiate either to granulocyte-like or to monocyte/macrophage-like cells depending on the nature of the inducing agent. Polar-planar compounds such as DMSO and dibutyryl cyclic AMP, and other compounds such as retinoic acid and actinomycin D induce differentiation to granulocytes. This can be observed as a progressive decrease in cell size and nuclear/cytoplasmic ratio, as well as appearance of kidney-shaped nuclei

and later lobed nuclei, characteristic of banded and segmented neutrophils. On the other hand, compounds such as 1,25-dihydroxy-vitamin D_3, phorbol esters like phorbol-myristrate-acetate (PMA), and sodium butyrate induce differentiation to monocytes or macrophages. Within 24h of compound addition, the cells start to clump and adhere to the substrate, then spread out and acquire pseudopodia, whereas the nuclei remain rounded. When cells commit to a differentiation pathway, they are no longer immortal, and will eventually stop proliferating and become senescent (Gallagher *et al.*, 1979; Collins, 1987; Birnie, 1988).

HL60 cells were routinely grown in RPMI-1640 complete media which comprised of RPMI-1640 (Sigma-Aldrich, USA) with 10% FBS (Lonza, Switzerland), 2 mM L-Glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all Sigma-Aldrich, USA). For details on differentiation procedure see Chapter 3.2.

2.1.4.2 HMEC-1

HMEC-1 cell line (ATCC® CRL-3243™) was established from dermal microvascular endothelial cells isolated from human foreskins that were transfected with pSVT vector, a PBR-322-based plasmid containing the coding region for the simian virus 40 A gene product, large T antigen (Ades *et al.*, 1992). They are adherent cells, with population doubling times 24-48 hours .They retain cobblestone morphology when cultured as monolayers, typical for normal endothelial cells, express von Willebrand's Factor (vWF), cell adhesion molecules ICAM-1 and CD44 and are capable of acetylated LDL uptake and rapid tube formation on matrigel. They retain many of the morphological, phenotypical and functional characteristics of primary human microvasular endothelial cells, so they are a good model for their replacement (Ades *et al.*, 1992).

HMEC-1 cells were cultured in complete MCDB 131 medium, which comprised of MCDB 131with L-glutamine in powder form (Sigma-Aldrich, USA), dissolved in sterile water with 15.7ml/L sodium bicarbonate solution 7.5% w/v (Sigma-Aldrich, USA) and filtered through 0.2μm pore membrane (Filtropur, Sarstedt, Germany), with added 10ng/mL Epidermal Growth Factor (EGF), 1 μg/mL Hydrocortisone, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all Sigma-Aldrich, USA) and 10% FBS (Lonza, Switzerland). For dissociation of cells to subculture, the Trypsin-EDTA method was used (subchapter 2.1.3). For treatment of cells with specific reagents, a serum-free version of the complete medium was used, in which the 10% FBS was replaced by 0.5% Bovine Serum Albumin (BSA) fatty acid-free (Sigma-Aldrich, USA).

2.1.4.3 A549

The A549 cell line (ATCC® CCL-185™) was derived from the explanted tumor of a 58-year old human male with alveolar cell lung carcinoma (Giard *et al.*, 1973). They are adherent cells with a population doubling of about 22 hours. A549 are human alveolar basal epithelial cells with squamous morphology that contain multilamellar cytoplasmic inclusion bodies typical of those found in type II alveolar epithelial cells of the lung. They have the ability to synthesize lecithin with a high percentage of disaturated fatty acids utilizing the cytidine diphosphocholine pathway (Lieber *et al.*, 1976). This is a hypotriploid cell line with a modal chromosome number of 66, although cells with 64, 65 and 67 chromosomes can also occur (Giard *et al.*, 1973).

A549 cells were culture in complete Dulbecco's Modified Eagle's Medium (DMEM), comprised of DMEM (Sigma-Aldrich, USA) supplemented with 10% FBS (Lonza, Switzerland), 2 mM L-Glutamine, and 100 U/ml penicillin and 0.1 mg/ml streptomycin (all Sigma-Aldrich, USA). To subculture them, they were dissociated from substrate using the Trypsin-EDTA method as described above (subchapter 2.1.3). When A549 cells were treated with specific reagents, a serum-free version of the complete medium was used, in which the 10% FBS was replaced by 0.5% BSA fatty acid free (Sigma-Aldrich, USA).

2.1.4.4 EA.hy926

The EA.hy926 cell line is a somatic cell hybrid of A549 alveolar epithelial cells with primary Human Umbilical Vein Endothelial Cells (HUVEC) (Edgell *et al.*, 1983). They are adherent cells with endothelial cell characteristics including the expression of factor VIII-related antigen or von Willebrand factor and the presence of Weibel-Palade bodies (Edgell *et al.*, 1990). They are also capable to exert functions common for differentiated endothelial cells such as angiogenesis in the form of tube formation in matrigel (Bauer *et al.*, 1992; Rieber *et al.*, 1993). They have maintained chromosomes from both cell precursors, leading to a modal chromosome number of 80. Population doubling is around 15 hours (Edgell *et al.*, 1983).

EA.hy926 cells were cultured in complete DMEM media, like their precursors A549, and were detached from substrate for subculturing using the Trypsin-EDTA method (subchapter 2.1.3). To treat them, the serum-free version of DMEM was used, as with A549.

2.1.5 Primary cells

2.1.5.1 Neutrophil isolation

Primary human neutrophils were isolated from human peripheral blood taken by phlebotomy performed by trained individuals from healthy volunteers, who had read and signed the appropriate consent form. Blood samples of 10-30 ml were immediately transferred into 50ml falcon tubes containing 1ml of sodium citrate 3.8% (Sigma-Aldrich, USA) per 10ml blood. After gentle mixing, the tube was centrifuged at 300 x g with no brake for 20 minutes. The separated plasma (upper layer) was discarded and 2.5ml of pre-warmed (in a 37 ^{o}C waterbath) solution of 6% Dextran in 0.85% NaCl (saline) solution (both Sigma-Aldrich, USA) per 10ml cell pellet were added. Pre-warmed saline solution (Sigma-Aldrich, USA) was further supplemented until reached the initial blood level (e.g. 20ml if started with 20ml whole blood). Cells were allowed to sediment at room temperature for approximately 30 minutes, with the tube's lid unscrewed. Percoll Plus (GE Healthcare, UK) and 10x Dulbecco's Phosphate Buffered Saline without Ca^{2+} or Mg^{2+} (D-PBS; Sigma-Aldrich, USA) or its 1x dilution were used to create a percoll gradient. First, 90% Percoll was created by adding 0.6ml 10x D-PBS in 5.4ml Percoll. This was then used to create 55%, 70% and 81% Percoll, as described in the table below (table 2.1).

	1x D-PBS added	**90% Percoll added**
55% Percoll	1.125ml	1.375ml
70% Percoll	0.75ml	1.75ml
81% Percoll	0.475ml	2.025ml

Table 2.1. Creation of Percoll Plus gradient solutions. Each Percoll gradient solution was created by combining the specified amounts of 1x D-PBS and 90% Percoll.

After Dextran sedimentation, the leukocyte rich upper layer was transferred to a new tube and saline added up to 50ml. The tube was centrifuged at 200 x g for 5 minutes. The supernatant was discarded and the cells were resuspended in 2.5ml of 55% Percoll. The 70% Percoll was transferred carefully on top of the 81% Percoll, and then the 55% Percoll with the cells was added above that. The gradient tube thus contained approximately 2.5ml from each of the different Percoll solutions (55%, 70% and 81%).

The gradient was centrifuged at 700 x g with no brake for 20 minutes. After centrifugation, two bands of cells were visible, while the red blood cells were pelleted at the bottom of the tube. The top band (55/70 layer) contained the mononuclear cells whereas the bottom band (70/81 layer) contained the polymorphonuclear cells (PMN), or neutrophils. Usually only the PMN band was collected and the rest were discarded. The collected cells were washed in Hank's Balanced Salt Solution without Ca^{2+} or Mg^{2+} (HBSS; Sigma-Aldrich, USA), at 200 x g for 5 minutes. Cells were resuspended in HBSS again and counted using a haemocytometer. For viability measurements trypan blue was added. Cells were then made to a density of 1 x 10^6 cells/ml and separated in different tubes, according to the experiments planned. After the separation, one last wash with HBSS was performed and cells were resuspended in serum-free medium usually RPMI-1640, which comprised of RPMI-1640 medium with 0.5% BSA fatty acid-free, 2 mM L-Glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all Sigma-Aldrich, USA), with added reagents if needed for current experiment. To assess neutrophil purity after isolation, cells were routinely checked by flow cytometry, with anti-CD3 and anti-CD66b staining and Propidium Iodide (PI) addition for extended viability assessment. Sometimes, cytospins were made that were stained with Giemsa stain to further observe purity of neutrophil populations. Only populations with more than 95% purity and viability were used.

2.1.5.2 HUVEC

Human Umbilical Vein Endothelial cells (HUVEC) are primary endothelial cells, isolated from the vein of the human umbilical cord (Jaffe *et al.*, 1973; Baudin *et al.*, 2007). These were bought from Promocell as proliferating cells from a single donor (Promocell, Germany). They are adherent cells with cobblestone morphology, which express von Willebrand factor and CD31, can uptake Dil-Ac-LDL, and contain Weibel-Palade bodies. They can survive for at least 15 population doublings, with doubling time of around 92 hours, after which they may start to become senescent (Jaffe *et al.*, 1973).

HUVEC cells were cultured in complete Endothelial Cell Growth Medium 2 (Promocell, Germany), which contains 2% Foetal Calf Serum (FCS), 5 ng/ml EGF, 10 ng/ml Basic Fibroblast Growth Factor (bFGF), 20 ng/ml Insulin-like Growth Factor (Long R3 IGF), 0.5 ng/ml Vascular Endothelial Growth Factor 165 (VEGF), 1 μg/ml Ascorbic Acid, 22.5 μg/ml Heparin and 0.2 μg/ml Hydrocortisone. This media was

chosen instead of the Endothelial Cell Growth Medium from the same company, since it was better defined and did not contain Endothelial Cell Growth Supplement (bovine hypothalamic extract) that might contain S1P or other substances that could interfere with experiments. For treatment of cells with specific reagents, a serum-free version of the complete medium was used, in which the 2% FCS was replaced by 0.5% BSA fatty acid free (Sigma-Aldrich, USA). When subculturing HUVEC, the Trypsin-EDTA method was used for cell dissociation (subchapter 2.1.3). However, since HUVEC complete media contains only 2% serum that is not enough for trypsin inactivation, another complete media was used in this step instead (usually complete DMEM).

2.2 Reconstitution of S1P Receptor Ligands

2.2.1 S1P

Sphingosine-1-phosphate, D-*erythro* was the synthetic solid form of S1P used (Enzo Life Sciences, USA). Before being utilized in any experiment, the powder was first dissolved in methanol at 1 mg/ml by heating to 65 ^{o}C until the solution was clear and colourless. S1P solution was then aliquotted in glass vials, 0.1mg per vial, and methanol was evaporated using a stream of nitrogen gas. What remained was a thin film of S1P on the sides of the vial that could be stored at -80 ^{o}C long term.

To use the dried S1P, it was first dissolved in 0.01 M NaOH at 1 mg/ml by heating to 80 ^{o}C until the solution was clear and colourless. Next, the solution was further diluted in PBS with 0.5% BSA fatty acid-free (Sigma-Aldrich, USA) to a concentration of 125μM S1P, by heating and vortexing until a fully homogeneous solution was achieved. The final S1P solution was aliquotted appropriately and used immediately or stored at -20 ^{o}C for up to one month. Before using the stored solution it was first heated in a 37 ^{o}C waterbath and mixed by vortexing.

2.2.2 CYM-5442

CYM-5442 is a novel S1PR1 agonist which is a chemically optimized version of another agonist (CYM-5181) that was discovered through high throughput screening (Gonzalez-Cabrera *et al.*, 2008; Schurer *et al.*, 2008). It was chosen because of its high potency and high selectivity for S1PR1, with an EC_{50} of 1.35nM compared with no activity up to 10μM for the other S1P receptors, as well as its high solubility in water solvents. It can effectively induce phosphorylation, internalization and ubiquitination of S1PR1 *in vitro* and can cause peripheral lymphopenia *in vivo*. Moreover, it can successfully penetrate the Blood-Brain-Barrier (BBB), making it a successful candidate for targeting the central nervous system. It binds to a different hydrophobic pocket in the receptor than the orthosteric site that S1P uses which requires specific headgroup interactions (Gonzalez-Cabrera *et al.*, 2008).

CYM 5442 hydrochloride was purchased from R&D Systems in solid form (R&D Systems, USA). For use in *in vitro* experiments it was first dissolved in DMSO (Sigma-Aldrich) at a concentration of 100mM, by warming and vortexing until solution was clear. This was further diluted in sterile water at 1mM final concentration and aliquotted

for short-term storage at -20 ºC. Before using the frozen solution, it was warmed in a 37 ºC waterbath and vortexed until completely homogenous.

2.2.3 CYM-5541

CYM-5541 was the result of optimization of molecules discovered through the same screening process that lead to the eventual creation of CYM-5442 (Schurer *et al.*, 2008; Jo *et al.*, 2012). CYM-5541 is a potent and selective agonist for S1PR3, with EC_{50} of between 72 and 132 nM whereas for S1PR1 EC_{50} > 10 μM, for S1PR2 EC_{50} > 50 μM, for S1PR4 EC_{50} > 50 μM, and for S1PR5 EC_{50} > 25 μM. Unlike CYM-5442 that is only an alternative binding mode agonist, CYM-5541 is a true allosteric agonist, which occupies a different chemical space in the ligand binding pocket of S1PR3 than S1P does (Jo *et al.*, 2012).

CYM-5541 was purchased from Sigma in powder form (Sigma-Aldrich, USA). In order to use it, it was dissolved in DMSO at a concentration of 10mM, by continuous heating and vortexing until solution was clear. This was then aliquotted and stored at -20 ºC for up to three months. Before use, it was warmed in a 37 ºC waterbath and vortexed.

2.2.4 SEW2871

SEW2871 is a potent and selective S1PR1 agonist with an EC_{50} of 13nM, with no activity for the other S1P receptors at up to 10μM (Hale *et al.*, 2004). It was only used at some optimization experiments before being replaced with the more potent and water soluble novel S1PR1 agonist CYM-5442.

SEW2871 (Sigma-Aldrich, USA) was dissolved in DMSO (Sigma-Aldrich, USA) at a concentration of 2 mg/ml (or 4.54 mM) by warming in a 37 ºC waterbath and repeatedly vortexing until the solution was clear and colourless. The solution was then aliquotted into glass vials and stored at -20 ºC in the dark for up to three months. Before use, the frozen SEW2871 solution was brought to 37 ºC in a waterbath and vortexed to ensure total homogenization.

2.2.5 FTY720-P

FTY720 (or fingolimod) is a molecule that can be phosphorylated *in vivo* into FTY720-phosphate (FTY720-P), an S1P analogue that is a potent agonist for S1PR1, S1PR4 and S1PR5, with EC_{50} values of around 0.3-0.6 nM, as well as S1PR3 with EC_{50} of about 3 nM, but does not activate S1PR2 (Brinkmann *et al.*, 2002; Mandala *et al.*, 2002).

FTY720 has been approved as a medicine for MS. It has the ability to cause retention of lymphocytes to lymphoid organs by activation and internalization of the S1PR1 (Brinkmann *et al.*, 2010). For its use *in vitro*, the already phosphorylated form FTY720-P was chosen to make sure it would be active even in the absence of sphingosine kinases.

FTY720 (S)-Phosphate was purchased from Cambridge Bioscience (Cambridge, UK). It was dissolved in chloroform at a concentration of 0.5 mg/ml then aliquotted for storage at -20 °C. Before use it was warmed at a 37 ºC waterbath and vortexed.

2.3 Flow cytometry

In flow cytometry, cells are stained with specific antibodies for surface or intracellular proteins, directly or indirectly conjugated with fluorochromes, which are molecules that absorb specific wavelengths of light and then emit light at a different wavelength spectrum. This allows for measurement of the relative expression of the specific target protein. The flow cytometer can measure the optical properties of single cells as they pass through the core of the instrument. Laser light is focused on the cells and is then detected by photomultiplier tubes (PMTs) as it passes through or is scattered by each cell. One PMT can detect light that is in line to the laser, giving a measurement called forward scatter (FSC), and another detects light perpendicular to the laser, giving a side scatter (SSC) value. FSC can be loosely correlated with size of individual cells, whereas SSC can be loosely correlated with granularity. Additional lasers in the instrument are used to excite fluorochromes or other fluorescent molecules, with perpendicular PMTs that detect the fluorescently emitted light. These PMTs are covered by band-pass filters that allow only a very narrow range of light spectrum to pass through. This allows the detection of light emitted by only one fluorescent molecule at a time. See table 2.2 for a list of the fluorescent molecules used in this study and the cytometer settings asscociated with each. Except for fluorochromes, conjugated with specific antibodies, fluorescent viability dyes were also used. These bind to nucleic acids or other cellular targets that are only exposed for binding in dead cells. This allows for distinction of alive from dead cells in the sample.

2.3.1 Staining protocol

Cells for staining were counted and separated in flow cytometry tubes (12 x 75 mm round bottom polystyrene tubes; BD Biosciences, USA), with around 100,000 – 500,000 cells per tube. Cells were washed in FACS buffer (PBS with 2% FBS) by centrifugation at 500 x g for 5 minutes. Supernatants were removed and Fc receptor blocking solution was added (Human TruStain FcX; Biolegend, USA), 5 µl per tube, and incubated at room temperature for 15 minutes. Without washing, appropriate amounts of primary antibody solution was added, according to manufacturer's instructions, and incubated at 4 ^{o}C for 30-45 minutes in the dark. Cells were then washed twice with 1ml FACS buffer by centrifugation at 500 x g for 5 minutes. If the primary antibody was not directly conjugated with fluorochrome, a secondary antibody was then added that was conjugated with fluorochrome and was raised against the

species of the primary antibody. Before addition, Fc receptor blocking was performed again. The cells were then incubated with the secondary antibody at 4 ºC for 45 minutes in the dark. After another two washes, the stained cells were finally resuspended in 200µl FACS buffer and taken to the flow cytometer for acquisition. At this point, the viability dye Propidium Iodide (PI) was added, when a measurement of cell viability was required. Acquisition occurred in a BD FACSCanto II instrument running BD FACSDiva software (BD Biosciences, USA). As controls, there was an unstained sample and samples stained with species and isotype matched antibodies conjugated with the same fluorochromes as the primary antibodies, or a secondary antibody only stained sample in the case of indirect flow cytometry. Data from flow cytometry were analysed using FlowJo 7.6 software (Treestar, USA). A list of all the antibodies used in this project can be seen at table 2.3.

Fluorochrome or viability dye	Excitation laser wavelength (nm)	Band-pass filter (nm)
FITC (Fluorescein)	488	530/30
PE (Phycoerythrin)	488	585/42
PerCP (Peridinin Chlorophyll)	488	670LP
PerCP/Cy5.5	488	670LP
PE/Cy7	488	780/60
PI (Propidium Iodide)	488	585/42
APC (Allophycocyanin)	635	660/20
Brilliant Violet 421	405	450/50
Zombie Aqua	405	510/50

Table 2.2. Fluorochromes and associated cytometer settings used. Laser wavelength and cytometer band-pass filter used for each fluorochrome and the viability dyes PI and Zombie Aqua in a FACSCanto II instrument (BD Biosciences).

Target	Conjugate	Clone	Isotype	Source
Human CD45	APC	2D1	IgG1	R&D Systems
Human CD11b	PE	238446	IgG2b	R&D Systems
Human CD69	APC	FN50	IgG1,κ	BD Biosciences
Human CXCR1	PE	42705	IgG2a	R&D Systems
Human S1PR1	APC	218713	IgG2b	R&D Systems
Mouse/Human S1PR3	-	polyclonal		Alomone Labs, Israel
Human CD45	PerCP	2D1	IgG1	BD Biosciences
Human CD66b	FITC	G10F5	IgM	Biolegend
Human CXCR1	FITC	42705	IgG2a	R&D Systems
Human CXCR2	FITC	48311	IgG2a	R&D Systems
Human CD3	APC	UCHT1	IgG1	R&D Systems
Human CD54 (ICAM-1)	PE	HA58	IgG1,κ	Biolegend
Human CD106 (VCAM-1)	APC	STA	IgG1,κ	Biolegend
Rabbit IgG	APC	polyclonal		R&D Systems
- (isotype control)	APC	MOPC-21	IgG1,κ	BD Biosciences
- (isotype control)	PE	133303	IgG2b	R&D Systems
- (isotype control)	PE	20102	IgG2a	R&D Systems
- (isotype control)	FITC	MM-30	IgM	Biolegend
- (isotype control)	FITC	20102	IgG2a	R&D Systems
- (isotype control)	PE	MOPC-21	IgG1,κ	BD Biosciences
Mouse CD3ε	PerCP/Cy5.5	145-2C11		Biolegend
Mouse CD19	APC	6D5		Biolegend
Mouse/Human CD11b	PE/Cy7	M1/70		Biolegend
Mouse Ly-6G	Brilliant Violet 421	1A8		Biolegend
Mouse F4/80	PE	BM8		Biolegend
Mouse CD11c	FITC	N418		Biolegend

Table 2.3. List of flow cytometry antibodies used. Reference table with all the antibodies used in this thesis, their conjugated fluorochrome (if any), clone number and company they were purchased from.

2.4 Chemotaxis assays

To measure the chemotactic motility of cells, a modified Boyden chamber assay was used (Boyden, 1962). For this assay, transwell cell culture inserts with permeable membranes are placed into the wells of cell culture plates, splitting them into two chambers, a top and a bottom one, separated by the membrane. The membrane has pores that allow easy diffusion of chemoattractants and other reagents, but can inhibit cells from passing through freely, depending on the pore size. However, cells can actively pass through the pores when stimulated by appropriate chemotactic gradients. Cells are added on the top chamber, whereas the bottom chamber usually contains the chemoattractant of interest. Chemotactic ability is then measured by counting the cells that passed through the filters and into the wells, after a defined amount of time. This allows measurement of the directional movement of cells towards chemotactic gradients. Alternatively, adding the chemoattractant into both the top and bottom chambers, allows the measurement of chemokinesis, which is then non-directional cell motility stimulated by certain molecules. Chemotaxis assays can be performed with bare membranes, mentioned as trans-membrane chemotaxis or with endothelial cells growing on top of the membranes, so the cells would have to pass through the endothelial layer and the membrane to reach the bottom chamber, mentioned as trans-endothelial chemotaxis assays (figure 2.1).

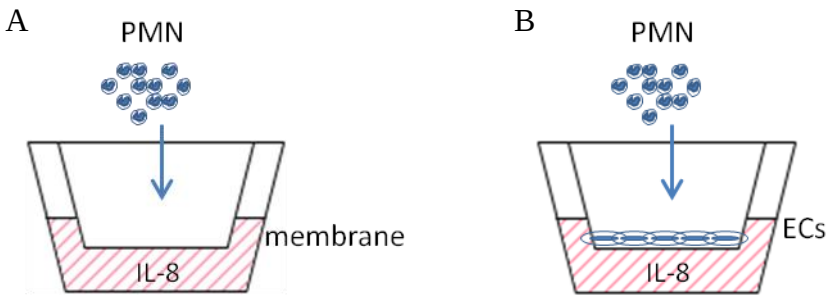


Figure 2.1. Chemotaxis assay set-up. Diagrammatic illustration of the set up of a trans-membrane (A) or trans-endothelial (B) chemotaxis assay. PMN: neutrophils, ECs: endothelial cells.

2.4.1 Trans-membrane chemotaxis

Cells were incubated in serum-free medium before the assay, from 30 minutes (for primary neutrophils) to 2 hours or overnight (for HL60). According to the experiment, they were pretreated or not with S1P or other reagents. Before starting the chemotaxis, the 24-well companion plate (BD Biosciences, USA) that was going to be used in the assay was blocked for 30 minutes with serum-free medium (RPMI 1640 with 0.5% BSA fatty acid-free). Then, 600μl of serum-free medium with or without chemoattractant were added to each well. Cell culture inserts, with 3μm pore diameter membranes (BD Biosciences), were inserted in the wells. Cells were resuspended to a concentration of 1 x 10^6 cells/ml and 100μl of cell suspension (100,000 cells) were added in each insert. Cells were left to migrate at 37^{o}C, 5% CO_2 for 60 - 120 minutes. Then the inserts were removed and 400-500μl of cell suspension from each well were transferred into flow cytometry tubes. A specific amount of counting beads (CountBright™ Absolute Counting Beads; Invitrogen) was added to the cells, and enumeration was achieved using a flow cytometer.

Firstly, the beads were gated on the forward scatter towards side scatter axis. The cells were also gated on the same axis. At least 1,000 bead events (usually 2,000) were acquired to assure statistically significant calculations. In order to calculate the cell concentration as cells/μl of cell suspension, the following equation was used:

$$\frac{number\ of\ cell\ events}{number\ of\ bead\ events} \times \frac{bead\ count\ used\ (lot\ dependent)}{volume\ of\ cell\ sample} = cells/\mu l$$

Example calculation: 500μl of cell suspension were used and then 20μl of bead suspension were added, with a lot concentration of 49,000 beads/50μl (so the 20μl would contain 19,600 beads).

$$\frac{6645\ cells}{2002\ beads} \times \frac{19600\ beads}{500\ \mu l} = 130.11\ cells/\mu l$$

In order to then calculate the total number of cells that had passed to the bottom chamber, the concentration was multiplied by the total volume of cell suspension in the bottom chamber (600μl). So, for the above example, the total migrated cell count would be:

130.11 cells/μl x 600 μl = 78,066 cells.

In some experiments the cell culture inserts were fixed and stained with haematoxylin, to count the cells that adhered on the bottom side of the membranes. This was used as a verification method for bead counting. At the end of chemotaxis, media was removed from both chambers and the inserts were fixed in cold methanol at -20^{o}C overnight, inside the 24-well plate. Methanol was removed and the filters were washed with water. Water was removed and 1.5ml haematoxylin was added to each well, for 30 minutes at room temperature. Haematoxylin was removed then, and the filters were washed with water, vigorously under tap. Scotts Tap Water was added for 2 minutes in room temperature. Scotts Tap Water was removed and 1ml of 50% ethanol was added to each well. After 2 minutes in room temperature, 50% ethanol was replaced with 75% ethanol. After another 2 minutes, 75% ethanol was replaced with 90% ethanol. Finally, 90% ethanol was replaced with 100% ethanol. Filters were left to air dry, then were excised using a scalpel and mounted onto slides (basal side facing up) using DPX mountant and coverslip. They were observed under a microscope, and the cells that were on the correct side of the membrane were counted.

2.4.2 Trans-endothelial chemotaxis

For trans-endothelial chemotaxis, endothelial cells in 500μl complete culture media were placed on top of cell culture inserts with 3μm pores, in 24-well companion plate and left to grow to confluency in a 37 ^{o}C with 5% CO_2 atmosphere. When confluent, they were treated with desired reagents, by removing media, adding 500μl serum-free media (their complete culture media with 0.5% BSA fatty acid-free instead of FBS) with treatment and incubating overnight. The wells of the 24-well plate that were going to be used for chemotaxis were blocked with serum-free media for 30 minutes, before adding 800μl serum-free media with chemokine (10ng/ml IL-8). The media with the treatment was removed and the cell culture inserts with the endothelial cells were transferred in the specific wells of the 24-well plate containing the chemoattractant. Isolated human neutrophils were added on top, 200,000 cells in 500μl serum-free media (the same media used for the endothelial cells and the chemokine), and incubated at 37 ^{o}C, 5% CO_2 for 2 hours. The inserts were removed and 400 μl of the cell suspension in the bottom chamber were transferred into flow cytometry tubes. The cells were then counted by flow cytometry using a defined amount of counting beads (CountBright™ Absolute Counting Beads; Invitrogen), following the same method as above.

2.5 Quantitation of gene expression

Gene expression is the transcription of genomic DNA (gDNA) to RNA, and then translation of messenger RNA (mRNA) to protein, though other RNAs like ribosomal RNA are the final product themselves. Measuring the abundance of a specific mRNA gives an indication of a gene's expression, although this does not always translate accurately to protein quantitation. It can be used however to compare gene expression between different cell types or before and after treatment in the same cells. Total RNA needs to be isolated first from the cells of interest. This is then used as a template for cDNA synthesis by reverse transcription. Finally, quantitation of gene expression is achieved by real-time PCR, which uses the cDNA to amplify specific target genes using primers and probes.

2.5.1 RNA isolation

Total RNA was isolated using the TRI Reagent (Sigma-Aldrich, USA) method or the RNeasy Plus Mini Kit (QIAGEN). The whole procedure was performed carefully, using sterile, RNAse-free materials and wearing clean gloves and protective clothing to avoid contaminating with RNases.

For the TRI Reagent method, cells in suspension were pelleted by centrifugation and 1ml of TRI was added per 5-10 x 10^6 cells. After repetitive pipetting to lyse the cells, they were incubated for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. Next, 0.2ml of chloroform (Sigma-Aldrich) per 1ml TRI were added. After capping the tubes securely, they were shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 minutes. Centrifugation of the samples followed, at 12,000 x g for 15 minutes at 4^{o}C. After the centrifugation, the mixture was separated in a lower red, phenol-chloroform phase, a white interphase that contains the genomic DNA and a colourless upper aqueous phase where the RNA resides. The aqueous phase was transferred in a new tube and the rest discarded. To precipitate the RNA from the aqueous phase, 0.5ml of isopropyl alcohol (2-propanol; Sigma Aldrich) per 1ml TRI used were added. After mixing the sample, it was incubated for 10 minutes at room temperature and then centrifuged at 12,000 x g for 10 minutes at 4^{o}C. The RNA precipitate could be seen as a gel like pellet at the bottom and side of the tube. The supernatant was discarded and the RNA pellet washed with 1ml of 75% ethanol per 1ml TRI used by vortexing and then centrifuging at 7,500 x g for 5 minutes at 4^{o}C. After removing the ethanol, the pellet was left to air-dry for 5-10

minutes and then was dissolved in RNase-free water. The amount and quality of RNA was measured by spectrophotometry using a Nanodrop instrument (Thermo Scientific). Only samples with absorbance 260/280 ratio higher than 1.8 were used for reverse transcription and PCR. The RNA was used immediately or stored at -80^{o}C for up to a few weeks.

When RNeasy Plus Mini Kit (QIAGEN) was used, RNA isolation was performed following the manufacturer's instructions. Cells were pelleted and lysed, using 600μl Buffer RLT Plus with 1% β-mercaptoethanol (Sigma-Aldrich) for 5-10 x 10^6 cells. For adherent cells grown into tissue culture plates, lysis was performed in the plates, by removal of media and addition of 600μl Buffer RLT Plus with 1% β-mercaptoethanol per well of a 6-well plate. Lysate was transferred into microcentrifuge tubes and homogenized using a needle and syringe. Homogenised lysate was then transferred into a gDNA eliminator column placed into a 2ml collection tube. This column binds the genomic DNA in the samples, which is then discarded, allowing for a gDNA free RNA. Centrifugation was performed at 8000 x g for 30 seconds. Column was discarded and one volume of 70% ethanol was added to the flow through. After mixing by pipetting, the sample was transferred into an RNeasy spin column placed in a 2 ml collection tube, 700μl at a time, and centrifuged at 8000 x g for 15 seconds. Flow through was discarded and this process was repeated until all the sample had been transferred in the column. The RNeasy spin columns selectively bind only RNA, which can then be collected by elution. Rneasy spin column was washed with 700 μl Buffer RW1by centrifugation at 8000 x g for 15 seconds and flow through was discarded. Column was then further washed with 500 μl Buffer RPE, diluted in 100% ethanol as instructed, by centrifugation at 8000 x g for 15 seconds. Flow through was discarded and a final wash was performed with another 500 μl Buffer RPE and centrifugation at 8000 x g for 2 minutes. To ensure no residual Buffer RPE had remained in the column, the collection tube was replaced with a new one and centrifugation at 8000 x g for 1 minute was performed. Finally, to elute the RNA, the collection tube was replaced with a new 1.5 ml collection tube, 30-50 μl RNase-free water were added directly to the spin column membrane, and centrifugation was performed at 8000 x g for 1 minute. The column was discarded and the RNA was analysed using a Nanodrop. Only samples with absorbance 260/280 ratio close to 2 were used further. The RNA was used immediately or stored at -80^{o}C for up to a few weeks.

2.5.2 cDNA synthesis

The RNA isolated from the cells was then used to synthesize complementary DNA (cDNA), by reverse transcription (RT). The enzyme reverse transcriptase is an RNA dependent DNA polymerase which is able to use RNA as a template to create cDNA. These enzymes can be found in many RNA viruses that need them to create cDNA as a part of their replication cycles.

About 1µg of total RNA was usually used for each RT reaction. The AffinityScript Multiple Temperature cDNA synthesis kit (Agilent, USA) was used, and the company's optimized protocol was followed. According to this, the reaction was prepared in a microcentrifuge tube by adding in order: 1µg of total RNA, RNase-free water to a total volume of 15.7µl, and 3µl of random primers (0.1µg/µl). The reaction was incubated at 65 ^{o}C for 5 minutes, and then cooled at room temperature for about 10 minutes to allow the primers to anneal to the RNA. After that, 2µl of 10x AffinityScript RT Buffer, 0.8µl of dNTP mix (25mM each dNTP), 0.5µl of RNase Block Ribonuclease Inhibitor (40U/µl), and 1µl of AffinityScript Multiple Temperature RT (reverse transcriptase) were added in the reaction in order. After mixing the components gently, the reaction was incubated at 25 °C for 10 minutes to extend the random primers, followed by incubation at 50 °C for 60 minutes for cDNA synthesis and 15 minutes at 70 ^{o}C to terminate the reaction. The cDNA was used immediately for subsequent qPCR amplification or stored at -80 °C to be used at a later time.

For Chapter 5 of this thesis, the Tetro cDNA Synthesis kit (Bioline, UK) was used instead. This allows for faster one-step cDNA synthesis. The reaction was again prepared in a microcentrifuge tube by addition of 1µg RNA, 1µl random hexamer primer mix, 1µl 10mM dNTP mix, 4µl 5x RT Buffer, 1µl RiboSafe RNase Inhibitor (10U/µl), 1µl Tetro Reverse Transcriptase (200U/µl) and RNase-free water up to 20µl. After mixing the reaction gently by pipetting, it was incubated for 10 minutes at 25°C followed by 30 min at 45°C and finally 5 min at 85°C to terminate the reaction. Reaction was chilled on ice and used immediately for PCR or stored at -80 °C to be used at a later time.

2.5.3 Real-time PCR

Polymerase chain reaction (PCR) is an enzyme-catalyzed amplification of a specific DNA sequence. A heat-stable DNA-dependent DNA polymerase is used, such as *Taq* polymerase, with specific 5' and 3' primers that will bind to the DNA template.

In real-time quantitative PCR (qPCR), a DNA sequence is being amplified and, simultaneously, real-time quantitation of the number of amplicons is performed. This can be achieved with the use of primer-probe technology, such as the TaqMan Gene Expression assays (Applied Biosystems, USA), which contain a set of specific gene primers and fluorogenic probe that target the same gene. The probes have the fluorescent dye FAM at their 5' end, and the quencher MGB at their 3' end. When the probe is intact, it cannot fluoresce due to the proximity of the fluorescent dye and the quencher. The probe anneals to the DNA sequence to be amplified just like the primers do, and when the *Taq* polymerase reaches it while synthesizing the complementary strand, it uses its endogenous 5' nuclease activity to cleave the probe, releasing the FAM dye that can now fluoresce separated from the quencher. With each subsequent PCR cycle, more dye is released, causing an increase in fluorescent intensity that is relatively proportional with the amount of amplicons synthesized. The qPCR instrument can detect this fluorescence and report it during the reaction process. Real-time qPCR can be used in conjunction with reverse transcription, to measure relative expression of a specific RNA by target cells, and compare expression after specific treatment.

Usually 1μl of cDNA mixture (100ng equivalent of RNA) was used per qPCR reaction. This was mixed with RNase-free water, to a total volume of 9μl. Then, 10μl of qPCR master mix (TaqMan® Gene Expression Master Mix 2X; Applied Biosystems, USA or SensiFAST™ Probe Hi-ROX Master Mix; Bioline, UK) were added, containing the DNA polymerase, dNTPs and buffers needed for a PCR reaction. Finally, 1 μl of the specific Taqman Gene expression assay or equivalent was added, containing primers and probe set for the target gene. The mix was transferred to a qPCR 96-well plate, and the reaction took place in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA) instrument. The program used was: first cycle, 2 minutes at 50 °C, then 10 minutes at 95 °C (for activation of enzymes); next forty cycles, 15 seconds at 95 °C (for denaturation of double strands), then 1 minute at 60 °C (for annealing and extention). As endogenous controls to compare gene expression, the house-keeping genes 18S ribosomal RNA or GAPDH were used. In all the experiments a negative

control containing no cDNA template was included, as well as an RT control, which contained RNA that had not been reverse transcribed into cDNA. All samples were run in triplicate, using the same amount of cDNA. The primers used can be seen on table 2.4.

Target gene	TaqMan Gene Expression Assay (or equivalent)	Source
S1PR1	Hs00173499_m1	Applied Biosystems
S1PR3	Hs01019574_m1	Applied Biosystems
S1PR4	Hs02330084_s1	Applied Biosystems
S1PR5	Hs00928195_s1	Applied Biosystems
CD69	Hs00934033_m1	Applied Biosystems
CXCR1	Hs01921207_s1	Applied Biosystems
IL-8	Hs00174103_m1	Applied Biosystems
18S	Hs99999901_s1	Applied Biosystems
GAPDH	Hs02758991_g1	Applied Biosystems
S1PR2	Hs_S1PR2_FAM_1	QIAGEN

Table 2.4. List of primers used. All primers for real-time PCR used were TaqMan Gene Expression Assays (Applied Biosystems) except for S1PR2 that was purchased from QIAGEN.

To analyse the qPCR results, the comparative C_T method was used. The C_T value (or threshold cycle) is defined as the intersection of the threshold line with the amplification plot for each sample. The threshold line is set automatically above the background and within the exponential growth phase of the amplification curve, and is the same for all samples. Data were analysed either using the expression of one of the genes as a reference, for relative expression of genes in one or more types of cells, or, when treated and untreated cells needed to be compared, the untreated cells' gene expression was used as a reference. To calculate fold difference in expression between treated and untreated samples or between genes in the same type of cells, the $2^{-\Delta\Delta C_T}$ format was used, where $\Delta\Delta C_T = \Delta C_{T\ target} - \Delta C_{T\ reference}$ and $\Delta C_T = C_{T\ target} - C_{T\ endogenous\ control}$ and C_T refers to the mean C_T value of the triplicate samples. Analysis was performed in Microsoft Office Excel 2007 (Microsoft).

2.6 Western blotting

Western blotting allows the relative measurement of expression of a target protein in a sample. Cell lysate is denaturated and loaded in SDS-PAGE gel, where the proteins in the sample are separated. The proteins in the gel are then transferred in a PVDF membrane by wet electroblotting. Finally, in order to detect target protein, the membrane is blotted with specific primary antibody followed by secondary antibody conjugated with horseradish peroxidase (HRP) that allows detection of chemiluminescence after addition of appropriate substrate.

2.6.1 Cell lysis and protein quantification

Cells were treated according to experiment, usually washed with ice cold PBS to rapidly stop treatment and pelleted by centrifugation. Cells were then lysed in around 60μl per 5 x 10^6 cells lysis buffer (CelLytic M; Sigma-Aldrich, USA), containing protease inhibitors (cOmplete Protease Inhibitor Cocktail; Roche, Switzerland), and if phosphorylated targets were examined, phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail; Pierce, Thermo Scientific, USA) as well. For complete lysis, cell lysate was incubated for 10 minutes on ice and then sonicated. Centrifugation at high speed was performed to pellet debris and DNA, which are then discarded by transfer of the supernatant to a new tube. Samples were kept on ice until use, or if longer storage was needed, they were kept at -20 °C for a few days.

For total protein quantification, a Bicinchoninic Acid (BCA) assay kit (Pierce, Thermo Scientific, USA) was used. This assay is based on the Biuret reaction, where peptide bonds reduce Cu^{2+} cations to Cu^{1+} when in an alkaline solution. The Cu^{1+} cation then chelates with BCA molecules, forming an intense purple-colored reaction product. The colorimetric absorbance at 562nm can then be measured using a protein standard of known concentrations to calculate sample protein concentration.

Samples or BSA standard concentrations (0, 125, 250, 500, 1000 and 2000 μg/ml BSA) were assayed in duplicate. Usually, 5 μl per sample or standard were added in 200μl BCA reagent mix in a 96-well plate, and incubated for 30 minutes at 37 °C. Absorbance at 562nm was measured using a plate reader, and unknown concentrations were calculated by creating a standard curve fit for BSA concentrations, using linear regression, in Prism 3 (Treestar) software.

2.6.2 SDS-PAGE

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) is a method used to separate protein molecules according to their size in a porous acrylamide matrix. The reducing agent β-mercaptoethanol is used to denature proteins, breaking the disulphide bonds that hold their tertiary structures, allowing the anionic detergent SDS to associate with the polypeptide chain. The number of bound SDS molecules is proportional to the length of the chain, so the negative electrical charge SDS provides is approximately proportional to the protein size. When electrical charge is applied then, the proteins in the sample run through the gel according to their size, towards the anode, with smaller proteins running faster than larger ones.

Usually between 20-40 μg of total protein were loaded per sample, in the wells of a 12% polyacrylamide gel. The gel consists of two parts, a stacking gel and a resolving gel. The first part of the gel is the stacking gel (5% polyacrylamide), with larger pores, where the samples move faster and are stacked so they all enter the resolving gel at the same time. The resolving gel (12% polyacrylamide) is the main part of the gel where the proteins are separated and run according to their size. The recipe for the creation of the two parts of the gel can be seen in table 2.5. Polymerization starts with addition of ammonium persulfate (APS) and is accelerated when N,N,N,N – tetramethylethylenediamine (TEMED) is added. Usually the ingredients for the two parts were mixed beforehand, without addition of TEMED. The resolving gel was prepared first and left to polymerize, with 2-butanol on top to level the gel surface. When polymerization was complete, 2-butanol was removed and washed with deionized water, then the stacking gel was prepared and added on top of the resolving gel, and a comb was placed on top to create the wells. When the stacking gel polymerized, the gel was ready to be used for western blot immediately, or was stored at -4 °C until used.

The samples were mixed 5 parts lysate with one part 6 x loading buffer (300 mM Tris (pH 6.8), 600 mM dithiothreitol, 12 % w/v SDS, 0.6 % bromophenol blue, 60 % glycerol; all Sigma-Aldrich, USA) with 30% β-mercaptoethanol (Sigma-Aldrich, USA) and were heated at 99 °C for 10 minutes, before being loaded in the gel. A protein ladder was also loaded (Prestained Protein Marker, Broad Range; New England Biolabs, UK) in at least one well per gel, so the molecular weights of the target proteins could be determined. The marker contains known molecular weight proteins conjugated with blue or red dye so they are visible in the gel and membrane. A mini vertical

electrophoresis unit (SE260, GE Life Sciences) was used to run the gel, at 30mA constant current for about 1 hour, until marker was well separated and loading front dye almost reached the end of the gel. Electrophoresis buffer used contained 0.025 M Tris, 0.192 M Glycine, and 0.1% (w/v) SDS, with pH 8.3.

12% resolving gel (10ml)	5% stacking gel (6ml)
3.3ml dH_2O	4.1ml dH_2O
4ml 30% (w/v) acrylamide / bisacrylamide mix	1ml 30% (w/v) acrylamide / bisacrylamide mix
2.5ml 1.5M Tris (pH 8.8)	750µl 1M Tris (pH 6.8)
100µl 10% SDS	60µl 10% SDS
100µl 10% APS	60µl 10% APS
4µl TEMED	6µl TEMED

Table 2.5. Recipe for creation of SDS-PAGE.

2.6.3 Electroblotting

After gel electrophoresis was finished, proteins were transferred in PVDF membrane using wet electroblotting. Membrane was cut at appropriate size and activated by immersing in methanol for 10 seconds, then washed twice with deionized water for 5 minutes each and finally in transfer buffer (0.025 M Tris, 0.192 M Glycine, 10 % methanol) for 5 minutes. A transfer "sandwich" was prepared, with sponge, 2 pieces of whattman paper, the gel, PVDF membrane, 2 more pieces of paper and another sponge, all clamped tightly together after ensuring no air bubbles are trapped between the gel and membrane. The sandwich was then placed in a tank transfer unit (TE22, GE Life Sciences) filled with transfer buffer and run at 30V constant voltage overnight, or 250mA constant current for 2 hours when rapid transfer was desired, both with water cooling and stirring.

2.6.4 Immunoblotting

When transfer was over, the membrane was removed from the device and was washed in PBS with 0.1% Tween-20 (PBS-Tween) for 5 minutes on an orbital shaker. To verify transfer was successfully, the membrane was stained with Ponceau-S which is a temporary protein stain that allows visualization of proteins in the membrane. Ponceau

solution was added for 2 minutes and then the membrane was washed with water until the red protein bands were visible. For destaining, membrane was washed with PBS-Tween until the colour had faded sufficiently. Membrane was then blocked with 3-5% BSA in PBS-Tween (blocking buffer), for 1 hour at room temperature on an orbital shaker. Then, membrane was incubated with primary antibody, diluted in blocking buffer, overnight at 4 °C on an orbital shaker.

Membrane was washed 3 times for 5 minutes each in PBS-Tween on an orbital shaker, then incubated with appropriate secondary antibody, HRP conjugated, diluted in blocking buffer, for 1 hour at room temperature, shaking. After another 3 washes with PBS-Tween, chemiluminescence substrate was added (Pierce SuperSignal West Pico Substrate, Thermo Scientific), incubated for 2-5 minutes and signal was detected using film (Kodak) developed in ready-made developer and fixer (Tentenal, Germany).

2.6.5 Stripping and reprobing

The PVDF membrane can be stripped to remove bound primary and secondary antibodies in order to then reprobe it with new antibodies for a different target, usually the loading control antibody. A low pH mild stripping buffer was used, containing 1.5% w/v Glycine, 0.1 % w/v SDS, 1 % Tween-20, with pH 2.2 (all Sigma-Aldrich, USA). The membrane was incubated 3 times for 10 minutes each with stripping buffer, at room temperature on an orbital shaker. Then it was washed 3 times for 5 minutes each with PBS-Tween, and was ready for reprobing. Before adding the new antibodies, blocking has to be repeated, since it was also stripped from the membrane.

2.7 Cellix VenaFlux flow-based adhesion assays

The Cellix VenaFlux platform (Cellix Ltd., Ireland) is an in vitro microfluidics system that can be used to investigate cell interactions and cell adhesion to adhesion molecules or endothelial cell monolayers under flow conditions (figure 2.2). It consists of a nanopump that controls the flow rate, with very low flow rate capabilities of 5 pL/min to 10 μL/min, which can mimic physiological blood flow shear stresses of between 0.05 – 20 dyne/cm^2. The system is attached to a microscope with bright field and fluorescence capabilities, a motorized stage, a cage incubator to control temperature and a camera to capture images of cell adhesion. The whole system is connected to a computer with VenaFlux Software which controls all functions.

There are two types of biochips that were used in this project. The Vena8/Vena8 Fluoro+ biochips (the former have been discontinued and replaced by the latter, with their only difference being that the latter are optimized for fluorescence) comprise of 8 microcapillary channels that are of similar dimensions with *in vivo* blood vessels, which they simulate (figure 2.3). Each channel is 100μm deep, 400μm wide and 28mm long, and it can be coated with recombinant adhesion molecules to examine leukocyte rolling or adhesion under flow. The VenaEC biochip can be used to culture endothelial cells creating a more physiological blood vessel-like channel, where the adhesion of cells on endothelial monolayers can be assessed. It comprises of two parts: a tissue culture treated substrate on which the endothelial cells are grown to confluency before the experiment, and a biochip part that is assembled on top of the substrate to create two microcapillary channels, each 120μm deep, 600μm wide and 20mm long (figure 2.4).

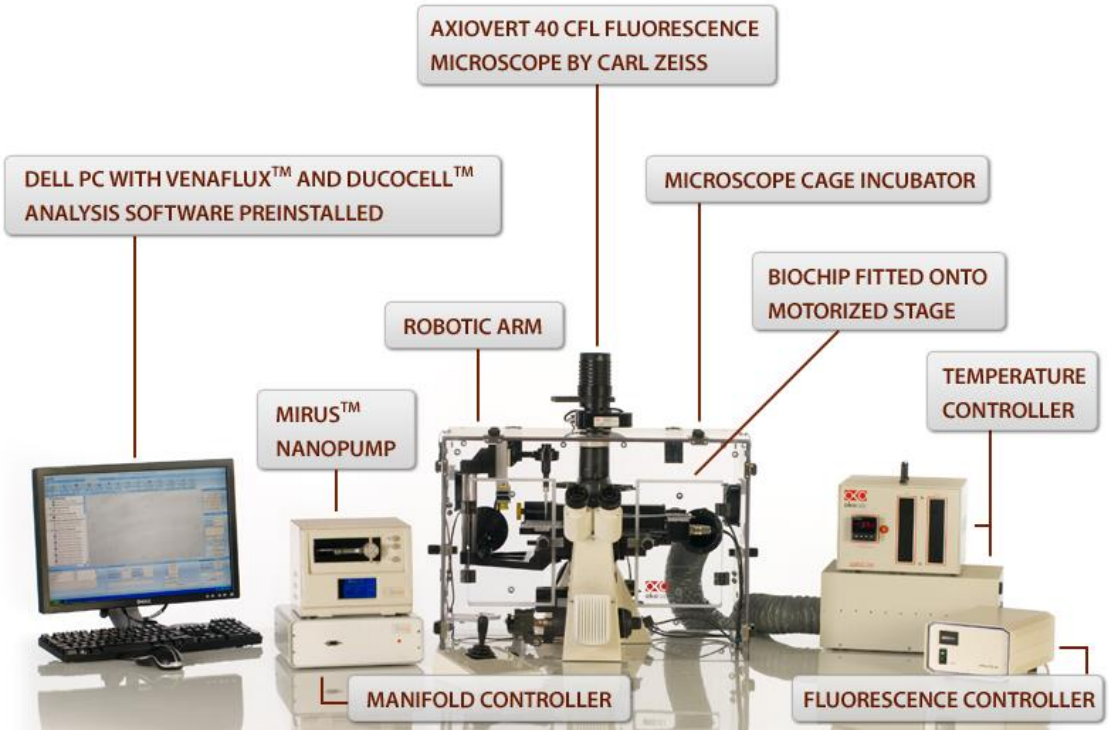


Figure 2.2. Cellix VenaFlux platform configuration.

2.7.1 Adhesion molecule - coated biochips

The channels of Vena8 or Vena8 Fluoro+ biochips were coated with recombinant human ICAM-1 Fc or VCAM-1 Fc (R&D systems) both 10µg/ml diluted in PBS. Around 12µl of ICAM-1 or VCAM-1 were pushed through the channel port with a pipette, making sure liquid could be seen at the other side. The biochip was incubated overnight at 4 °C in a sealed humidified container to prevent it from drying. Before use, the channels were blocked with 1% BSA in PBS for 30 minutes at room temperature, to prevent unspecific adhesion. In some cases, chemokine stimulant was added in the channel instead of with the cells, by adding 12µl of 500ng/ml chemokine (usually IL-8) in the channel and incubating for 30 minutes – 2 hours before the blocking step.

Differentiated HL60 or primary human neutrophils were resuspended in serum-free RPMI-1640 media at a density of 1 x 10^6 cells/ml and incubated for 30 minutes – 2 hours. If needed, they were treated with 1µM S1P for 1 hour in serum free media. Instrument was switched on and initialized, by running setup step in VenaFlux software, then adjusting camera image. The system was washed using pump washout step, first with sterile deionized water, then with 70% ethanol twice and finally water again, making sure no air bubbles were trapped in the plumbing. A final pump washout was

made with prewarmed RPMI-1640 basal media, which was also the flow medium, to prime the system. The biochip was then mounted on the microscope stage, and the channel to be observed was washed with 40µl medium at 40 dynes/cm^2 using chip washout step. Excess media at the end of the channel was removed and 100µl of cell suspension (100,000 cells) were added in its place. For stimulation of cells with chemokine, S1P or $MgCl_2$, appropriate amount of stimulant was added in the cell suspension just before addition in the channel. Flow was initiated at a shear stress of 10 dyne/cm^2 for 10 seconds to diffuse the cells into the channel, then continued at 0.5 dyne/cm^2 for 5 minutes to assess adhesion. At the last minute images were captured, at 4-8 different fields of view along the length of the channel. This procedure was repeated for all the channels of the biochip used in the experiment. For each image captured, cells that had adhered were counted and analysed using Prism3 software (GraphPad Software Inc).

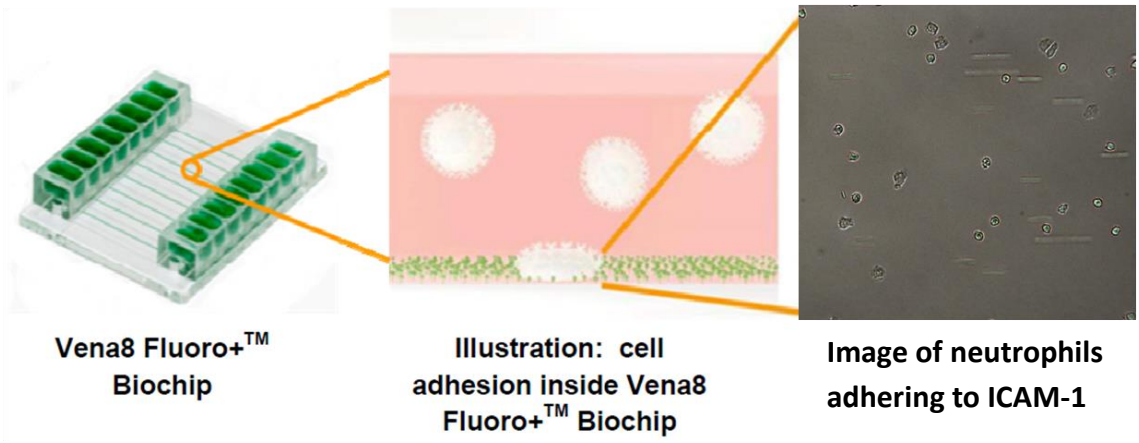


Figure 2.3. Vena8 Fluoro+ Biochip. The Vena8 Fluoro+ biochip has 8 channels that can be coated with adhesion molecules to investigate cell adhesion.

2.7.2 Endothelial cells - coated biochips

VenaEC substrates were placed in the wells of a 6-well plate, treated side up, and were sterilized with UV light for 30 minutes before use. Sometimes, they were coated with 20ng/ml fibronectin for 1 hour at room temperature, to allow endothelial cells to adhere more firmly. HMEC-1 or HUVEC cells were then subcultured on the substrates, at 37 °C, 5% CO_2, until they reached confluency. They were treated overnight in serum-free media with 10µM S1P or 100ng/ml TNF-α or both.

Isolated human neutrophils were stained with CFSE before being used in the Cellix assay, to distinguish them from the endothelial cells by fluorescence. Neutrophils were resuspended in PBS at a density of 5-10 x 10^6 cells/ml and CFSE was added at a final concentration of 20 μM. Cells were incubated for 10 minutes at room temperature in the dark and then cold complete RPMI-1640 media was added to stop labelling. Neutrophils were washed by centrifugation at 300x g for 5 minutes and resuspended in serum-free media at a density of about 2 x 10^6 cells/ml, to be used on the Cellix.

The instrument was initiated as was described above for adhesion molecule coated biochips. The fluorescence bulb was switched on as well, to take fluorescent images of CFSE labelled neutrophils. VenaEC biochip was assembled by clamping together with substrate and was mounted at microscope stage. The channel was washed as above, although care had to be taken not to disrupt the endothelial monolayer (lower dynes were tried first to make sure the layer was stable enough). Neutrophil suspension was added in 100μl (around 200,000 cells) and flow was initiated as described above. Images were captured at both bright field and fluorescent mode for each field of view, for a total of 4-9 fields along the length of the channel. This was repeated for the second channel of the biochip and then substrate was replaced with another one and the whole process was repeated. Adhered neutrophils were counted in fluorescent images, using the bright field images for verification, and data were analysed using Prism3 (Graphpad).

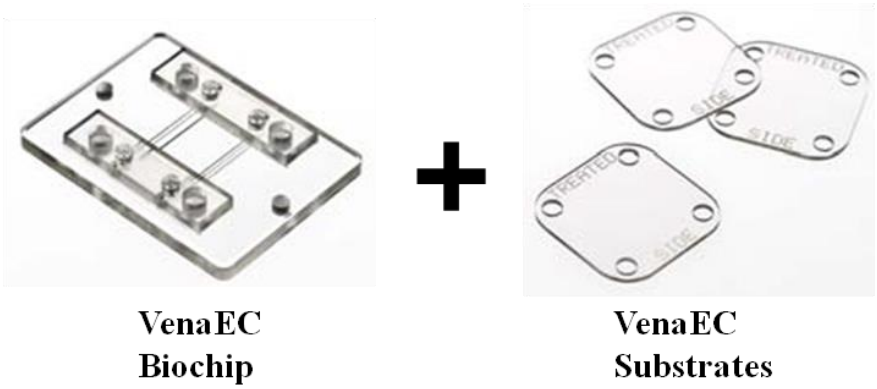


Figure 2.4. Vena EC Biochip. Endothelial cells are grown on the VenaEC substrates. The VenaEC biochip is then assembled on top of the substrate to create the microcapillaries.

2.8 Statistical analysis

Data were graphed and analysed using Prism3 software (GraphPad Software Inc) or Microsoft Office Excel 2007 (Microsoft, USA). For comparison between two groups, the two-tailed unpaired student's t-test was used, with significant differences when $p<0.05$ (*), highly significant when $p<0.01$ (**), and extremely significant when $p<0.001$ (***). For comparison between many groups, one-way analysis of variance (ANOVA) was used. Flow cytometry data were analysed using FlowJo 7.6 software (Treestar). Image analysis was made using ImageJ software. PCR data were analysed using Microsoft Office Excel 2007 (Microsoft, USA).

CHAPTER 3

HL60 cell line as a neutrophil model

Chapter 3. HL60 cell line as a neutrophil model

3.1 Introduction

The first "immortal" cell line was established in 1951, when human tumor cells from a case of cervical cancer were isolated and cultured in the lab to create the HeLa cell line (Scherer *et al.*, 1953; Lucey *et al.*, 2009). Since then, cell lines are routinely used not only to investigate diseases but also to imitate the "normal" cell types they are derived from. It is not always easy for a scientist to use *ex vivo* primary cells for his studies. These cells are hard or even impossible to maintain in culture, they are usually non-proliferating terminally differentiated cells that will not survive in culture without using very specialized, often expensive, culture media with added nutrients that is complicated to create. Even if they can survive, they cannot divide and need to be used immediately for experiments since they will not last more than a few days at most. There are some exceptions to this, like the HUVEC cells that can survive for 5-7 passages, but they will still undergo apoptosis afterwards. The procedures utilized to obtain *ex vivo* cells are usually quite complicated and time consuming, and the fact that the cells have to be used soon after their isolation makes their routine handling in the lab quite challenging. The fact that these procedures will have to be repeated every time a new experiment needs to be set up complicates things even more.

For all the above reasons, scientists often rely on cell lines to perform their experiments. These might be cancerous forms of the type of cell they are interested in, or immortalized counterparts created *in vitro*. The main advantage of a cell line is its immortality, allowing them to be grown almost indefinitely in culture and stored for future use. Another important advantage, however, is the fact that all of the cells are identical, derived from the same cell precursor, from the same donor, allowing for more controlled, easily repeatable experiments, whereas *ex vivo* cells derived from different donors can vary significantly. On the other hand though, immortalization may have caused changes to the cells, making them less comparable to their primary counterparts. It is important, therefore, for a scientist that decides to use a cell line, to take this into consideration, and if possible investigate the differences between the cell line and the primary cells.

In this study, neutrophils were the main cells of interest. Isolation of primary human neutrophils, however, is a relatively long, time consuming procedure (see chapter 2).

Moreover, neutrophils are very short lived cells, surviving only for a few hours *ex vivo*, meaning they have to be used the same day they were isolated. Furthermore, neutrophils derived from multiple donors may have significant differences in how they behave during assays and what they express. For these reasons, the use of a cell line as a neutrophil model was investigated. The cell line chosen was the HL60 cells, of human promyelocytic leukaemia origin, which have the capacity to differentiate into neutrophil-like cells under the appropriate conditions. HL60 differentiation was characterized and the expression of different markers and S1P receptors was examined. Moreover, their chemotactic responsiveness to IL-8 and their adhesive capabilities under flow conditions were investigated.

3.2 Materials and Methods

3.2.1 HL60 cell culture and differentiation

HL60 is a human cell line of promyelocyte origin that was established from a 36-year old woman with acute promyelocytic leukaemia (Collins *et al.*, 1977)(see chapter 2.1). HL60 can be induced to differentiate into granulocyte-like cells or to monocyte/macrophage-like cells, depending on the compound used to stimulate differentiation. In this study they were differentiated into granulocyte-like cells and were used as a neutrophil model.

The cells were routinely cultured in pre-warmed complete medium which comprised of RPMI 1640 medium supplemented with 10 % foetal bovine serum (FBS), 2 mM L-Glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all Sigma-Aldrich). They were seeded to a density of 3-5 x 10^5 cells/ml and incubated at 37 °C with 5% CO_2. They were passed every 3-4 days into fresh medium.

For differentiation, about 1.25 x 10^6 cells were transferred into a 25cm^2 flask, with complete RPMI medium supplemented with 1.25% DMSO which induces differentiation (differentiation medium). When these cells were split, they were re-cultured in fresh differentiation medium. In some experiments, differentiated or undifferentiated HL60 were incubated overnight in serum - free RPMI 1640 medium with or without 1.25% DMSO respectively. Serum - free RPMI 1640 medium was the same as the complete medium except FBS was replaced by 0.5% fatty acid-free BSA. Differentiated cells were used 4-6 days after DMSO addition, unless otherwise specified.

3.2.2 Immunocytochemistry

With immunocytochemistry, a cell sample can be fluorescently labelled with antibodies that detect a protein of interest, then visualised using fluorescent or confocal microscopy. Cells need to be cytospun onto a slide and stained with the appropriate primary and secondary antibodies to detect expression by fluorescence. A DNA dye is usually used as well in order to stain the nucleus of the cell.

Cells were washed with PBS + 1% BSA and resuspended in a density of about 5 x 10^5 cells/ml. Then, 200µl of cell suspension (100,000 cells) were cytospun at 1000rpm for 3 minutes onto glass slides, then left to air dry. The cells were fixed by immersing the

slides into ice cold methanol and leaving them for 10 minutes at -20 °C. After drying, the cell section was circumscribed with a hydrophobic barrier pen. The cells were blocked with PBS + 1% BSA for 1 hour and then stained overnight with a primary antibody diluted accordingly in PBS + 1% BSA, in a humidified atmosphere, at 4 °C. The next day the cells were washed twice with PBS + 1% BSA for 5 minutes. Then an appropriate fluorescently conjugated secondary antibody was added, diluted in PBS + 1% BSA. The cells were stained with the secondary antibody for 3 hours, in the dark, at room temperature, and then washed 3 times with PBS for 5 minutes each. Finally, DAPI, a DNA dye, diluted 1μM in PBS was added and incubated for 2 minutes, then washed once more with PBS. The slides were mounted with coverslip using a fluorescent mounting medium (Dako). They were stored at 4 °C in the dark until observed with a confocal microscope.

3.2.3 RT - qPCR

RNA was isolated with the TRI Reagent (Sigma-Aldrich) method (see chapter 2.5.1). Reverse transcription for production of cDNA was performed using Agilent's AffinityScript Multiple Temperature cDNA synthesis kit (chapter 2.5.2). The cDNA was then used for detection of gene expression by real time PCR, using TaqMan® Gene Expression assays (Applied Biosystems) as primers (see chapter 2.5 for full list), with the TaqMan® Gene Expression Master Mix 2X (Applied Biosystems) in an Applied Biosystems qPCR machine. The house-keeping gene *18S* was used as the reference gene. Data were analysed using the $\Delta\Delta C_T$ method (chapter 2.5.3).

3.2.4 HL60 chemotaxis

In chemotaxis assays, cells pass through a porous membrane into a bottom chamber containing a chemoattractant molecule (see chapter 2.4). For HL60 chemotaxis, the chemokine IL-8, important for neutrophil migration was used as the chemoattractant. Differentiated or undifferentiated HL60 cells were incubated in serum-free media for 2 hours or overnight, and were then added on top of a 3μm membrane (BD Biosciences), usually $1x10^5$ cells per well, and left to migrate towards 50ng/ml IL-8 in serum-free RPMI 1640 media for 2 hours. Cells that had passed in the bottom chamber were measured by flow cytometry using counting beads.

3.2.5 Cellix *VenaFlux* flow-based adhesion assays

Channels on Vena8 biochips were coated with 10µg/ml VCAM-1 Fc or ICAM-1 Fc overnight (see chapter 2.7). Before using them in experiments, they were blocked with 1% BSA for 30 minutes. Cellix device and *VenaFlux* software were switched on and initialised. After washing the pump connections, biochip was mounted on the microscope stage and the channel to be used was briefly washed. Differentiated HL60 cell suspension in medium was added in one end of the channel, with or without chemokine or other stimulant and flow was initiated (0.5 dyne/cm^2). Several (4-8) representative fields of view were captured along the channel's length. This was repeated for all the channels used in the experiment. For each image captured, adherent cells were measured and results analysed using Prism3 software (GraphPad Software Inc).

3.3 Results

3.3.1 HL60 characterization and differentiation

HL60 are normally promyelocytic cells, so in order to be used as a neutrophil model they need to be differentiated into granulocyte-like cells. The compound used for this purpose was DMSO, at 1.25%, and their differentiation was investigated. Morphologically, after 4-5 days in DMSO cells became notably smaller in size, and their proliferation rate decreased gradually, until they finally appeared to have become senescent (data not shown).

HL60 cells were incubated for 3-9 days with differentiation medium (RPMI-1640 complete with 1.25% DMSO) and then characterised by flow cytometry. They were stained for CD45, CD11b and CD69 surface expression at different time points and the mean fluorescence intensity (MFI) was measured (figure 3.1). CD45 was expressed at high levels even by undifferentiated cells, but its expression increased after DMSO incubation, with a peak expression at 5-7 days of differentiation (figure 3.1A). CD11b is an early marker of HL60 differentiation. It was not expressed at all by undifferentiated cells, but started being produced even after only 3 days of DMSO incubation, peaking at 7 days of differentiation (figure 3.1B). On the other hand, CD69 expression by HL60 appeared to be variable. It started high in undifferentiated cells and was decreasing after differentiation, although at 5-7 days of DMSO incubation it increased again slightly (figure 3.1C). This is in contrast with primary neutrophil CD69 expression, which is non-existent on resting neutrophils cell surface, but can be induced by various compounds (Atzeni *et al.*, 2002).

Next, the cells were stained for S1PR1 and S1PR3, as well as the CXCL8 chemokine receptor CXCR1, normally expressed by primary neutrophils. In order to avoid decreased surface expression due to internalization of the receptors after binding to their ligand, the cells were incubated overnight in serum-free medium. Undifferentiated HL60 cells as well as cells induced with DMSO for 6 days were used. CXCR1 and S1PR1 were not expressed on HL60 cells' surface, even after their differentiation (figure 3.2A-D). On the other hand, S1PR3 was expressed at a relatively high percentage of undifferentiated cells, but was downregulated after differentiation (figure 3.2E-F).

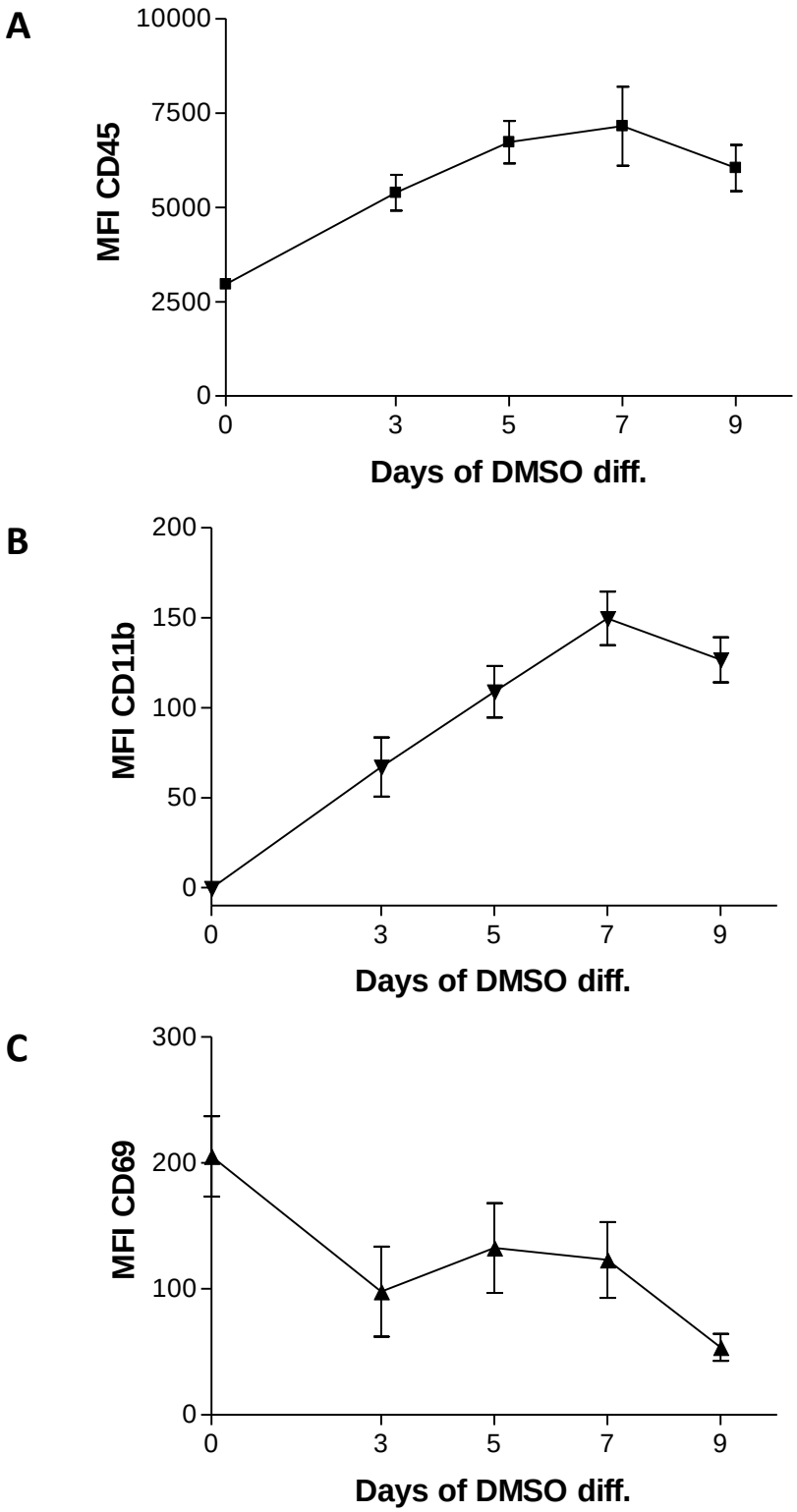


Figure 3.1. Characterisation of HL60 differentiation by DMSO. HL60 cells were incubated for 0, 3, 5, 7, and 9 days with 1.25% DMSO, then stained for different cell surface antigens and analysed by flow cytometry. (A-C) The mean fluorescence intensity (MFI) of CD45, CD11b and CD69 respectively, normalized for the appropriate isotype control, in accordance with days of differentiation. The data represent means ± SEM from three independent experiments.

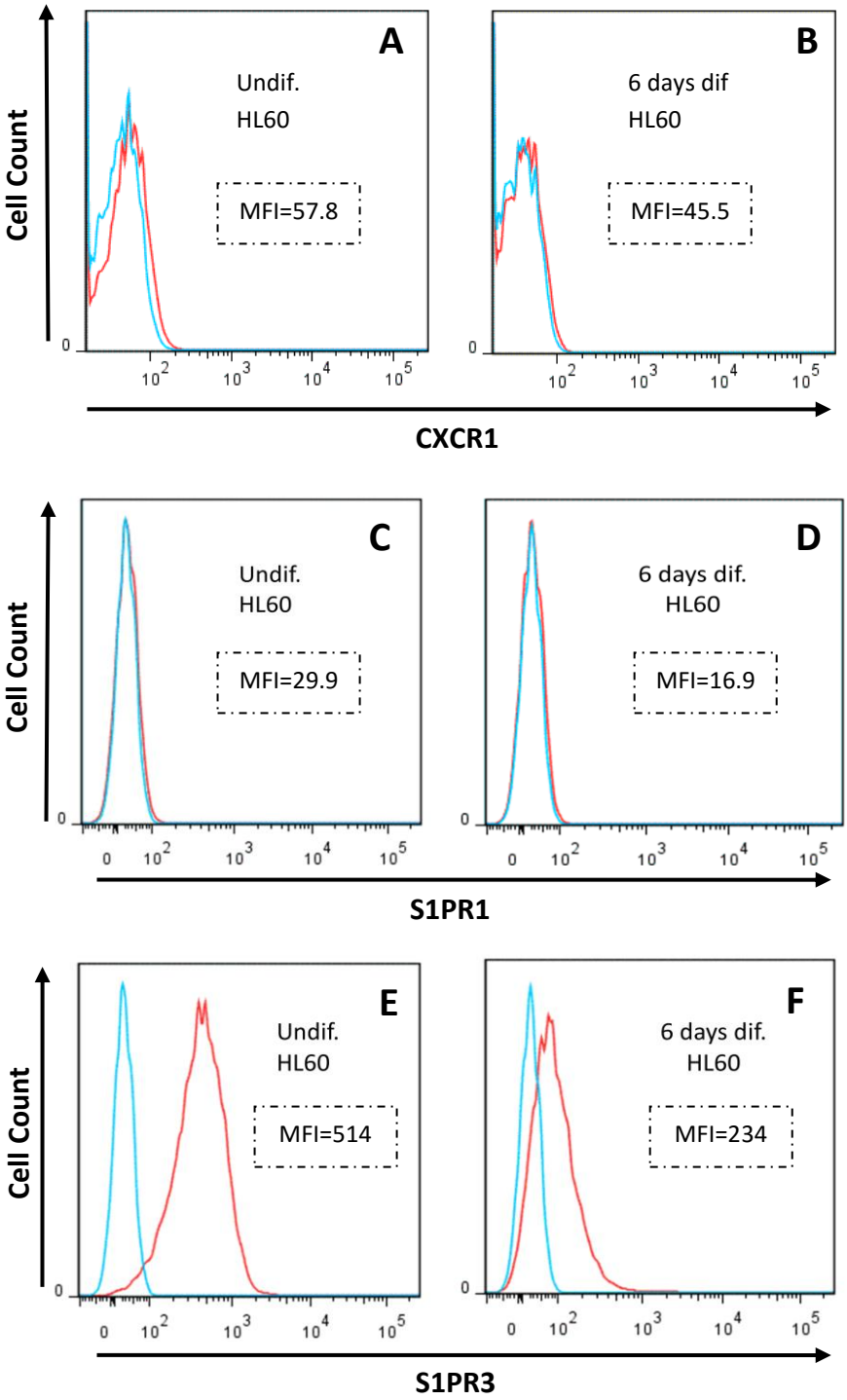


Figure 3.2. HL60 expression of chemokine and S1P receptors before and after differentiation. HL60 cells were incubated for 6 days with 1.25% DMSO for differentiation. Differentiated or not cells were incubated overnight in serum-free medium, and then stained with fluorescently - labelled monoclonal antibodies (red) or appropriate isotype control antibodies (blue). (A-B) CXCR1 expression in undifferentiated and differentiated HL60 cells respectively. (C-D) S1PR1 expression in undifferentiated and differentiated HL60 cells respectively. (E-F) S1PR3 expression in undifferentiated and differentiated HL60 cells respectively. Data are representative of 4 independent experiments. MFI: mean fluorescence intensity

The expression of the S1P receptors S1PR1 and S1PR3 was also measured using immunofluorescent staining of differentiated or undifferentiated HL60 cells. Confocal images from cells stained for S1PR1 show some S1PR1 expression in the cytoplasm and granules close to the nucleus (figure 3.3A), with decreased intensity of stain after differentiation (figure 3.3B). S1PR3 on the other hand, shows low intensity staining compared with S1PR1 (figure 3.3C), with even further decrease after differentiation (figure 3.3D). It is curious that we see S1PR1 stain with this technique, whereas there was none shown by flow cytometry, but this could be due to low surface expression, not enough to be detected by the antibody, or a fault with the immunofluorescense antibody detecting different targets.

To further investigate the S1P receptors expression by HL60 cells, their mRNA expression was measured using real time quantitative PCR. Total RNA was isolated from undifferentiated and differentiated HL60 and used in qPCR after reverse transcription into cDNA. The housekeeping gene *18S* was used for normalisation and the mRNA expression of *S1PR1, S1PR3, S1PR4, S1PR5, CD69* and *CXCR1* was calculated. Looking at the relative expression of these genes in undifferentiated cells, *CD69* was the most abundantly expressed, with *S1PR4* second and *S1PR3* following (figure 3.4A). *S1PR5* and *CXCR1* were barely expressed, with *S1PR5* expression slightly exceeding that of *CXCR1*, whereas *S1PR1* could not be detected at all by the qPCR instrument (figure 3.4A). After differentiation, HL60 cells greatly downregulated *S1PR3* expression, though *CD69* was expressed in only slightly lower levels than before differentiation (figure 3.4B). On the other hand, there was a 3-fold increase in *S1PR4* expression, whereas *S1PR5* and *CXCR1*, barely detected before, were upregulated 100 and 40 times respectively (figure 3.4B). *S1PR1* was still not detected even after differentiation (data not shown).

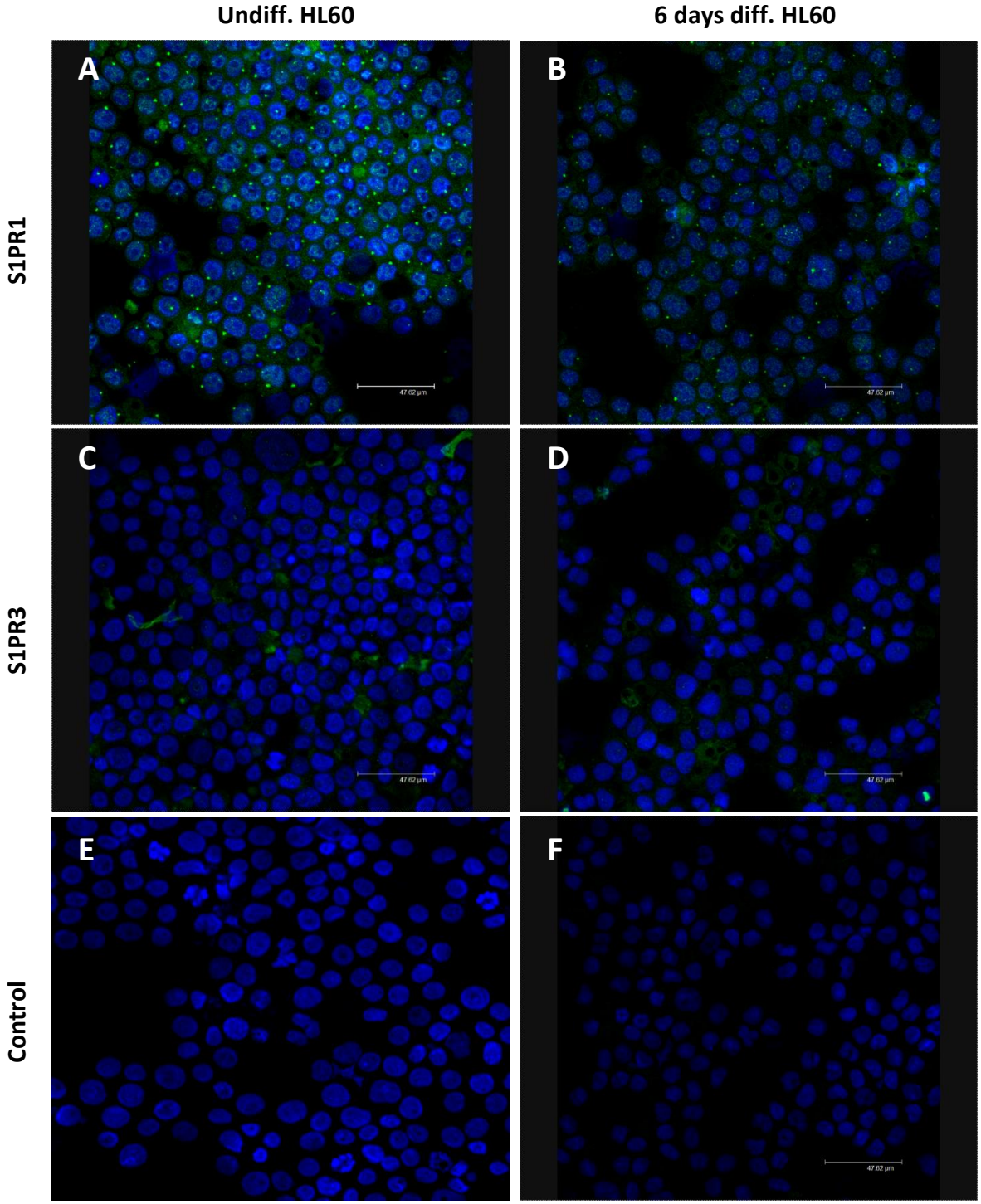


Figure 3.3. Immunofluorescent expression of S1PR1 and S1PR3 in undifferentiated and DMSO differentiated HL60 cells. Undifferentiated HL60 (A, C, E) and 6 days differentiated HL60 cells (B, D, F) were incubated overnight in serum-free conditions. Then they were cytospun onto slides, fixed, and stained with primary polyclonal antibodies for S1PR1 (A, B) and S1PR3 (C, D), and appropriate secondary antibodies, or secondary only as controls (E, F). DAPI was also used to stain the nucleus (genomic DNA). They were observed in a confocal microscope. Pictures are representative of two independent experiments. Green: antibody, blue: DAPI. Scale bars: 47.62μm

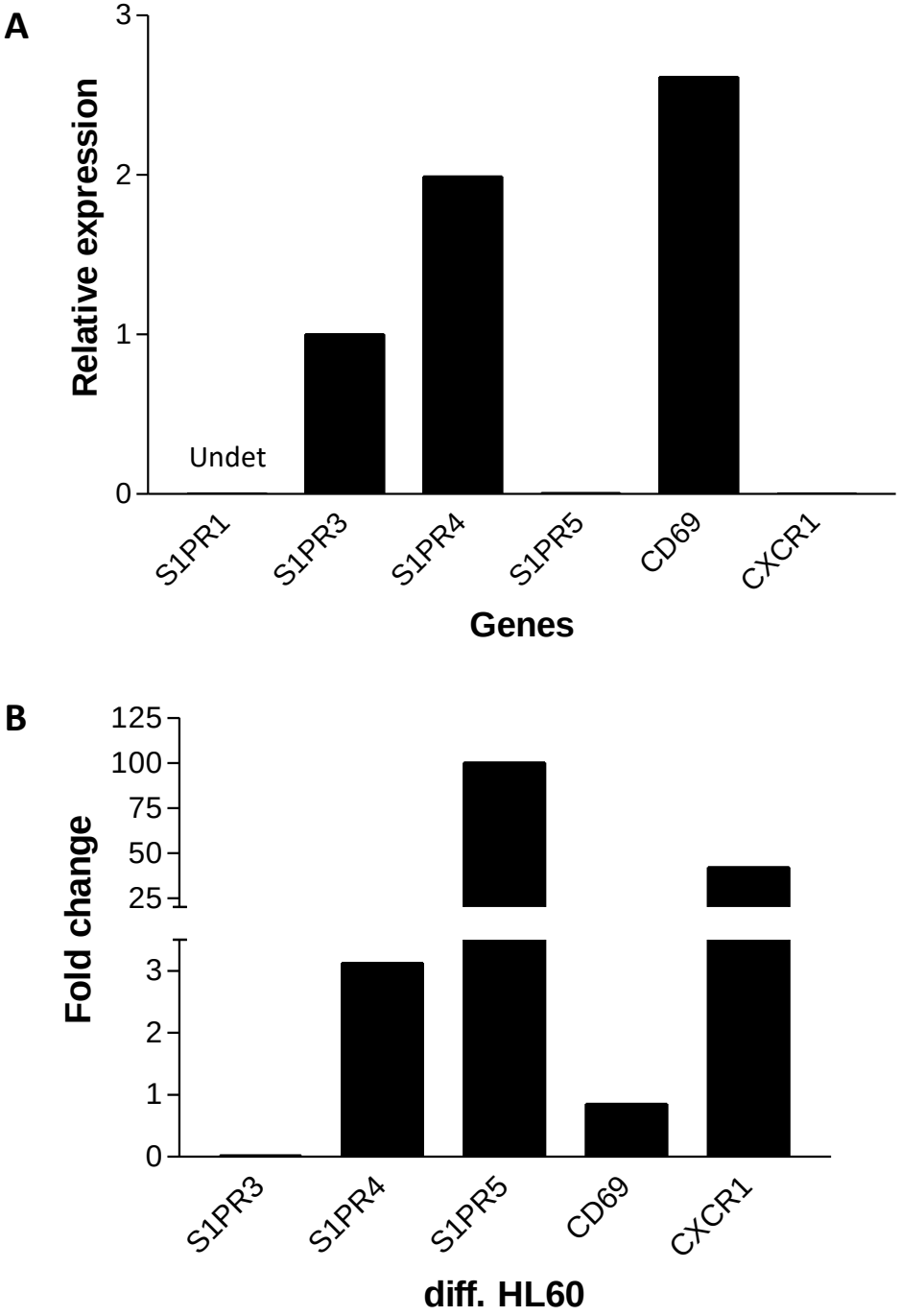


Figure 3.4. mRNA expression of S1P receptors, CD69 and CXCR1 by undifferentiated and differentiated HL60 cells. mRNA expression was measured by real time PCR. Data are normalized for the housekeeping gene 18S. (A) Relative expression of *S1PR1, S1PR3, S1PR4, S1PR5, CD69* and *CXCR1* mRNAs by HL60 cells, using *S1PR3* expression as a reference. (B) Fold change of *S1PR3, S1PR4, S1PR5, CD69* and *CXCR1* mRNA expression after differentiation of HL60 cells, using undifferentiated cells' expression as a reference. Undet: undetected mRNA expression

3.3.2 HL60 chemotaxis

To further investigate the usefulness of HL60 as a neutrophil model for this project, their ability to migrate towards the important neutrophil chemoattractant CXCL8 (IL-8) was assessed. However, it was already implied that since the important IL-8 receptor CXCR1 is not expressed in undifferentiated HL60, there would be no migration towards IL-8 observed, as was indeed the case (data not shown). Differentiated HL60 appeared to, at least, express the mRNA for CXCR1, but still when their migration towards IL-8 was measured, it was not significant over vehicle (figure 3.5).

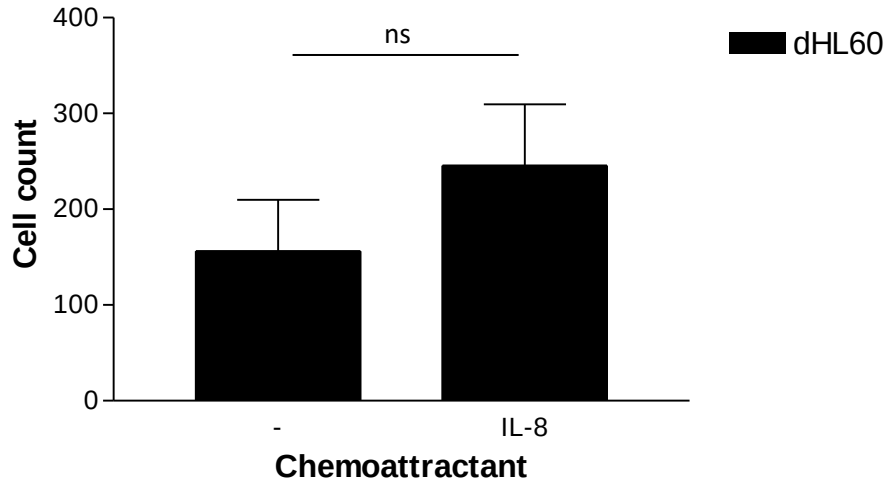
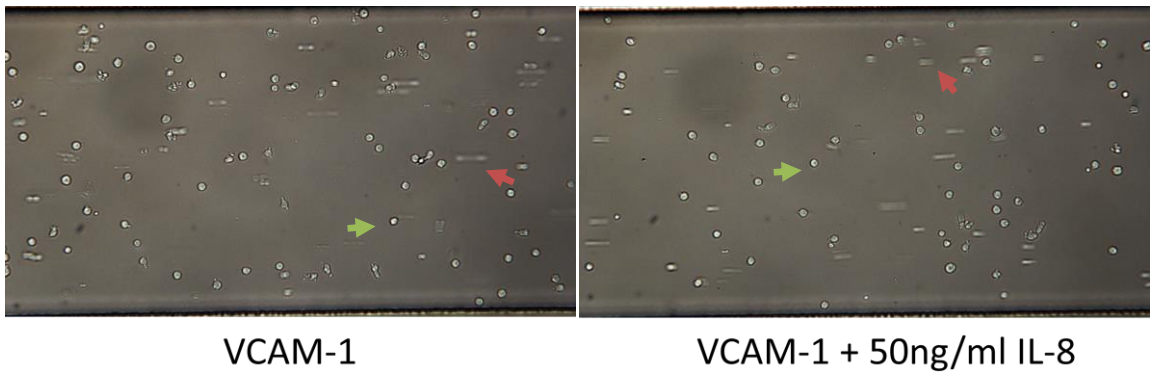


Figure 3.5. IL-8 chemotaxis of differentiated HL60 cells. About $1x10^5$ differentiated HL60 cells were left to migrate for 2 hours towards 50ng/ml IL-8 or vehicle (-). The cells that passed through the filter into the wells were measured by flow cytometry using counting beads. Data are representative of two independent experiments. ns $p \geq 0.05$

3.3.3 HL60 flow-based adhesion assays

In order to investigate HL60 adhesion to the endothelium, another aspect that is important for this project, the Cellix *VenaFlux* system was used. This system can be used to simulate blood flow, allowing for flow-based adhesion assays, which are more physiologically relevant. The channels were coated with VCAM-1 or ICAM-1 adhesion molecules instead of endothelial cells, to create a more controlled assay. In resting state, without any stimulation, the β_2-integrins on neutrophil surface bind to these molecules with low affinity. Stimulation with molecules such as the chemokine IL-8 though, changes integrin conformation, increasing its affinity for the adhesion molecules. Mn^{2+}can also activate integrins unspecifically, so $MnCl_2$ can be used as a positive control. Differentiated neutrophil-like HL60 cells (dHL60) were used in these assays, after 5 days DMSO treatment. Differentiated HL60 adhered to VCAM-1 quite well, even without any stimulation (figure 3.6). When they were stimulated with IL-8, S1P or $MnCl_2$, there was no significant difference in adhesion (figure 3.6). On the other hand, dHL60 did not adhere to ICAM-1 at all, and stimulation with IL-8 could not change that (figure 3.7). Stimulation with $MnCl_2$ caused some adhesion (figure 3.7), but very low compared with VCAM-1 adhesion (figure 3.6).



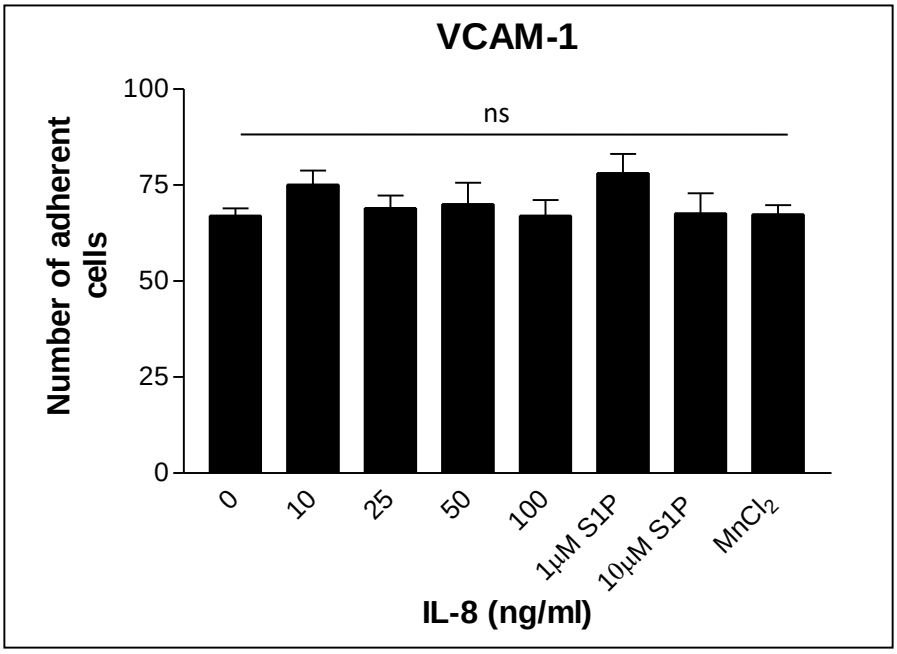
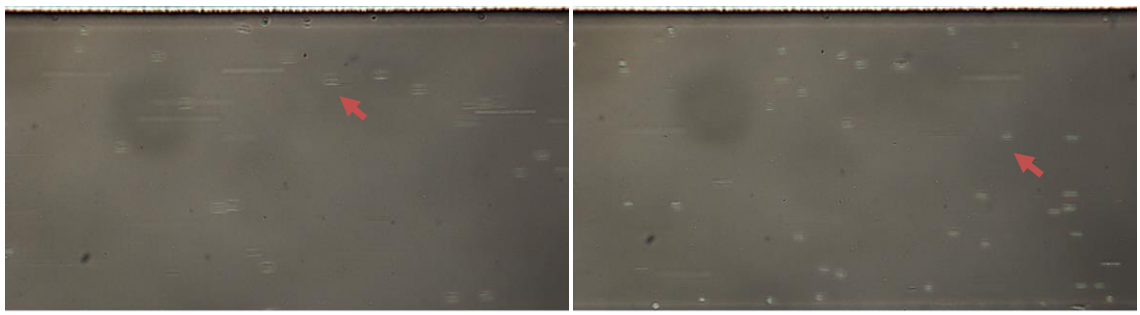


Figure 3.6. Flow-based adhesion assay to VCAM-1 with dHL60. Upper: Capture images from flow-based assay with dHL60 in VCAM-1 coated channels, untreated or treated with 50ng/ml IL-8. Clearly defined cells are adherent (green arrow), blurred cells are flowing (red arrow). Bottom: Differentiated HL60 were treated with different concentrations of IL-8, S1P, or 1mM $MnCl_2$ as a positive control, and used in VCAM-1 coated channels. Bars show means ± SEM from 5-7 captured fields of view. Data are representative of 4 independent experiments. ns $p>0.05$



ICAM-1

ICAM-1 + 50ng/ml IL-8

ICAM-1 + MnCl2

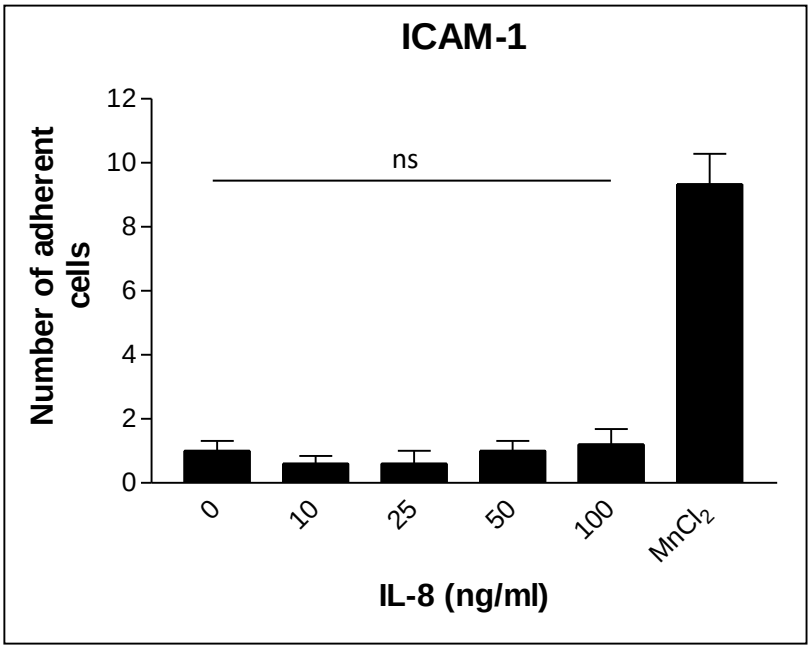


Figure 3.7. Flow-based adhesion assay to ICAM-1 with dHL60. Upper and middle: Capture images from flow-based assay with dHL60 in ICAM-1 coated channels, untreated or treated with 50ng/ml IL-8 or 1mM $MnCl_2$. Clearly defined cells are adherent (green arrow), blurred cells are flowing (red arrow). Bottom: Differentiated HL60 were treated with different concentrations of IL-8, or 1mM $MnCl_2$ as a positive control, and used in ICAM-1 coated channels. Bars show means ± SEM from 5-7 captured fields of view. Data are representative of 2 independent experiments. ns $p>0.05$

3.4 Discussion

HL60 cells have the capacity to differentiate into granulocyte-like cells, making them appropriate to be used as a neutrophil model. However, as with all cell lines, they can be different than the primary cells in many ways, so it is important to investigate before using them in a study. Depending on what one is planning to use them at, they can prove useful for one project but not so useful for another.

HL60 differentiate after a few days in DMSO into cells that look like neutrophils, and have other neutrophil-like qualities, like decreased survivability (Collins, 1987). Like ageing mature neutrophils, terminally differentiated HL60 start to show signs of programmed cell death or apoptosis (Martin *et al.*, 1990). HL60 express CD45 in higher levels after differentiation, which agrees with what Boss et al. had found (Boss *et al.*, 1980). Mature neutrophils also have increased CD45 expression compared to their precursors (Lacombe *et al.*, 1997). CD11b, an important neutrophil adhesion molecule (see chapter 1), is an early differentiation marker for HL60, and they start expressing it immediately after differentiation is initiated, as has been shown before (Hickstein *et al.*, 1987). On the other hand, they express high levels of CD69, which is not normally expressed by neutrophils unless induced (Atzeni *et al.*, 2002).

Notably, HL60 do not express any surface CXCR1, an important neutrophil chemokine receptor. However, there is some CXCR1 mRNA expression detected after HL60 differentiation, which agrees with previous findings (Hauert *et al.*, 2002). This was not enough though to induce chemotaxis towards IL-8 on differentiated HL60 cells, although primary neutrophils are highly chemotactic towards this chemokine. Different studies are in disagreement on whether dHL60 can migrate towards neutrophil chemoattractants, such as IL-8 and fMLP; even when they appear to be chemotactic though, their migration is much lower compared with primary neutrophils (Fontana *et al.*, 1980; Niedel *et al.*, 1980; Sirak *et al.*, 1990; Hauert *et al.*, 2002). There are also reports of CXCR4 expression and CXCL12 (SDF-1) induced chemotaxis in granulocyte-like but not monocyte-like differentiated HL60 cells (Gupta *et al.*, 1999; Gupta *et al.*, 2001).

For this project, the expression of S1P receptors by HL60 was important. Neither differentiated nor undifferentiated HL60 express S1PR1, which according to Rahaman and colleagues is one of the major S1P receptors expressed by primary neutrophils

(Rahaman *et al.*, 2006). This was confirmed by the current study, which showed that neutrophils mainly express S1PR1 and S1PR4 (see chapter 4). HL60 do express S1PR4, but this might not be enough to show if S1P has an effect on neutrophils, by using HL60 as a model. Furthermore, HL60 express S1PR3 before differentiation, but it is highly downregulated after their differentiation into neutrophil-like cells. Primary neutrophils do not appear to express S1PR3 either (chapter 4; Rahaman *et al.*, 2006). In agreement with our findings, Sato et al. showed that S1PR3 mRNA expression is downregulated after HL60 differentiation, whereas S1PR1 and S1PR2 are not expressed at all before or after differentiation (Sato *et al.*, 1998). S1PR1 is one of the major S1P receptors and has been implicated with cell trafficking for many different types of immune cells (see chapter 1), so its absence from HL60 cells would be a major drawback in their usefulness as a neutrophil model in this project. On the other hand, it has been shown that exogenous S1P causes calcium mobilization through phospholipase C activation in HL60 cell line, indicating there is some S1P signalling in these cells (Okajima *et al.*, 1996).

Another neutrophil function that was important for this study is neutrophil adhesion. Differentiated HL60 showed a different adhesion pattern than expected, when used in flow-based adhesion assays. Neutrophils would be expected to adhere mainly to ICAM-1, through their β_2 integrin CD11b/CD18. However, differentiated HL60 do not adhere to ICAM-1 even when stimulated by IL-8 although they express CD11b. This might mean they lack CD18 expression, or their integrin might not be activated, resulting to low affinity for ICAM-1. Since we already know their IL-8 signaling is impaired, this could be the reason IL-8 stimulation does not have an effect. Generally, what one would expect for adhesion to VCAM-1 and ICAM-1, would be to be low without any stimulation and increased after stimulation. However, dHL60 greatly adhere to VCAM-1 without stimulation, with no significant effect after stimulation, whereas they do not adhere at all to ICAM-1, and stimulation does not change that significantly.

In conclusion, it appears that HL60 are not a good enough model for the purpose of this project. IL-8 signalling is important for neutrophil chemotaxis and other related functions such as adhesion, but it seems to be impaired in HL60. Moreover, their S1P receptor expression profile appears to be different than primary neutrophils, with the important neutrophil S1P receptor S1PR1 missing completely from HL60 cells. Finally, their adhesion to ICAM-1 and VCAM-1 seems to be abnormal, not allowing for investigation of normal effects of S1P on neutrophil adhesion. In many ways dHL60 are

very similar to neutrophils, and they might be useful as a neutrophil model for a different study, but they were not appropriate to be used in this study.

CHAPTER 4

Direct effects of S1P on neutrophils

Chapter 4. Direct effects of S1P on neutrophils

4.1 Introduction

Neutrophils or PMN are major mediators of inflammation and are involved in many pathological inflammatory conditions such as ischemia-reperfusion injury. There are indications that S1P can affect neutrophil migration into tissue and other functions of neutrophils such as the respiratory burst and degranulation (Rahaman *et al.*, 2006; Hao *et al.*, 2014).

Neutrophils appear to express transcripts of the S1P receptors S1PR1, S1PR4 and S1PR5, though in pneumonia S1PR3 is also expressed (Rahaman *et al.*, 2006). There are reports that S1P can have inhibitory effects on neutrophil migration towards IL-8 and fMLP *in vitro* (Kawa *et al.*, 1997; Rahaman *et al.*, 2006). A cross-talk between S1PR4 or S1PR3 and CXCR1, an IL-8 receptor, might be partly responsible for this effect (Rahaman *et al.*, 2006). Moreover, S1P lyase deficiency, which results in increased S1P levels, causes impaired neutrophil trafficking in mice, through an S1PR4 dependent mechanism, another indication that S1P might affect neutrophils (Allende *et al.*, 2011). The same effect on neutrophil trafficking is observed when animals are treated with S1P to attenuate myocardial ischemia-reperfusion injury (Theilmeier *et al.*, 2006), or ischemia-reperfusion injury in the lung after transplantation (Okazaki *et al.*, 2007). These *in vivo* effects though could be either direct or indirect, with S1P acting on neutrophils directly, or through a cascade of effects to other cells.

On the other hand, S1P has been shown to act through S1PR1 to cause or intensify hyperalgesia in mice in a neutrophil-dependent mechanism (Finley *et al.*, 2013). Furthermore, S1P seems to enhance immune-complex activation of neutrophils through Fcγ receptor, affecting Ca^{2+} mobilization, ROS generation, morphology and adhesion to endothelial surfaces under flow conditions (Florey and Haskard, 2009a). S1P also increases antineutrophil cytoplasmic antibody (ANCA)-mediated neutrophil activation (associated with systemic small vessel vasculitis), by enhancing respiratory burst and degranulation (Hao *et al.*, 2014). Similarly, S1P pre-treatment was shown to increase neutrophil respiratory burst activation mediated by fMLP, by stimulation of PI3K and Akt signalling pathways (Wang *et al.*, 2015).

It is clear that S1P might be able to affect neutrophils, but whether this effect is direct or not remains to be further investigated. Moreover, neutrophils can be affected in different

ways by S1P, positively or negatively, affecting their migration or activation, through different S1P receptor signalling and by stimulation of various signalling pathways. In this chapter, the direct effects S1P might have on neutrophils were investigated, focusing especially on any effects on neutrophil trafficking. For this reason, neutrophil S1P receptor expression was firstly examined, followed by the pathways through which S1P signals on neutrophils, and finally any direct S1P effects on neutrophil migration and adhesion were explored.

4.2 Materials and Methods

4.2.1 Whole blood flow cytometry

To use whole blood in a flow cytometry experiment, an extra red-blood-cell lysis step is added, and the whole procedure is carried out at room temperature rather than at 4^{o}C. A small amount of blood was added per tube (usually 100µl), Fc receptor blocking was performed (Human TruStain FcX^{TM}, BioLegend) and then stained as normally with fluorochrome conjugated primary antibodies or appropriate isotype controls, as described in chapter 2.3. After staining was completed, 2ml of red-blood-cell lysis buffer (FACS lysing solution, BD Biosciences) were added per tube and incubated for 10 minutes. After centrifugation and removal of the lysis buffer, a wash was performed and cells resuspended in PBS + 2% FBS to be examined in the flow cytometer (FACS Canto II, BD Biosciences). Data were analysed using FlowJo 7.6 (Treestar).

4.2.2 Neutrophil isolation and CD69 expression stimulation

Neutrophils were isolated from whole human blood using the Percoll method as described in chapter 2.1.5. Sometimes the final wash step was performed at a higher centrifugation speed of 400 x g instead of 200 x g, to avoid losing many cells. However, this lead to higher levels of neutrophil activation, and neutrophil profile was slightly different, so in later isolations it was avoided. Cells were separated as needed and used immediately for experiments, or cultured for 30 minutes in serum-free RPMI-1640 medium (RPMI-1640 with 0.5% BSA fatty-acid free) first. For stimulation of CD69 expression, neutrophils were incubated for 18 hours in serum-free medium with 500U/ml GM-CSF (PeproTech). In some experiments, neutrophils were incubated for 1 hour in serum-free medium with 5ng/ml PMA (PeproTech) instead. CD69 expression was measured using flow cytometry with anti-CD69 APC conjugated antibody (BD Biosciences; see chapter 2.3). Data analysed using FlowJo 7.6 (Treestar).

4.2.3 RT - qPCR

RNA was isolated with the TRI Reagent (Sigma-Aldrich) method, or using the RNeasy Plus Mini Kit (QIAGEN), following the company's protocol (see chapter 2.5.1). Reverse transcription for production of cDNA was performed using Agilent's AffinityScript Multiple Temperature cDNA synthesis kit (chapter 2.5.2). The cDNA was then used for detection of gene expression by real time PCR, using TaqMan® Gene

Expression assays (Applied Biosystems) as primers (see chapter 2.5 for full list), with the TaqMan® Gene Expression Master Mix 2X (Applied Biosystems) in an Applied Biosystems qPCR machine. The house-keeping gene *18S* was used as the reference gene. Data were analysed using the $\Delta\Delta C_T$ method (chapter 2.5.3).

4.2.4 Phospho-Akt (S473) cell-based ELISA

The Cell-Based ELISA kit for Human/Mouse/Rat Phospho-Akt (S473) from R&D Systems was used, following the protocol the company provided. Cell-based Enzyme Linked Immunosorbant Assay (ELISA) allows detection of two target proteins at the same time on whole cells, with no need for cell lysis. Around 100,000 cells in 100µl were seeded in each well of the provided 96-well plate, incubated for 1 hour or overnight at 37 °C, 5% CO_2, then stimulated for desired times with different reagents (S1P, IL-8 or H_2O_2), and 8% formaldehyde was added for 20 minutes at room temperature for fixation. Alternatively the fixed cells could be stored at 4 °C overnight or for up to 2 weeks before continuing the procedure. Formaldehyde was removed and the plate was washed with wash buffer 3 times for 5 minutes under gentle shaking on a rocker platform. Quenching buffer was added then, 0.6% H_2O_2 in wash buffer, and incubated for 20 minutes at room temperature. Another 3 washes were performed then for 5 minutes each with gentle shaking. Next, cells were incubated with blocking buffer for 1 hour at room temperature and then washed 3 times as before.

Primary antibody mixture was added (rabbit anti-pAkt (S473) and mouse anti-total Akt in blocking buffer) and incubated overnight at 4 °C. After washing 3 times, the secondary antibody mixture (anti-rabbit IgG HRP-conjugated and anti-mouse IgG AP-conjugated diluted in blocking buffer) was added and incubated for 2 hours at room temperature. The cells were then washed 2 times with wash buffer followed by 2 washes with PBS, all for 5 minutes with gentle shaking. Finally, the fluorogenic substrates were added; first substate F1, and after 20-60 minutes incubation substrate F2 followed by 20-40 minutes incubation, all while protected from light. Fluorescence was measured in a plate reader at 530nm excitation / 590nm emission for phospho-Akt and 360nm excitation / 460nm emission for total Akt. Results were normalized for background, which was the fluorescence from secondary only wells, and presented as ratio of phospho-Akt / total Akt. For neutrophils, however, a modification to the original protocol had to be made, specifically for the quenching buffer step that was changed from 0.6% H_2O_2 to 2.4% H_2O_2, with 1 hour incubation instead of 20 minutes.

The reason for this was the endogenous peroxidase activity of neutrophils that had to be completely quenched in order to get appropriate measurements.

4.2.5 Western blotting

Cells were treated or pre-treated with S1P, IL-8 or H_2O_2 in serum-free media for appropriate time, then equal volume of ice cold PBS was added and incubated on ice for 2 minutes to stop treatment. Cells were then lysed and protein measured with BCA protein assay (see chapter 2.6). Samples with loading buffer were loaded on a poly-acrylamide gel together with a protein ladder; same amount of protein was loaded per sample. Gel was run at 30mA for about an hour. Western blotting "sandwich" was prepared, with sponges, whattman paper, the gel and a piece of PVDF membrane, and run in transfer buffer. Membrane was blocked and stained with primary antibody against phospho-ERK1/2 (phospho-p44/42 (Thr202/Tyr204); Cell Signaling, USA) overnight. After washing the membrane, appropriate secondary antibody, anti-rabbit HRP conjugated, was added and incubated for 1 hour. Finally, membrane was washed, incubated with substrate and developed. Membrane was then stripped and stained again with appropriate loading control antibody, usually total ERK1 or GAPDH.

4.2.6 Phospho-kinase array

The Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems) was used, following the protocol described at the company manual provided. The array can detect relative phosphorylation levels of 43 kinase phosphorylation sites and 2 related total proteins, with antibodies spotted in duplicate on two parts nitrocellulose mebranes. Isolated human neutrophils were treated for 3 minutes with 1μM S1P or 100ng/ml IL-8, or pre-treated with 1μM S1P for 1 hour then treated with 100ng/ml IL-8 for 3 minutes, all in serum-free media, and were then lysed using the provided lysis buffer. The membranes were blocked with provided block buffer for 1 hour at room temperature on a rocking platform. Lysates were prepared by dilution in block buffer and then added to the membranes and incubated overnight at 4 ^{o}C on a rocking platform. The membranes were washed 3 times with provided wash buffer for 10 minutes on a rocking platform, and were then incubated with appropriate Detection Antibody Cocktail, diluted as instructed, for 2 hour at room temperature on a rocking platform. Next, they were washed another 3 times and incubated for 30 minutes with Streptavidin-HRP solution, diluted appropriately, on a rocking platform. Finally, they were washed and developed using Pierce chemiluminescent substrate (SuperSignal West Pico Substrate, Thermo

Scientific), and Kodak film, as in western blotting (see chapter 2.6.4). Results were analyzed by densitometry using ImageJ software.

4.2.7 Neutrophil chemotaxis

Isolated human neutrophils were added at the top of a modified Boyden chamber with a 3μm membrane and left to migrate towards different concentrations of the chemokine IL-8 (R&D Systems) for 60-90 minutes (see chapter 2.4). There were control wells with no chemokine, or chemokinesis control with chemokine on both chambers. In some experiments neutrophils were pre-treated with 1μM S1P in serum-free media for 30 minutes then left to migrate towards IL-8. For CD69 induction before chemotaxis, neutrophils were incubated for 18 hours with 500U/ml GM-CSF or media only, and then left to migrate towards IL-8 with or without S1P pre-treatment. For all experiments, cells that had passed at the bottom chamber were measured by flow cytometry using counting beads.

4.2.8 Cellix *VenaFlux* flow-based adhesion assays

Channels on Vena8 or Vena8 Fluoro+ biochips were coated with 10μg/ml VCAM-1 Fc or ICAM-1 Fc overnight (see chapter 2.7). Before using them in experiments, they were blocked with 1% BSA for 30 minutes. Cellix device and *VenaFlux* software were switched on and initialised. After washing the pump connections, biochip was mounted on the microscope stage and the channel to be used was briefly washed. Neutrophil cell suspension in medium was added in one end of the channel, with or without chemokine or other stimulant and flow was initiated (0.5 dyne/cm^2). Several representative fields of view were captured along the channel's length. This was repeated for all the channels used in the experiment. For each image captured, adherent cells were measured and results analysed using Prism3 software (GraphPad Software Inc).

4.3 Results

4.3.1 Neutrophil characterization and CD69 expression

4.3.1.1 Neutrophil characterization

In order to use primary neutrophils to test whether S1P has an effect on them, they have to be isolated from human blood. However, the isolation procedure has to be performed carefully, because neutrophils are fragile cells and need to be handled with care, otherwise they become activated and their profile changes significantly. They are also very short lived cells, starting to undergo apoptosis only a few hours after isolation.

The first step to make sure isolated neutrophils would be similar with blood neutrophils was to compare them with neutrophils in whole human blood. Whole human blood was used in flow cytometry, after red blood cell lysis had been performed. In order to differentiate the leukocyte populations, the leukocyte marker CD45 was used, whereas the neutrophil marker CD66b was used to identify the neutrophil population (PMN; figure 4.1). Next, the PMN population was tested for cell surface expression of the chemokine receptors CXCR1 and CXCR2, the adhesion molecule CD11b and the activation marker CD69. Both chemokine receptors and CD11b were expressed by almost the entire neutrophil population, whereas CD69 was not expressed at all (figure 4.2).

Neutrophils were isolated from whole human blood, and were stained for flow cytometry immediately after isolation. At the forward scatter (FSC) towards side scatter (SSC) graph, it is obvious that there is mainly only one uniform population (figure 4.3). Propidium iodide was used to assess the viability, which was over 95%, whereas the gated population was found to not express CD3, a T cell marker, at all, indicating the purity of the PMN population (figure 4.3). Isolated neutrophils were stained for CD45, CXCR1, CD11b and CD69. All of them express CD45 and CD11b as was expected, most of them express CXCR1, and CD69 is not expressed at all (figure 4.4). These results are similar to what was observed for whole blood neutrophils, showing that isolated neutrophils are suitable to be used in further experiments. Isolated neutrophils were also stained with S1P receptor antibodies, for a first assessment of the receptors they express, demonstrating an expression of S1PR3, but no expression of S1PR1 (figure 4.4E-F).

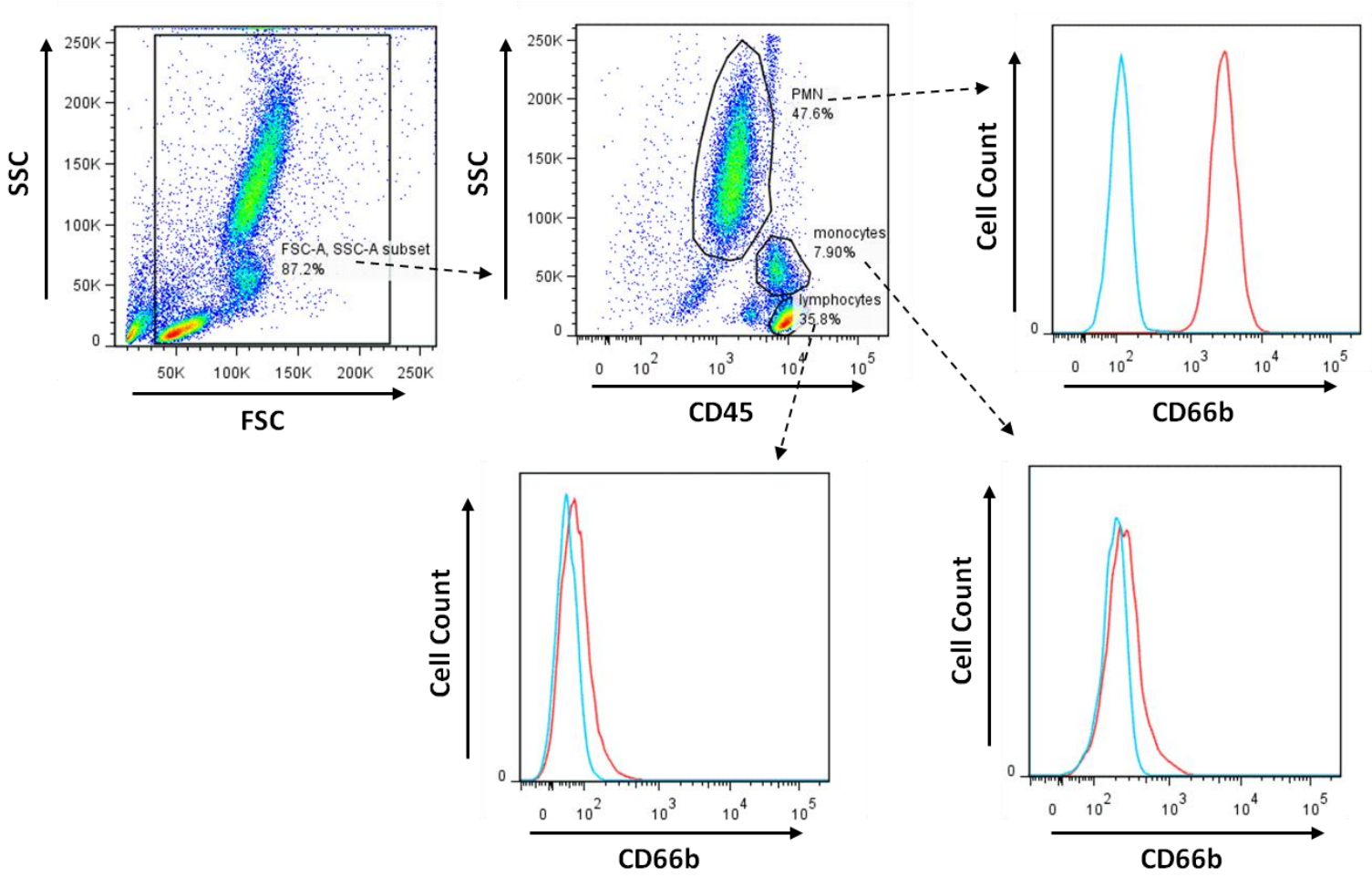


Figure 4.1. Whole blood flow cytometry. Gating strategy on neutrophils (PMN). CD45 is a leukocyte marker, CD66b is a neutrophil marker. Histograms: red line represents fluorescently conjugated monoclonal antibody, blue line represents isotype control.

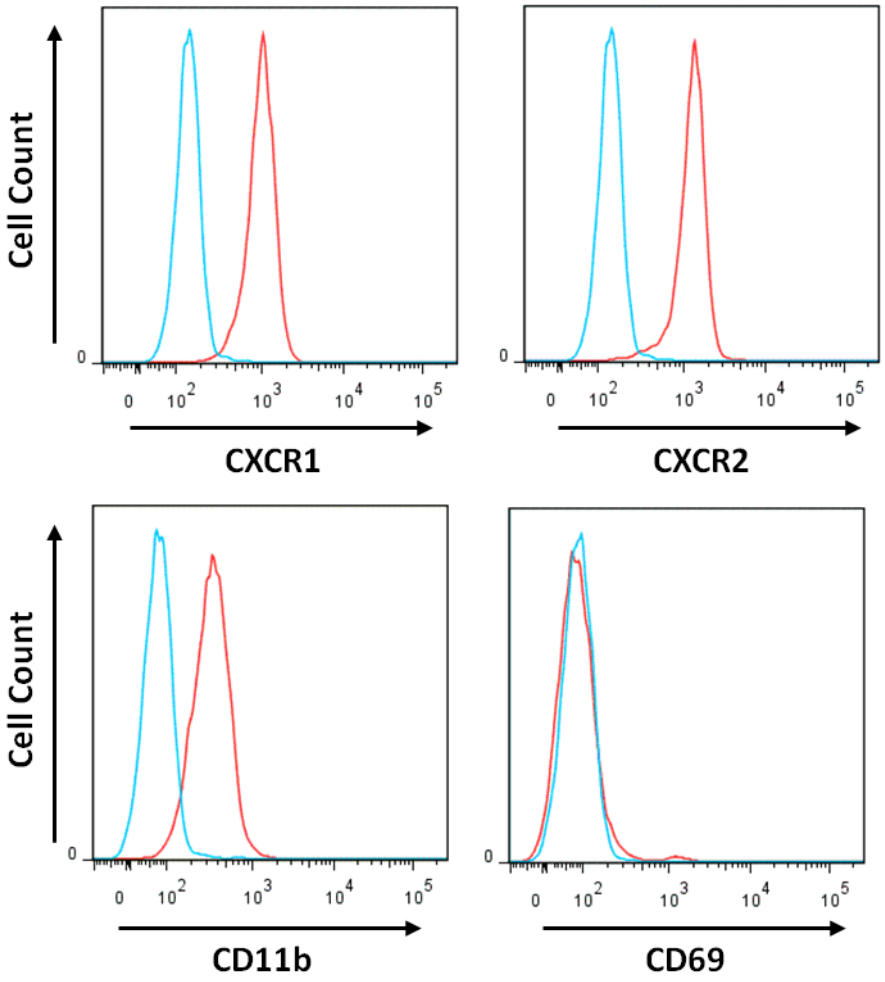


Figure 4.2. Flow cytometry analysis of PMN in whole blood. Whole blood flow cytometry was performed with gating to the neutrophil population. Cells were stained with fluorescently conjugated monoclonal antibodies for CXCR1, CXCR2, CD11b and CD69 (red) or appropriate isotype control antibodies (blue). Results are representative of 2 independent experiments.

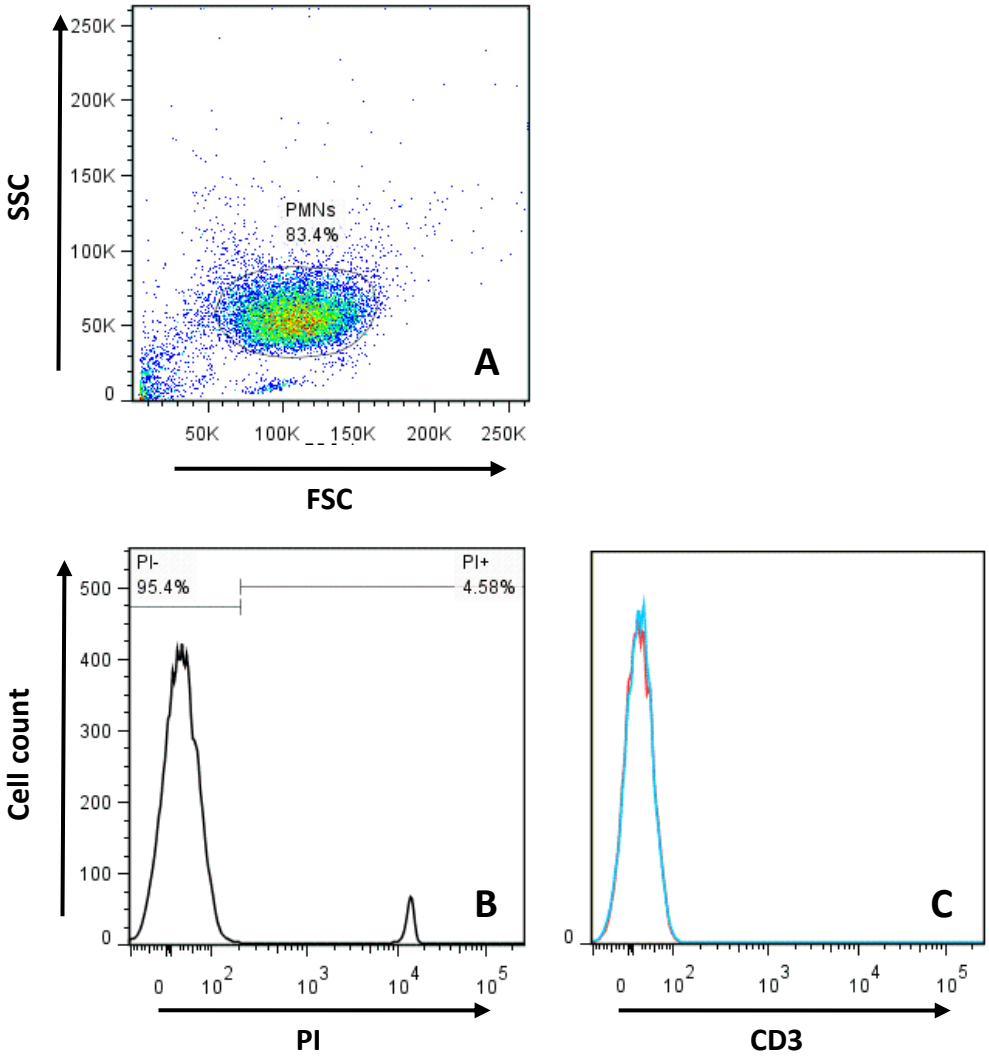


Figure 4.3. Neutrophil isolation assessment. PMNs were stained immediately after isolation. (A) Gating technique. (B) Propidium iodide (PI) staining to assess viability of cells. PI+ non viable, PI- viable cells. (C) Staining with monoclonal fluorescently conjugated anti-CD3 antibody (red) and appropriate isotype control (blue) to assess purity of cells. CD3 is a T lymphocyte marker.

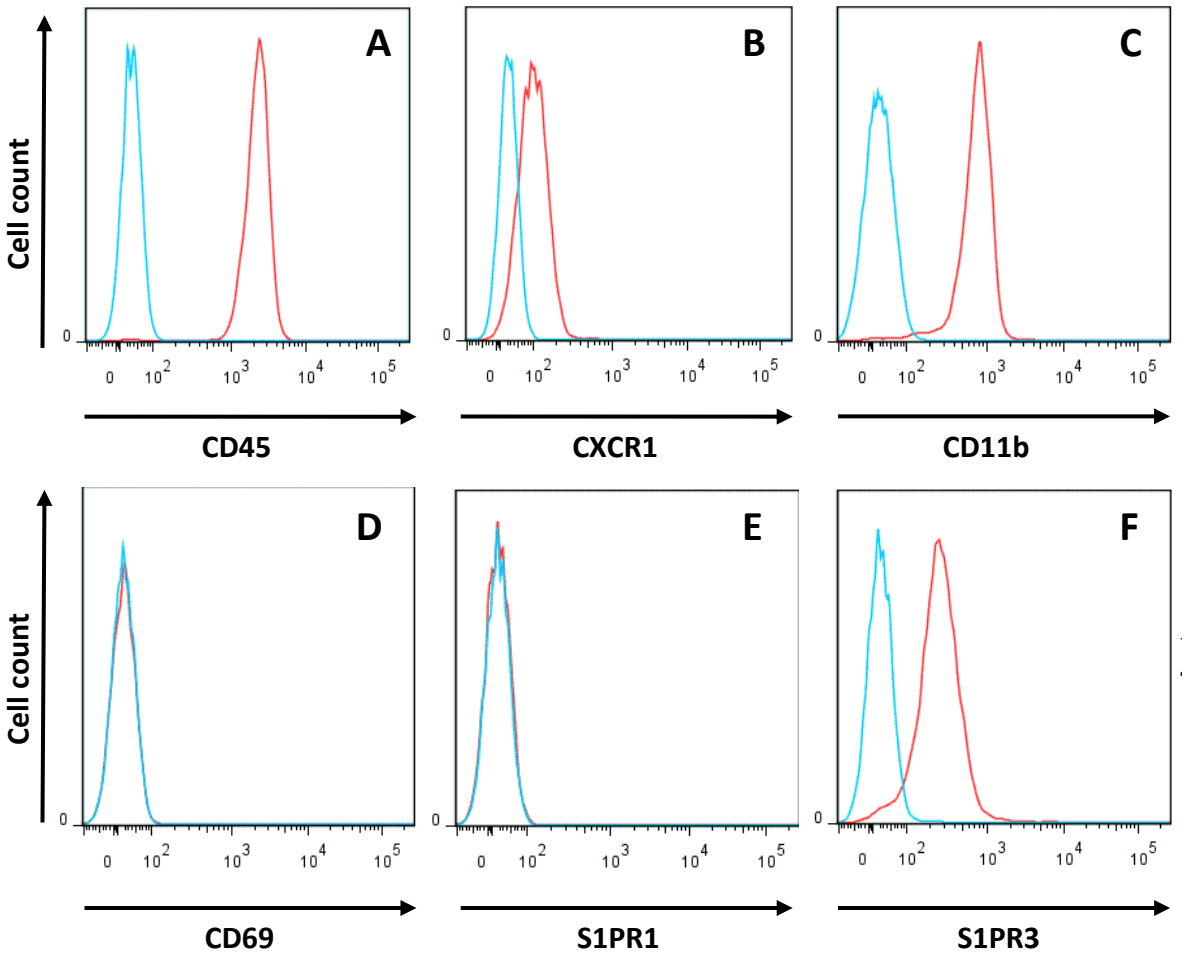


Figure 4.4. Neutrophil expression of cell surface antigens. Human peripheral blood PMNs were stained, immediately after isolation, with fluorescently conjugated monoclonal antibodies or non-directly conjugated polyclonal primary antibody (S1PR3) and appropriate conjugated secondary antibody (red), and separately with appropriate isotype control antibodies or secondary only (blue). Data are representative of n independent experiments. (A) Surface expression of CD45 antigen (n=1). (B) Expression of the chemokine receptor CXCR1 (n=4). (C) Expression of the adhesion molecule CD11b (n=4). (D) Expression of the CD69 antigen (n=4). (E) S1PR1 surface expression (n=2). (F) S1PR3 surface expression (n=3).

4.3.1.2 Neutrophil S1P receptors

To further investigate the S1P receptors that neutrophils express, real-time PCR was used. Total RNA was isolated from neutrophils and used in qPCR after cDNA synthesis. The S1P receptor genes' *S1PR1, S1PR3, S1PR4* and *S1PR5* mRNA expression was assessed, in parallel with the chemokine receptor's *CXCR1* and activation molecule's *CD69* mRNA expression. *S1PR1* and *S1PR4* are expressed in higher levels than the rest, even higher than *CXCR1* expression, with *S1PR4* being the highest; whereas *S1PR5* is expressed in very low levels and *S1PR3* is barely expressed at all (figure 4.5). *CD69* mRNA seems to also be present at relatively high levels (figure 4.5). These results differ from what was observed for surface expression of S1PR1 and S1PR3 (figure 4.4); this could be due to a fault of the flow cytometry antibodies, with low sensitivity in the case of S1PR1 antibody, and recognition of different targets (perhaps another S1P receptor) for S1PR3. On the other hand, mRNA expression does not necessarily translate to protein expression, so that could be another reason for these contradictory results. As for CD69 expression, it appears that might be the case, since we can observe some mRNA expression with no surface expression apparent (figures 4.4, 4.5). This could indicate a mechanism of *CD69* mRNA accumulation for a rapid expression after activation; or that CD69 protein is instead stored intracellularly to be transported to the cell surface after appropriate stimulation.

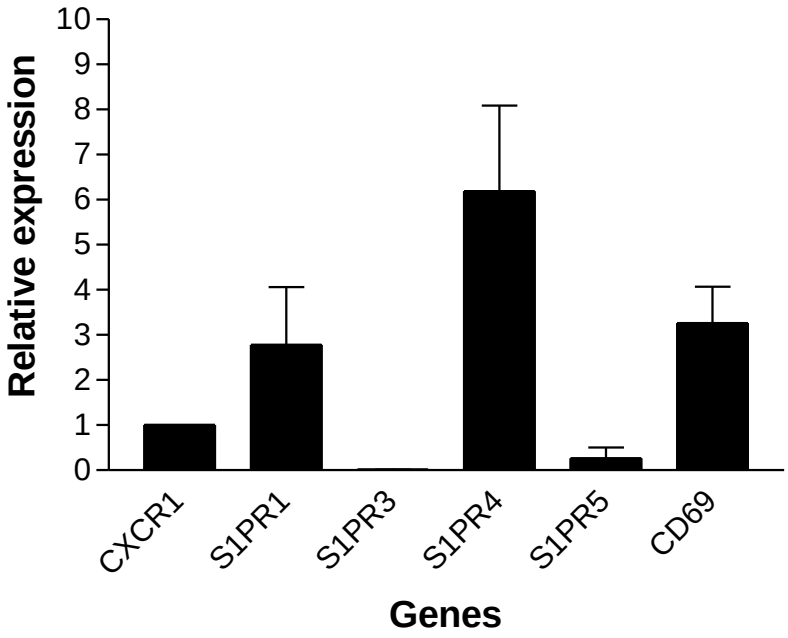


Figure 4.5. Relative mRNA expression of different genes by neutrophils. mRNA expression was detected using real-time PCR. Data are normalized for the housekeeping gene *18S* and presented as relative expression in comparison with *CXCR1* gene expression. Data represent mean ± SEM from two independent experiments.

4.3.1.3 Neutrophils and CD69

As has been previously observed, the activation molecule CD69 plays an important role in S1PR1 mediated lymphocyte egress from lymphoid organs (Shiow *et al.*, 2006; Swan *et al.*, 2012). However, CD69 can be expressed by neutrophils, too, after appropriate stimulation, though its role has not been thoroughly identified yet (Gavioli *et al.*, 1992; Atzeni *et al.*, 2002). In order to investigate whether CD69 interacts with S1P receptors in neutrophils, isolated neutrophils were treated with various molecules to stimulate CD69 expression.

According to one theory, CD69 is constitutively expressed in neutrophils, but is stored intracellularly until neutrophils are activated by appropriate compounds such as phorbol-12-myristrate 13-acetate (PMA) and fMLP, in which case it is rapidly relocated to the cell surface (Gavioli *et al.*, 1992; Noble *et al.*, 1999). This theory could not be reproduced in the current study, since when isolated neutrophils were treated for 1 hour with 5ng/ml PMA and then stained for CD69 surface expression and analysed by flow cytometry, no CD69 expression induction was observed (data not shown).

According to a different study though, brief incubation is not enough to cause CD69 surface expression in neutrophils, but an extended stimulation for 18 hours with GM-CSF, IFN-α or IFN-γ causes an induction of CD69 expression, with up to 60% of neutrophils expressing it on their surface (Atzeni *et al.*, 2002). The same study claims that this is due to new protein synthesis and an intracellular storage was not identified. For the current project, isolated neutrophils were incubated with 500U/ml GM-CSF for 18 hours and CD69 surface expression was assessed. This method indeed caused some CD69 expression on neutrophils' surface, which was observed only after incubation with GM-CSF and not after 18 hours in medium only (figure 4.6). The results varied, starting from 2.4% of neutrophils positive for CD69 expression with up to 40% positive in some cases. It is interesting to note that the method of isolation played a huge role in the capacity of neutrophils to express CD69 after incubation. When a washing step was altered to a higher centrifugation speed, neutrophil CD69 expression after 18 hours of GM-CSF incubation was lower, with 2 - 12% of neutrophils being positive, whereas in the non-altered method of neutrophil isolation, 12 - 40% of all neutrophils expressed CD69 on their surface (table 4.1).

Low expression group (n=6)	High expression group (n=8)
8.22%	30.5%
10.6%	20.1%
5.84%	12.3%
2.42%	15.8%
12.5%	20.1%
6.73%	37.6%
	40.6%
	30.5%
Mean ± SD = 7.72% ± 0.036	**Mean ± SD = 25.94% ± 0.103**

Table 4.1. Neutrophil CD69 surface expression after GM-CSF incubation. Percentage of cells positive for CD69 surface expression after 18 hours incubation with 500U/ml GM-CSF as was assessed by flow cytometry. Low expression group refers to neutrophils isolated with an altered method whereas high expression group refers to neutrophils isolated with the regular method of neutrophil isolation. SD: standard deviation

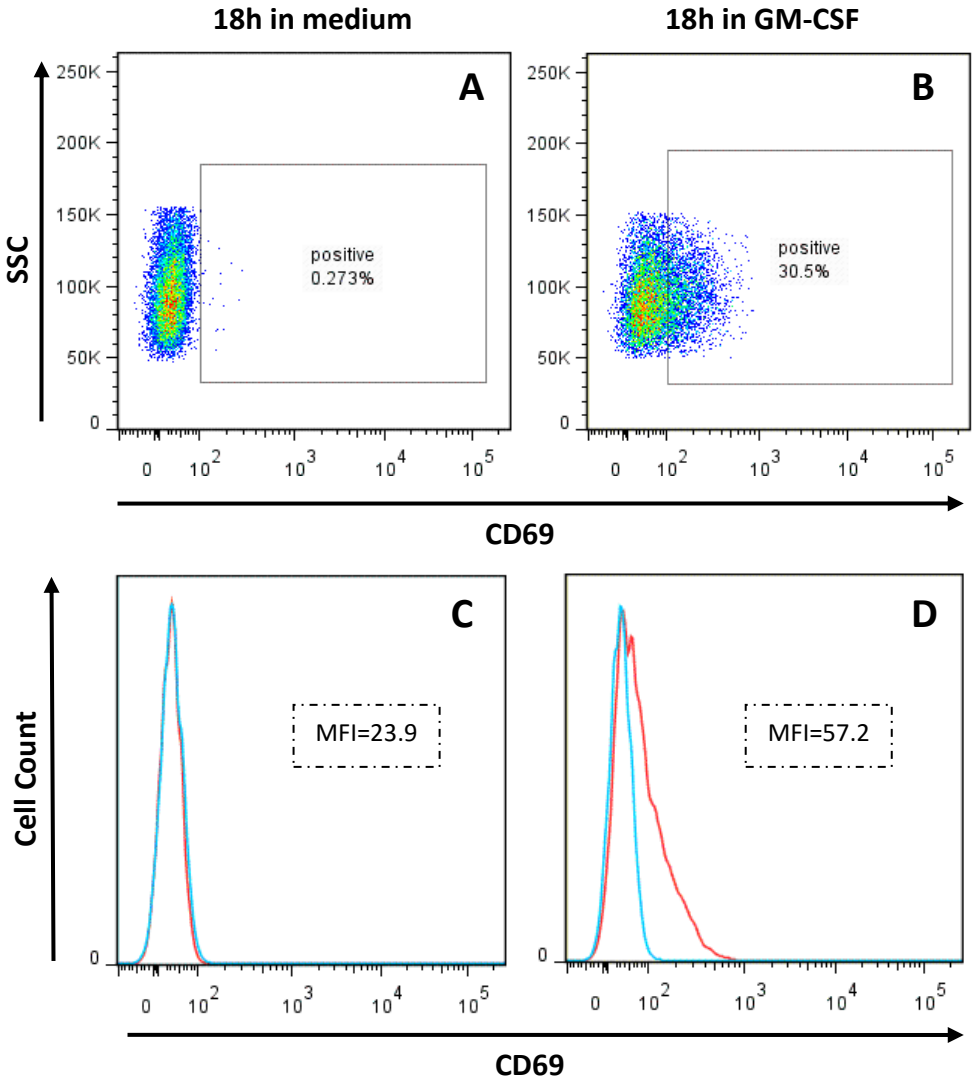


Figure 4.6. Expression of CD69 after GM-CSF incubation. Neutrophils were stained after 18h in medium only or after 18h in 500U/ml GM-CSF, with directly conjugated monoclonal antibody for CD69 (red) or appropriate isotype control antibody (blue). (A) Dot plot showing the percentage of cells expressing CD69 after 18h in medium. (B) Dot plot showing the percentage of cells expressing CD69 after 18h in GM-CSF. (C-D) Histograms for CD69 expression after 18h in medium and 18h in GM-CSF respectively. MFI: Median fluorescent intensity

Neutrophils incubated for 18 hours with GM-CSF or medium only were then assessed for the expression of important neutrophil molecules, such as chemokine receptors, adhesion molecules and of course S1P receptors. The IL-8 receptor CXCR1 was still expressed on the surface of neutrophils after 18 hours incubation either in medium or GM-CSF, at relatively similar levels as when freshly isolated (figure 4.7 A-C). CD11b, an adhesion molecule, was also expressed; however 18 hours after isolation its expression decreased, although GM-CSF incubation kept it at relatively higher levels (figure 4.7 D-F). S1PR3 which seemed to be expressed by freshly isolated neutrophils, as indicated by flow cytometry, appeared to greatly decrease after 18 hours, whereas GM-CSF incubation caused an even further decrease (figure 4.7 G-I).

To further assess the expression of S1P receptors, qPCR was used, to measure their mRNA expression after 18 hours incubation with GM-CSF or medium comparative to the expression by freshly isolated neutrophils (figure 4.8). *S1PR1* was one of the S1P receptors mainly expressed by fresh neutrophils, but after 18 hours incubation with GM-CSF it decreased considerably, at ¼ of its previous expression, something that was not observed as much with medium only. The other receptor expressed in high amounts by fresh neutrophils, *S1PR4*, underwent an ever more dramatic decrease, with only 10% expression of what was before remaining, both after 18 hours incubation with GM-CSF and with medium only. On the other hand, *S1PR5*, which was expressed at very low levels after isolation, increased 2-fold after 18 hours in medium and 2.5-fold after GM-CSF incubation. *S1PR3* was barely expressed after isolation, and its expression was even lower after 18 hours in medium or GM-CSF. Interestingly, *CXCR1* mRNA expression appeared to decrease significantly after 18 hours in medium or GM-CSF, although CXCR1 protein was observed at similar levels on neutrophil surface. Moreover, *CD69* mRNA did not appear to increase after 18 hours incubation in GM-CSF, but this is probably due to high levels of mRNA present before incubation although there was no surface expression. However, 18 hours incubation in medium only did cause a decrease in the mRNA levels of *CD69*.

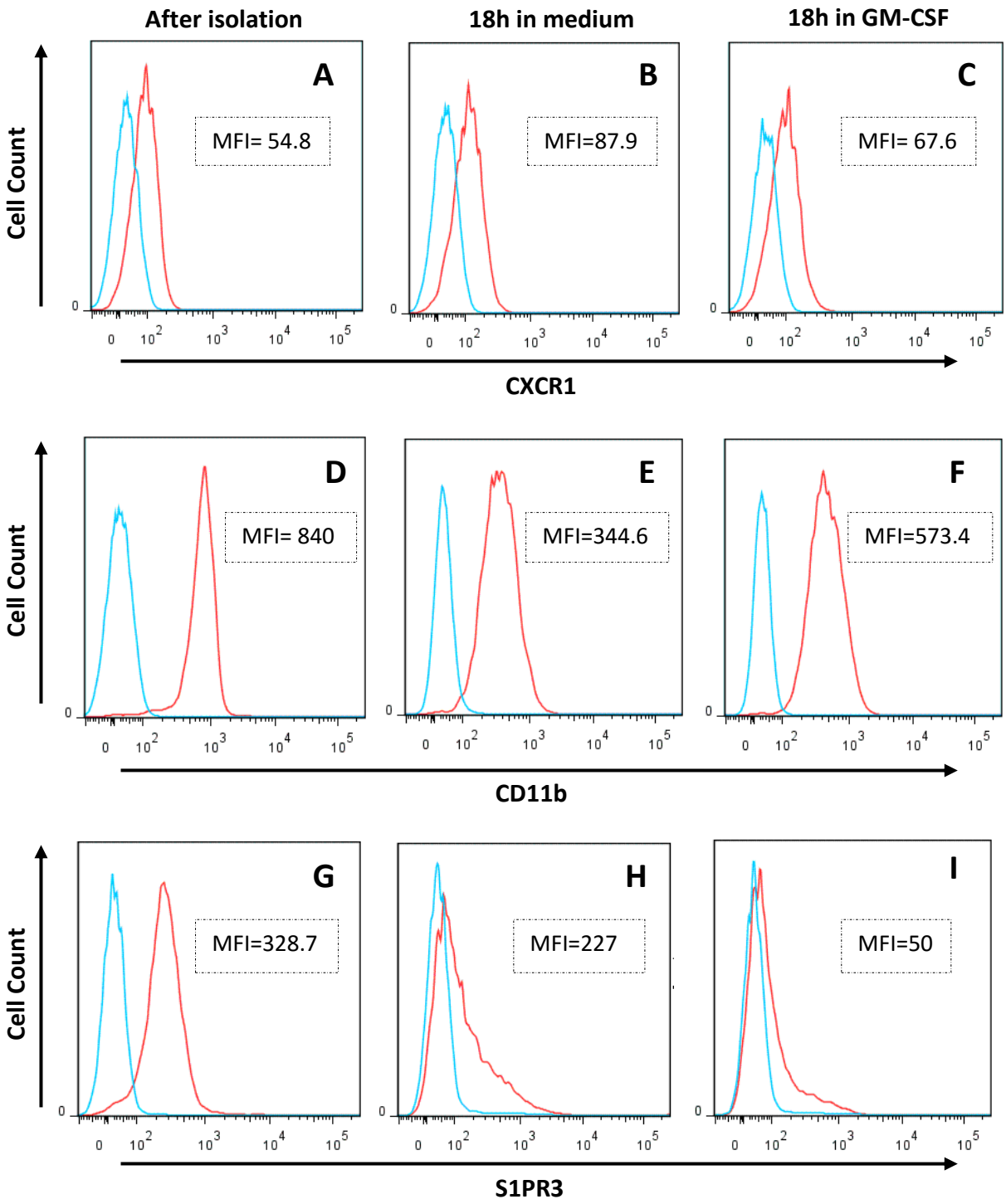


Figure 4.7. Variation in surface expression of CXCR1, CD11b and S1PR3 after GM-CSF incubation. Neutrophils were stained immediately after isolation, after 18h in medium only and after 18h in medium with 500U/ml GM-CSF. Red: specific antibody directly conjugated or with conjugated secondary antibody (for S1PR3), blue: appropriate isotype control or secondary only. Data are representative of n independent experiments. (A-C) CXCR1 expression immediately after isolation, after 18h in medium, and after 18h in GM-CSF respectively (n=2). (D-F) CD11b expression after isolation, 18h in medium, 18h in GM-CSF respectively (n=3). (G-I) S1PR3 expression after isolation, 18h in medium and 18h in GM-CSF respectively (n=3). MFI: mean fluorescence intensity, normalised for appropriate control.

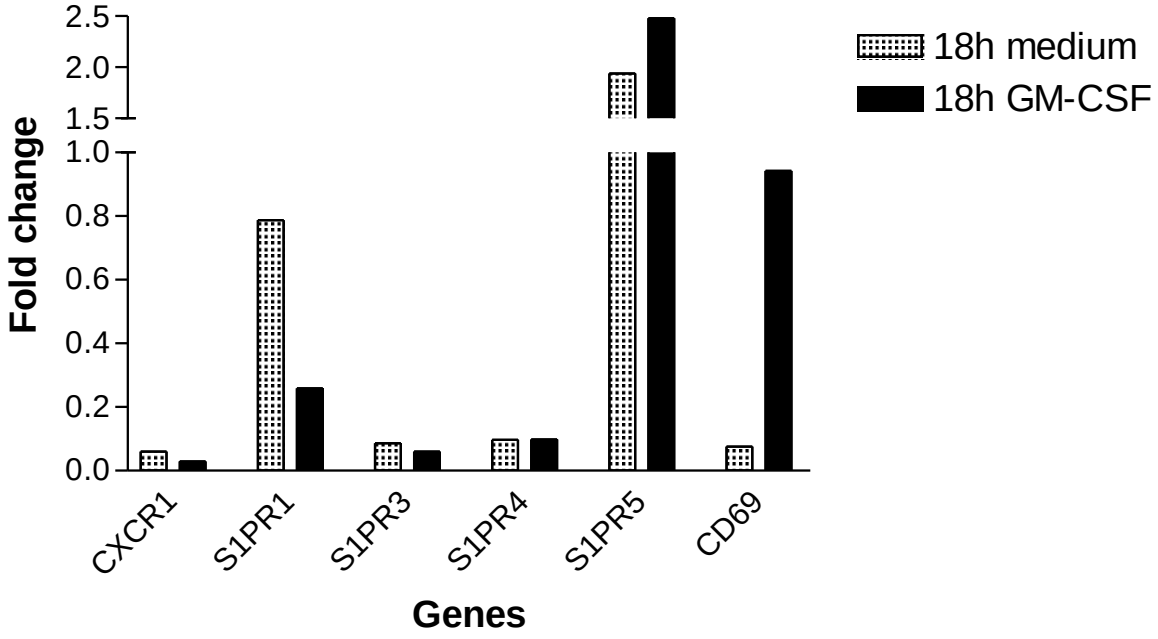


Figure 4.8. Differences in mRNA expression of different genes after GM-CSF incubation of neutrophils. mRNA expression was measured using real time PCR. Data show the fold change in neutrophil mRNA expression of different genes after 18h in medium only or 18h in 500U/ml GM-CSF, in comparison with the mRNA expression immediately after isolation.

4.3.2 Activation of phospho-kinase pathways in neutrophils

4.3.2.1 Phospho-kinase array

S1P signals through 5 G-protein coupled receptors leading to the activation of different signalling pathways. In order to investigate whether S1P can signal in neutrophils, after verifying that neutrophils express S1P receptors, the next step was to measure the phosphorylation of important kinases indicating a signalling cascade has been initiated. Thus, a phospho-kinase array was used, where neutrophils were treated with S1P or IL-8 as a control, and the phosphorylation of different kinases was compared to that on untreated cells (figure 4.9). Indeed, neutrophils treated with S1P expressed a lot of phosphorylated kinases, including GSK3α/β, MSK1/2, p53, RSK1/2/3, c-Jun, STAT4 and eNOS. Moreover, it was investigated whether S1P pretreatment has an effect on IL-8 signalling. When neutrophils were first treated with S1P before being treated with IL-8, there was an increase in phosphorylation of kinases MEK1/2 and MSK1/2 whereas the kinases GSK3α/β, RSK1/2 and STAT4, among others, were less phosphorylated (figure 4.9).

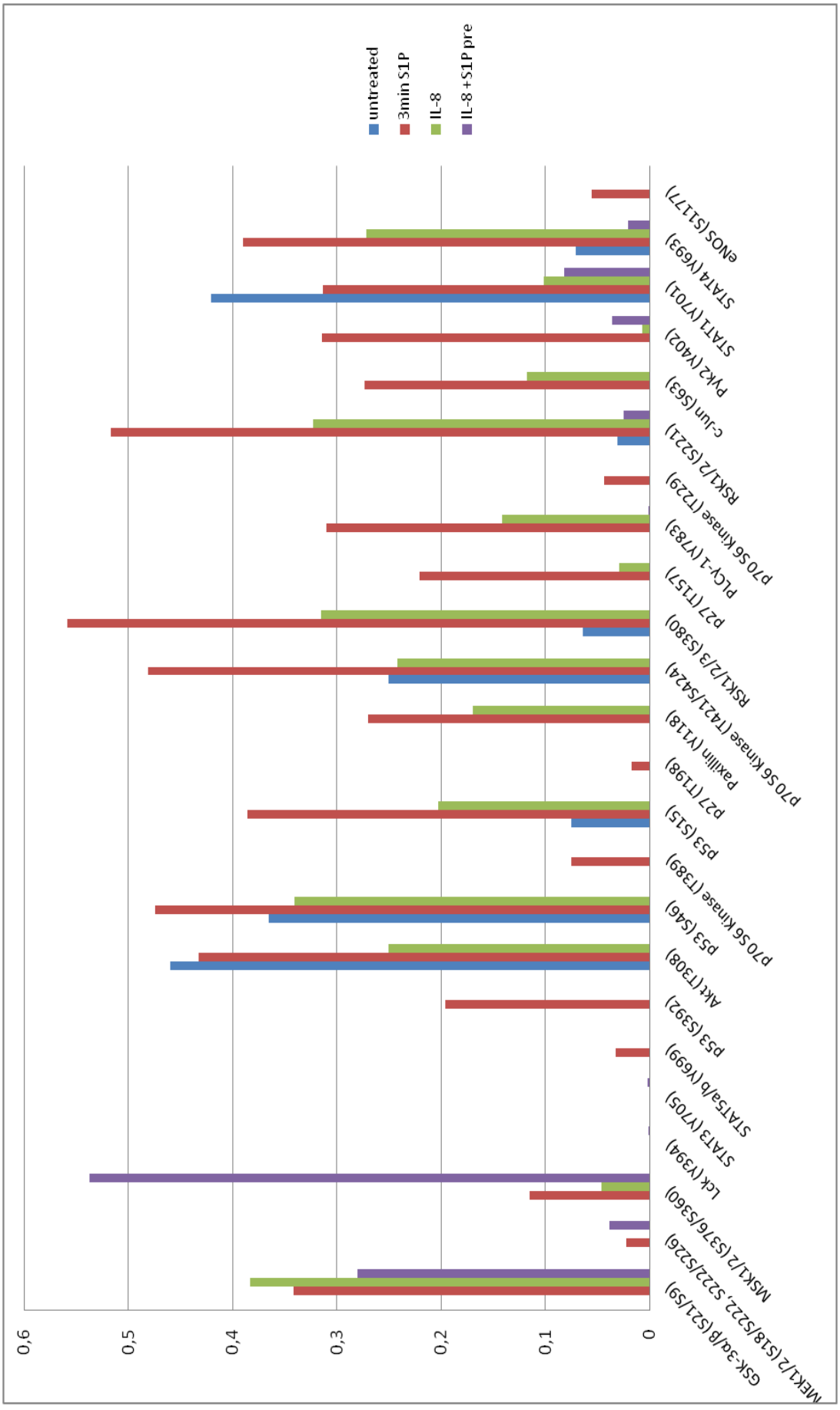


Figure 4.9. Phospho-kinase array with neutrophils. Neutrophils were untreated or treated for 3 minutes with 1µM S1P or 100ng/ml IL-8 or pre-treated with 1µM S1P for 1 hour then treated with 100ng/ml IL-8 for 3 minutes. They were then lysed and used in a phospho-kinase array.

4.3.2.2 p-ERK and p-Akt expression

To further investigate the phosphorylation of some important signalling kinases, cell-based ELISA and western blotting were used. Neutrophils were treated with different S1P concentration as well as IL-8 and H_2O_2 as controls, before being used in a cell-based ELISA for phospho-Akt (p-Akt). Although IL-8 and H_2O_2 caused an increase in p-Akt normalized for total Akt, S1P did not appear to have an effect (figure 4.10). On the other hand, when neutrophils were treated with 1μM S1P then used in western blotting for phospho-ERK1/2 (p-ERK1/2), there was a significant increase in ERK phosphorylation compared with untreated cells (figure 4.11). The optimal treatment time varied from 5-10 minutes, whereas IL-8 treatment for 3 minutes was enough to cause similar ERK phosphorylation. Again the results were diverse depending on the donor of the cells, with phosphorylation after IL-8 treatment sometimes being much more pronounced, comparable with the phosphorylation caused by H_2O_2, another positive control (figure 4.11). Moreover, S1P pretreatment before IL-8 stimulation was investigated, showing a significant increase in p-ERK1/2 phosphorylation in the pretreated compared with the non-pretreated cells (figure 4.12).

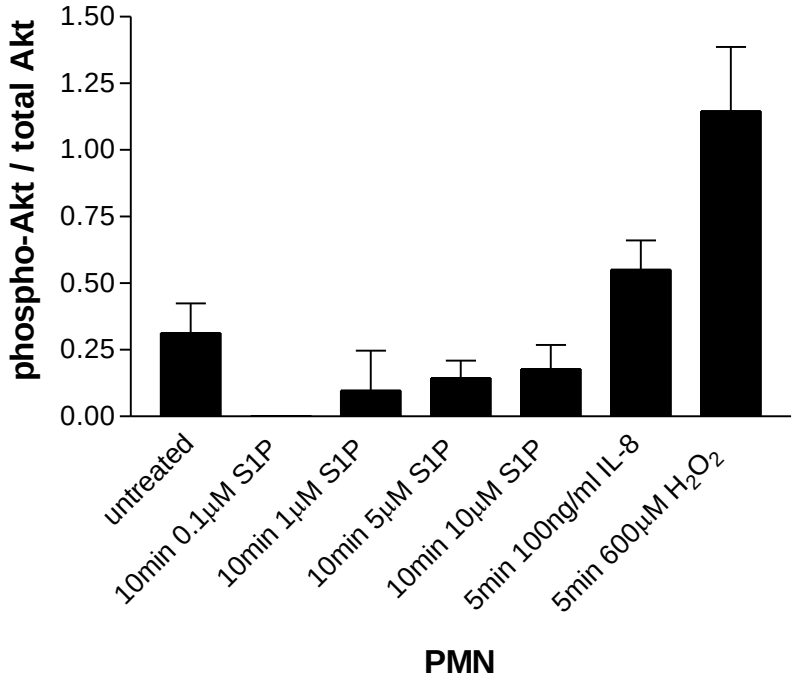


Figure 4.10. Phospho-Akt expression of neutrophils after S1P treatment. Neutrophils (PMN) where treated with different concentrations of S1P as well as IL-8 and H_2O_2 as controls, then used at a cell-based pAkt ELISA assay. Results are depicted as a ratio of phospho-Akt/total Akt.

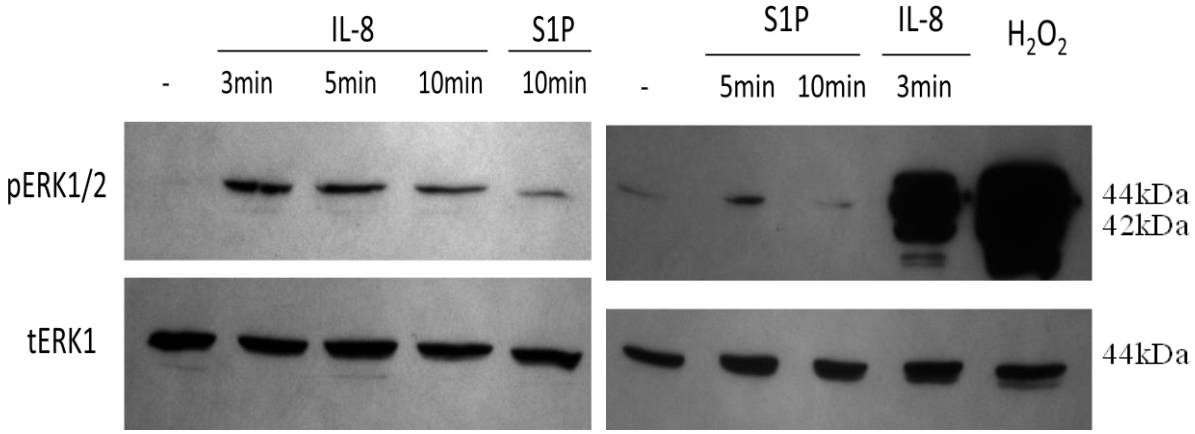


Figure 4.11. pERK1/2 expression of neutrophils after treatment with IL-8 and S1P. Isolated neutrophils were treated for different times with 100ng/ml IL-8 or 1μM S1P, or 600μM H_2O_2 for 5 minutes as control. Lysates where then prepared and used in western blotting experiments with pERK1/2 antibody, as well as total ERK1 antibody as loading control. Results are from two representative experiments out of 5 similar experiments.

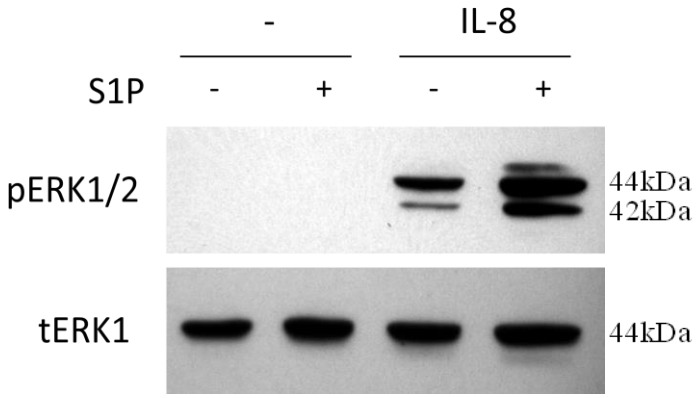


Figure 4.12. Effect of S1P pre-treatment on pERK1/2 expression induced by IL-8. Isolated neutrophils were either pre-treated with 1μM S1P for 1 hour or with medium only, then treated with 100ng/ml IL-8 for 3 minutes. Lysates were used for pERK1/2 western blotting (as above). Results are representative of two independent experiments.

4.3.3 Neutrophil chemotaxis

After it had been established that S1P can signal on neutrophils through S1P receptors, the effects this signaling has were investigated. Specifically, the effects it might have on neutrophil chemotaxis towards IL-8, which is the focus of this study, were explored. Neutrophils are very chemotactic towards IL-8, even at low concentrations, as can be seen in figure 4.13. However, when neutrophils were pre-treated with S1P for 30 minutes before being used for chemotaxis, there was no consistent effect observed. Again, the donor of the cells played a major role, leading to some expreriments showing a decrease in chemotaxis (figure 4.14), others an increase (data not shown), whereas in others there was no significant difference (figure 4.15). Interestingly, when a chemokinesis control was used, where IL-8 was added in both sides of the chemotaxis membrane, S1P seemed to cause a decrease in general chemokinetic capabilities of neutrophils (figure 4.15).

Furthermore, the effects CD69 might have on S1P mediated chemotaxis towards IL-8 were investigated. Neutrophils were treated with GM-CSF for 18 hours to induce CD69 surface expression. Untreated neutrophils, after 18 hours of incubation, were considerably less chemotactic towards IL-8 compared to fresh neutrophils. When untreated neutrophils were pre-treated with S1P before IL-8 chemotaxis however, there was an increase in chemotactic response (figure 4.16). Neutrophils treated with GM-CSF for 18 hours appeared to maintain more of their chemotactic ability; however, the effect of S1P on chemotaxis seemed to be ablated (figure 4.16). This might be an indication that CD69 expression on GM-CSF treated neutrophils interferes with S1P signalling, perhaps with a mechanism similar to that on lymphocytes. In this specific experiment, after GM-CSF incubation around 12.5% of the cells appeared to be positive for CD69 expression. Due to the relatively low percentage of CD69 positive cells, other mechanisms should also be considered.

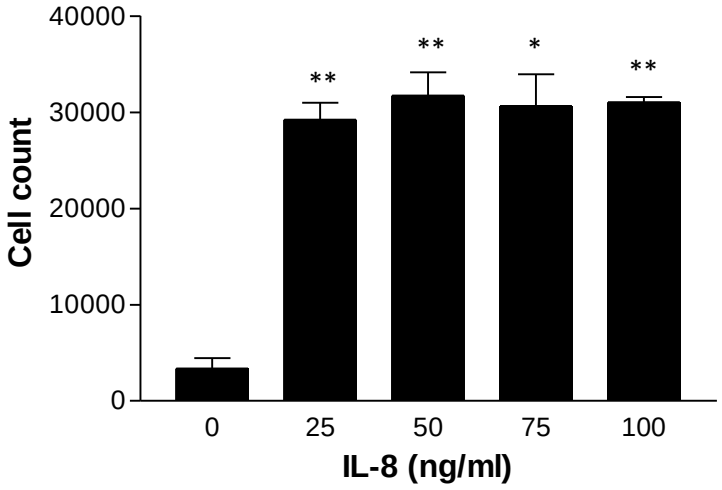


Figure 4.13. Neutrophil chemotaxis towards different concentrations of IL-8. Neutrophils were incubated in serum-free medium for 30 minutes then left to migrate towards different concentrations of IL-8 for 60 minutes. The cells that passed into the wells were measured by flow cytometry. Data representative of two independent experiments. *p<0.05, **p<0.01 compared with 0ng/ml IL-8.

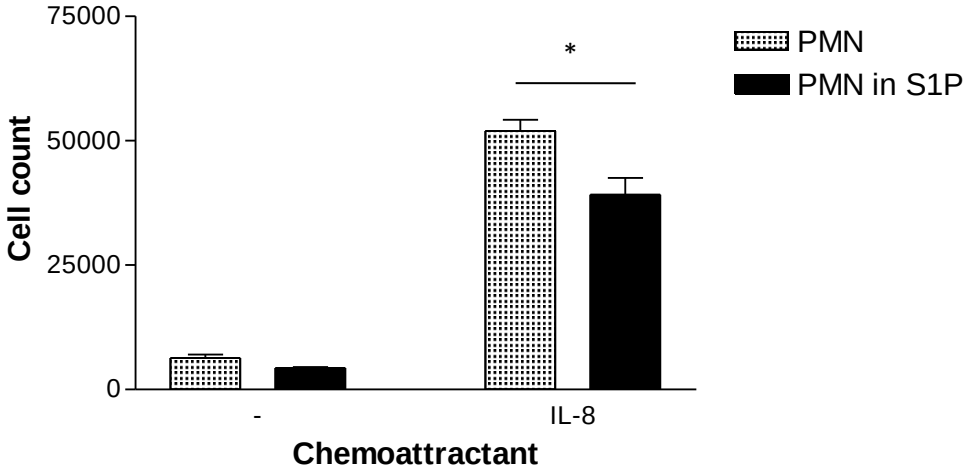


Figure 4.14. Effect of S1P pretreatment on neutrophil chemotaxis towards IL-8. Neutrophils (PMN) were incubated for 30 minutes in 1μM S1P in serum-free medium or medium only, then left to migrate for 60 minutes towards 50ng/ml IL-8 or vehicle (-). The cells that passed into the wells were measured by flow cytometry using counting beads. *p<0.05

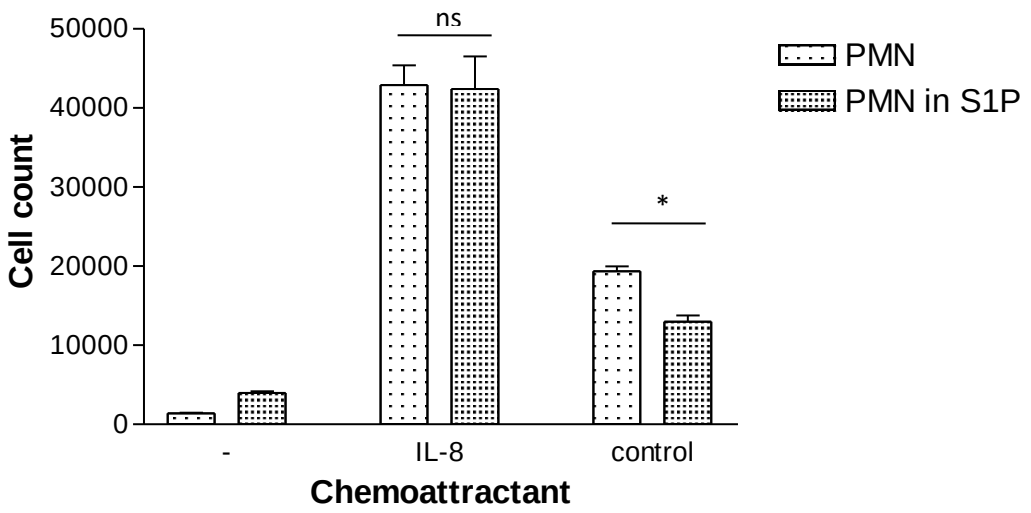


Figure 4.15. Effect of S1P pretreatment on PMN chemotaxis and chemokinesis. PMNs were incubated for 30 minutes in 1μM S1P in serum-free medium or medium only, then left to migrate for 90 minutes toward 50ng/ml IL-8 or vehicle(-). A chemokinesis control was included where 50ng/ml IL-8 were added in both sides of the chemotaxis filter (control). Cells that passed into the wells were counted by flow cytometry. *$p<0.05$, ns $p\geq0.05$

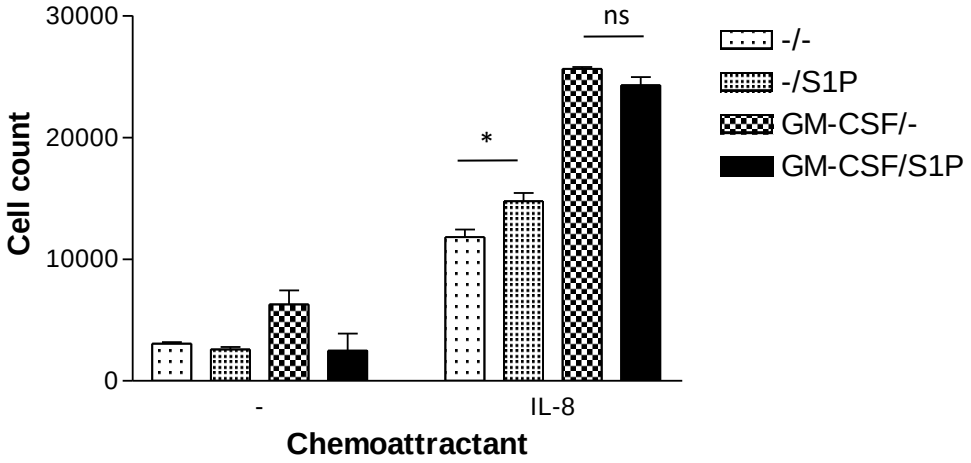


Figure 4.16. Effect of S1P on GM-CSF pretreated neutrophils. Neutrophils were incubated for 18h with medium only (-) or 500U/ml GM-CSF. Then, they were incubated for 30 minutes with 1μM S1P or medium only and left to migrate towards 50ng/ml IL-8 or vehicle for 90 minutes. Cells that passed into the wells were counted. *$p<0.05$, ns $p\geq0.05$

4.3.4 Neutrophil flow-based adhesion assays

The Cellix^{TM} system of flow-based adhesion was used to assess whether S1P has an effect on neutrophil adhesion to VCAM-1 and ICAM-1 adhesion molecules. For these assays, the channels were coated with the adhesion molecules VCAM-1 or ICAM-1, and neutrophils stimulated or not with IL-8 were used to flow through. Neutrophils show low adherence to VCAM-1 without any stimulation; however stimulation with IL-8 causes a major increase in adhesion, with 25ng/ml IL-8 being the optimal concentration (figure 4.17). On the other hand, neutrophils adhere to ICAM-1 even without any stimulation; again, however, IL-8 stimulation significantly increases adhesion, with 50ng/ml being the optimal concentration (figure 4.18). In several experiments, some neutrophils were pre-treated with 1μM S1P for 1 hour before being stimulated with various IL-8 concentrations and used in the assay (figure 4.19). S1P treatment appeared to have varied effects on neutrophil adhesion, so to further investigate, one "optimal" IL-8 concentration was chosen per adhesion molecule, 25ng/ml for VCAM-1 and 100ng/ml for ICAM-1, and the neutrophils were pre-treated with a range of S1P concentrations (figure 4.20). Again, the effects seemed to be varied; so, eventually, one S1P concentration, that seemed to have the most significant effect for both VCAM-1 and ICAM-1 was chosen, 5μM S1P, and it was used with one IL-8 concentration in a triple assay with 3 channels per adhesion molecule (figure 4.21). The conclusion after this assay was that S1P does not have a consistent significant effect on IL-8 induced neutrophil adhesion to VCAM-1 and ICAM-1.

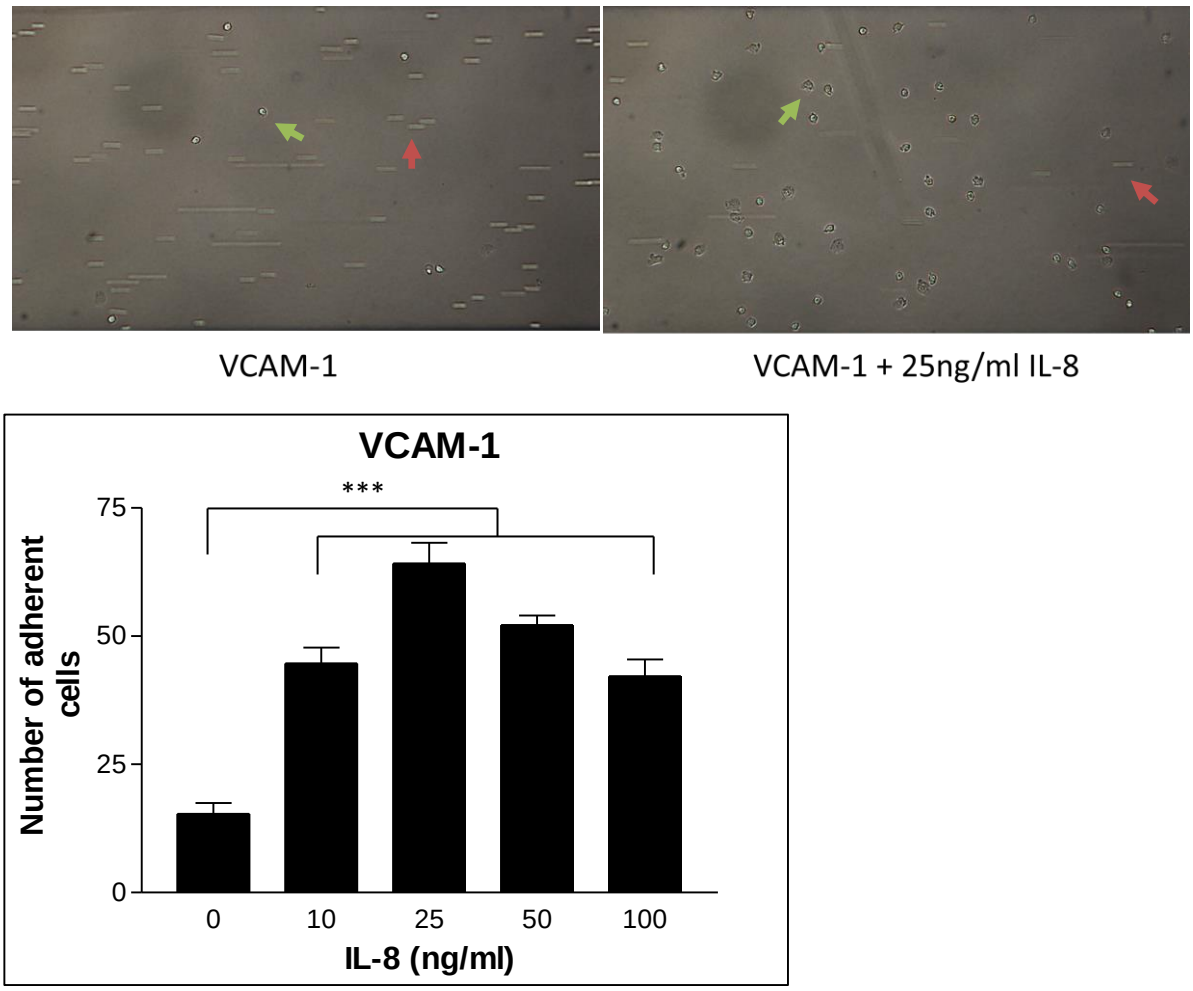
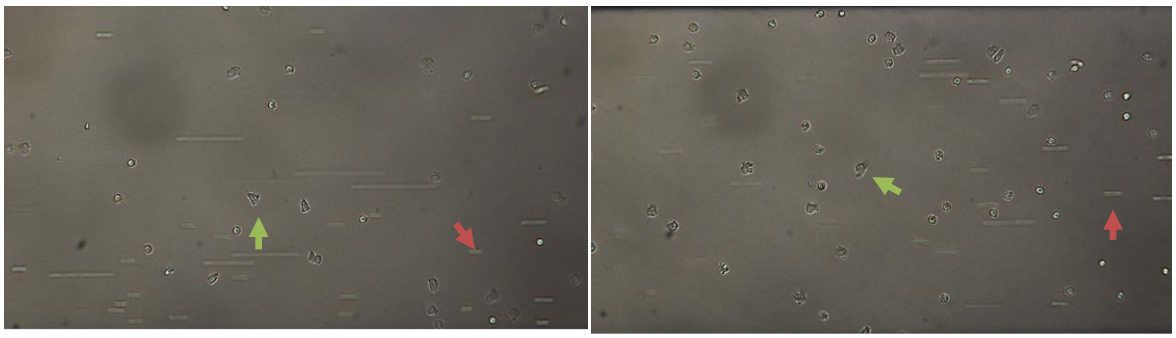


Figure 4.17. Flow-based adhesion assay to VCAM-1 with PMN. Top: Capture images from flow-based assay with neutrophils (PMN) in VCAM-1 coated channels, untreated or treated with 25ng/ml IL-8. Clearly defined cells are adherent (green arrow), blurred cells are flowing (red arrow). Bottom: Isolated neutrophils were treated with different concentrations of IL-8, and used in VCAM-1 coated channels. Bars show means ± SEM from 5-7 captured fields of view. Data are representative of 5 independent experiments. *** $p<0.001$ compared with 0 ng/ml IL-8.



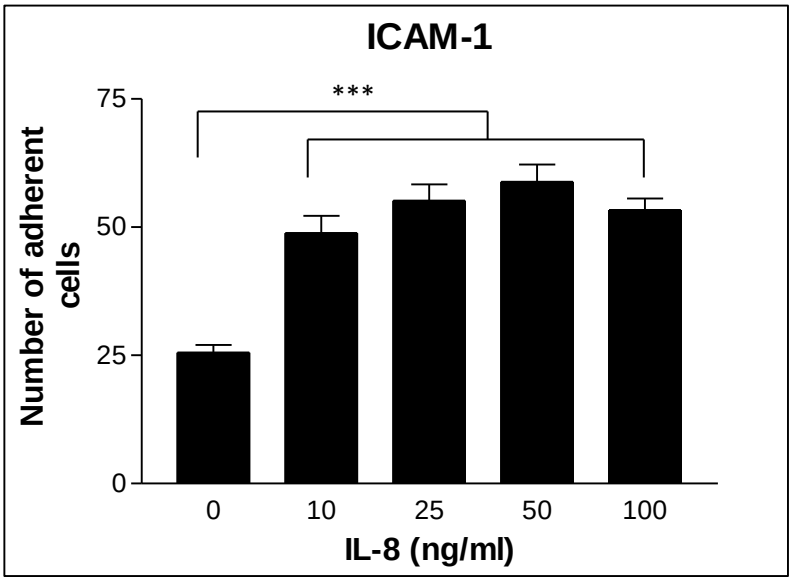


Figure 4.18. Flow-based adhesion assay to ICAM-1 with PMN. Top: Capture images from flow-based assay with neutrophils (PMN) in ICAM-1 coated channels, untreated or treated with 50ng/ml IL-8. Clearly defined cells are adherent (green arrow), blurred cells are flowing (red arrow). Bottom: Isolated neutrophils were treated with different concentrations of IL-8, and used in ICAM-1 coated channels. Bars show means ± SEM from 5-7 captured fields of view. Data are representative of 5 independent experiments. *** $p<0.001$ compared with 0 ng/ml IL-8.

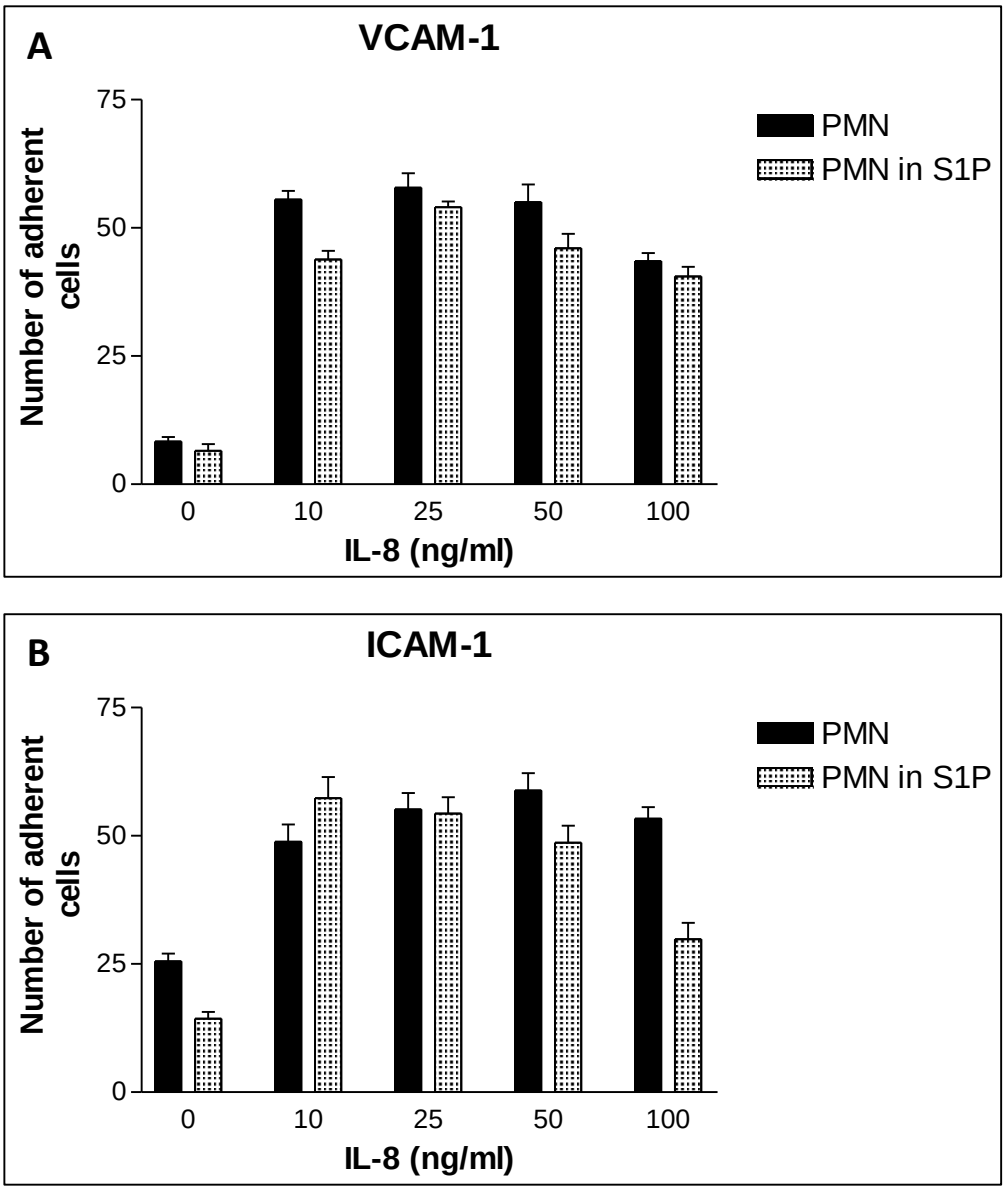


Figure 4.19. S1P pre-treatment effect on IL-8 stimulated flow-based adhesion of PMN. Isolated neutrophils were pre-treated with 1μM S1P or medium only for 1 hour, stimulated with different concentrations of IL-8, and then used in VCAM-1 (A) or ICAM-1 (B) flow-based adhesion assays. Bars show means ± SEM from 5-7 captured fields of view. Data are representative of 3 independent experiments.

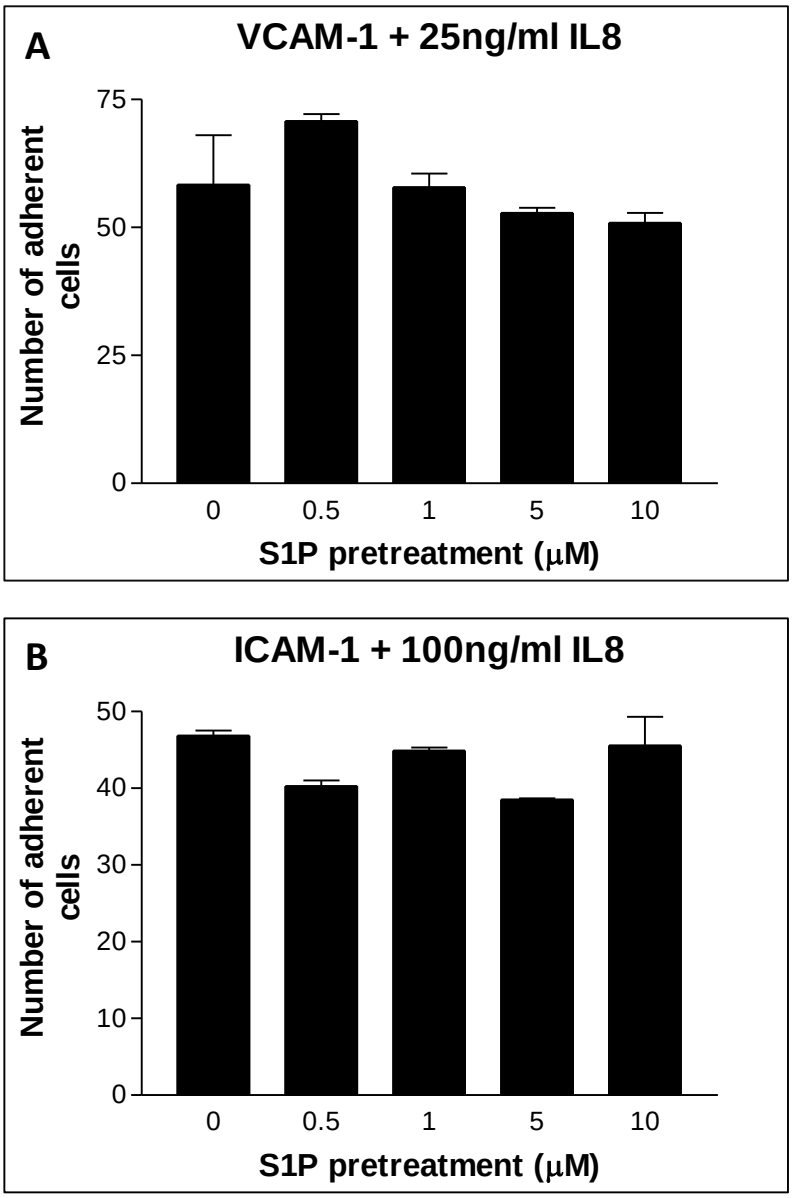


Figure 4.20. Effect of different concentrations of S1P pre-treatment on IL-8 stimulated flow-based adhesion. Isolated neutrophils were pre-treated with different concentrations of S1P for 1 hour, stimulated with 25ng/ml IL-8 for VCAM-1 or 100ng/ml IL-8 for ICAM-1, and then used in VCAM-1 (A) or ICAM-1 (B) flow-based adhesion assays. Bars show means ± SEM from 2 independent channels in the same experiment.

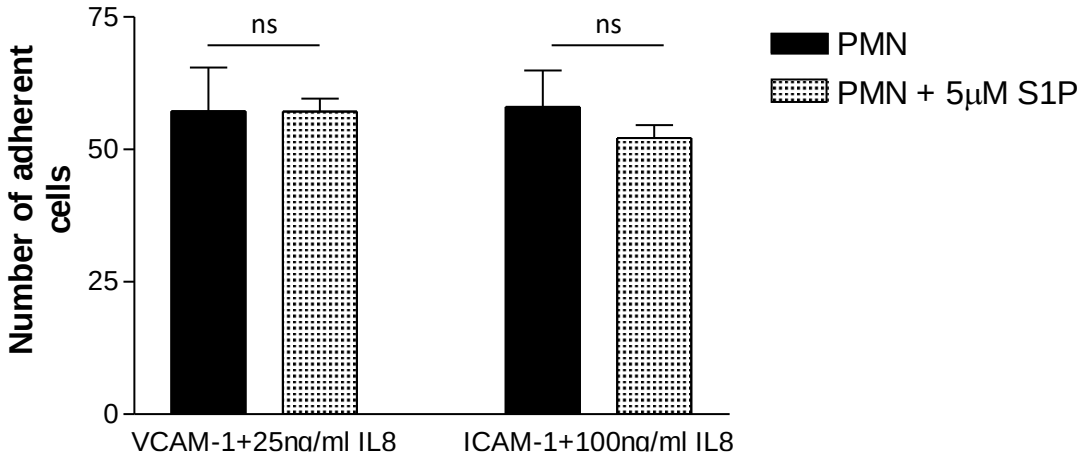


Figure 4.21. S1P pre-treatment and IL-8 stimulated flow-based adhesion. Isolated neutrophils were pre-treated with 5μM S1P or medium only for 1 hour, stimulated with 25ng/ml IL-8 for VCAM-1 or 100ng/ml IL-8 for ICAM-1, and then used in VCAM-1 or ICAM-1 flow-based adhesion assays. Bars represent means ± SEM from 3 independent channels in the same experiment. ns $p>0.05$

4.4 Discussion

Neutrophils isolated from human blood appeared to mainly express S1P receptors S1PR1 and S1PR4, at least at an mRNA level, with very low amounts of S1PR5. This result partly agrees with Rahaman et al., who suggested neutrophils express the receptors S1PR1, S1PR4 and S1PR5 (Rahaman *et al.*, 2006). CD69 is an early activation marker on T cells, which interferes with their egress from lymphoid organs by crosslinking with S1PR1 (Shiow *et al.*, 2006). In regards to CD69 expression by neutrophils, they appear to express it at transcript level, but not on the cell surface, immediately after isolation. This seems to agree with findings of an intracellular storage of CD69, so that it can be rapidly relocated to the cell surface after activation with PMA or fMLP (Gavioli *et al.*, 1992; Noble *et al.*, 1999). However, no such relocation was observed after short PMA treatment. On the other hand, longer treatment with GM-CSF appeared to induce CD69 surface expression, a result that had also been observed before by Atzeni et al. (Atzeni *et al.*, 2002). Interestingly for any connections between CD69 and S1P, when neutrophils were treated with GM-CSF, leading to an upregulation of CD69 on the cell surface, S1PR1 was downregulated on an mRNA level, a result reminiscent of the effect CD69 can have on S1PR1 expression on lymphocytes (Shiow *et al.*, 2006).

Neutrophils not only express S1P receptors, but when treated with S1P, downstream signalling can be observed as well. This was shown by phosphorylation of several kinases and signalling molecules. Moreover, S1P pre-treatment appears to enhance IL-8 modulated signalling. More specifically the ERK1/2 pathway appears to be mostly affected by S1P signalling. This pathway seems to be involved in several neutrophil functions, including neutrophil adhesion, degranulation, oxidative burst, the formation of neutrophil extracellular traps (NET), IL-8 production, etc (Sue *et al.*, 1997; Capodici *et al.*, 1998; Alvarez *et al.*, 2006; Hakkim *et al.*, 2011).

In regards to neutrophil chemotaxis towards IL-8, S1P did not appear to have a consistent effect. This was in contrast to what has been shown in the past, where S1P was suggested to inhibit neutrophil chemotaxis towards IL-8, as well as fMLP (Kawa *et al.*, 1997; Rahaman *et al.*, 2006). There was some inhibition shown in some experiments, but is seems that the donor of the neutrophils can affect the results. Moreover, different techniques for chemotaxis assays and different counting methods might account for the disagreement in our data. Interestingly, when neutrophils were

incubated overnight before being used for chemotaxis, S1P seemed to increase their chemotactic ability which was highly decreased compared to fresh neutrophils. However this effect was not seen when neutrophils were treated overnight with GM-CSF, causing an increase in their CD69 expression; indicating a possible link between CD69 and S1P signalling.

As with neutrophil chemotaxis, neutrophil adhesion under flow conditions did not seem to be affected, at least in a consistent manner, by S1P. This result is in agreement with the findings of Kawa et al, who did not observe any effects of S1P on neutrophil adhesion to HUVEC cells, although S1P inhibited neutrophil trans-endothelial migration through the same cells (Kawa *et al.*, 1997). On the other hand, Florey and Haskard found that S1P can enhance immune complex mediated neutrophil adhesion under flow conditions; although in the current study it could not enhance IL-8 mediated neutrophil adhesion (Florey and Haskard, 2009a).

To sum up, S1P can signal on neutrophils through the receptors S1PR1 and S1PR4 to cause activation of downstream signalling cascades, like ERK1/2. IL-8 mediated signalling is also enhanced by S1P. However, this signalling does not appear to translate into an effect to neutrophil migration or adhesion. The neutrophil functions that are affected by S1P signalling need to be further investigated.

CHAPTER 5

Effects of S1P on endothelial cells

Chapter 5. Effects of S1P on endothelial cells

5.1 Introduction

S1P can affect neutrophils directly or it can affect them indirectly, by having an effect on endothelial cells. This can result in either a negative or a positive outcome on neutrophil migration. S1P effects on endothelial cells that would have an impact on neutrophil trans-endothelial migration would be effects on endothelial chemokine production, adhesion molecule expression, or endothelial barrier function.

An important chemokine responsible for neutrophil migration to the sites of inflammation is CXCL8 (or IL-8) (see chapter 1). There are several studies reporting IL-8 inducing effects of S1P on many different types of cells (Lin *et al.*, 2006; Oz-Arslan *et al.*, 2006; Milara *et al.*, 2009). More thoroughly investigated are the cells of the respiratory system, with human bronchial epithelial cells (Cummings *et al.*, 2002), alveolar epithelial cells (Milara *et al.*, 2009), and airway smooth muscle cells (Rahman *et al.*, 2014) reported to produce increased IL-8 after S1P stimulation. However, S1P can also induce IL-8 production in completely diverse types of cells, from immature dendritic cells (Oz-Arslan *et al.*, 2006) to human retinal pigment epithelial cells (Qiao *et al.*, 2012). In regards to endothelial cells which are the focus of this study, there is a report of S1P inducing IL-8 production of human umbilical vein endothelial cells (HUVEC) (Lin *et al.*, 2006). Adhesion molecule expression of HUVEC cells has also been reported to be induced by S1P (Shimamura *et al.*, 2004), with the same being shown for other types of cells too, including vascular smooth muscle cells (Yogi *et al.*, 2011), alveolar epithelial cells (Milara *et al.*, 2009) and neuroblastoma cells (Costello *et al.*, 2011).

S1P may also affect endothelial cell barrier functions. According to several studies, exogenous S1P promotes endothelial barrier integrity and decreases endothelial permeability (Garcia *et al.*, 2001; Singleton *et al.*, 2005; Itagaki *et al.*, 2007). This effect appears to be mediated by S1PR1 signalling (Singleton *et al.*, 2005). On the other hand, S1PR2 and S1PR3 signalling appears to have the opposite effect, disrupting the endothelial barrier and increasing vascular permeability (Singleton *et al.*, 2006; Sanchez *et al.*, 2007). Furthermore, increased permeability can be also mediated by endogenously generated S1P, as was revealed by the use of a sphingosine kinase inhibitor, resulting in decreased endogenous S1P synthesis, and inhibition of thrombin -

and histamine - induced increase in permeability (Itagaki *et al.*, 2007). The mechanisms behind the effects of S1P on endothelial barrier integrity are not thoroughly understood, but it appears S1P can cause cytoskeletal rearrangement, and specifically actin filament assembly (Garcia *et al.*, 2001; Singleton *et al.*, 2005).

For the purpose of this study, S1P effects on endothelial cells that could indirectly affect neutrophil migration were investigated. Firstly, induction of IL-8 and other chemokines expression was examined. Moreover, the effects of S1P on endothelial barrier were studied, by neutrophil trans-endothelial migration and permeability assays. The mechanism for these effects was also explored. Finally, effects on neutrophil adhesion to endothelial cells were investigated, by measuring adhesion molecule expression of endothelial cells and performing flow-based adhesion assays.

5.2 Materials and Methods

5.2.1 ELISA

In Enzyme Linked Immunosorbant Assays (ELISA) of the "sandwich" format, an appropriate capture antibody is added to ELISA plate wells, followed by the sample that contains the protein of interest. The protein is captured by the antibody, and cannot be removed after washing, and then a biotinylated detection antibody is added that binds to the target protein as well, creating a "sandwich" of antibody-protein-antibody. Finally, avidin-peroxidase is added, which binds the biotinylated antibody, and allows detection after addition of appropriate substrate. A protein standard is used together with the samples, diluted in a series of known concentrations, to create a standard curve, in order to calculate protein concentration from absorbance values.

ELISA assay for IL-8 was performed using the Human IL-8 ELISA Development Kit from PeproTech (UK), following the instructions provided. Capture antibody (rabbit anti-hIL-8) was diluted appropriately in PBS and added to wells of a specially designed 96-well ELISA plate (Immulon, Fisher Scientific, UK). After overnight incubation at room temperature, plate was washed 4 times with wash buffer (0.05% Tween-20 in PBS) and blocked using 1% BSA in PBS as a block buffer, for 1 hour in room temperature. Plate was again washed 4 times and samples were added in triplicate in wells after being diluted in diluent (0.1% BSA in PBS-Tween) if needed. Recombinant human IL-8 was used as standard, diluted to five different concentrations from 1ng/ml to zero in diluent, which were also added in the plate in triplicate. After incubating for 2 hours at room temperature, plate was washed in the same manner and incubated with detection antibody (biotinylated rabbit anti-hIL-8), appropriately diluted in diluent, for another 2 hours. Plate was then washed and incubated with Avidin-Peroxidase (Avidin-HRP conjugate) diluted in diluent, for 30 minutes at room temperature. After another round of washes, plate was incubated with a substrate for peroxidase, O-phenylendiamine (OPD) solution (0.4 mg/ml OPD, 50 mM citrate pH 5.0 and 0.012 % (v/v) H_2O_2) for 5-15 minutes at room temperature. The reaction was terminated by addition of 2M H_2SO_4. Absorbance at 492 nm was measured at a plate reader. Standard curve was created and unknowns calculated using Prism 3.0 software (Graphpad).

5.2.2 RT-qPCR

RNA was isolated using the RNeasy Plus Mini Kit from QIAGEN, following the company's protocol (chapter 2.5.1). Reverse transcription for production of cDNA was performed using Tetro cDNA Synthesis kit (Bioline, UK; chapter 2.5.2). The cDNA was then used for detection of gene expression by real time PCR, using TaqMan® Gene Expression assays (Applied Biosystems) as primers (see chapter 2.5 for full list), with the exception of *S1PR2* primer-probe which was bought from QIAGEN (Hs_S1PR2_FAM_1 QuantiFast Probe Assay), using the SensiFAST™ Probe Hi-ROX Master Mix (Bioline, UK) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) qPCR instrument. The house-keeping gene *GAPDH* was used as the reference gene. Data were analysed using the $\Delta\Delta C_T$ method (chapter 2.5.3).

5.2.3 Chemokine array

The Human Chemokine Array Kit (R&D Systems, UK) was used, according to the instructions provided. HUVEC cells were treated overnight with 10μM S1P or left untreated, and their supernatants were collected to be used in the chemokine array. Array contains nitrocellulose membranes with capture antibodies for 31 different chemokines in duplicate. Membranes were blocked using provided block buffer for 1hour at room temperature on a rocking platform. Samples were prepared by appropriate dilution with provided buffers as per manufacturer's instructions. Detection antibody cocktail was added and the samples were incubated for 1 hour at room temperature. Blocking buffer was removed from membranes, and was replaced with samples in detection antibody cocktail. Membranes were incubated overnight at 4 ^{o}C on a rocking platform. They were then washed with provided wash buffer 3 times, and incubated with appropriately diluted Streptavidin-HRP solution in blocking buffer for 30 minutes at room temperature on a rocking platform. After washing again, membranes were incubated with provided chemiluminescence reagent mix and developed using X-ray film as in western blotting (chapter 2.6.4). Results were analysed by densitometry using ImageJ software.

5.2.4 Neutrophil trans-endothelial chemotaxis assay

HMEC-1 or HUVEC endothelial cells were cultured on top of 3μm pore membrane inserts (chapter 2.4). When confluent, they were treated overnight with different concentrations of desired molecules, usually S1P or analogs, in serum-free media (same

as their complete media, except FBS was replaced by 0.5% fatty acid free BSA). Isolated human neutrophils, around 200,000 cells, were added on the top chamber above the endothelial cells (media with treatment was removed first) and left to migrate towards the bottom chamber containing 10ng/ml IL-8 (R&D Systems) for 120 minutes (see chapter 2.4). Neutrophils that had passed through to the bottom chamber were measured by flow cytometry using counting beads (CountBright™ Absolute Counting Beads, Invitrogen).

5.2.5 Transmission Electron Microscopy

Samples were prepared as for trans-endothelial chemotaxis, with neutrophils added on top of 3μm pore membrane inserts coated with endothelial cells, and left to migrate for 2 hours. The inserts with the cells were then transferred into new plate with 4% glutaraldehyde in sodium cacodylate buffer for fixation. After 24-48 hours at 4 °C, the fixed inserts were given to Newcastle University Electron Microscopy Research Services, where they were further processed, resin embedded and cut into ultrathin sections for observation at a transmission electron microscope (TEM). Images of neutrophils in contact with endothelial cells were taken, focusing on the contact sites. No neutrophils passing through the cells and/or the membrane pores were found as was desired.

5.2.6 FITC-Albumin permeability assay

HMEC-1 or HUVEC endothelial cells were cultured to confluency on 3μm pore membrane inserts. They were treated overnight with 10μM S1P, 10μM CYM5442, 10μM CYM5541 or 10μM FTY720P, or left untreated. Bovine albumin conjugated with fluorescein isothiocyanate (FITC-albumin, Sigma-Aldritch) 50μM in medium was added on top of endothelial cells on inserts, replacing medium with treatment, and left to diffuse for 2 hours. FITC-albumin that had passed to the bottom chamber was measured by fluorescence at 485nm excitation / 528nm emission. A standard curve with known FITC-albumin concentrations was also created to calculate FITC-albumin concentration in samples. There was also a control insert without cells, to measure background membrane permeability.

5.2.7 Trans-Endothelial Electrical Resistance (TEER)

For measurements of trans-endothelial electrical resistance (TEER), the Millicel ERS-2 (Electrical Resistance System, Millipore Co) was used. This is a meter and electrode system that can be used to measure the electrical resistance of epithelial or endothelial cell monolayers. Cells were grown to confluency on 3 μm trans-well inserts (the same used for chemotaxis) and treated with desired agents overnight. An empty insert without cells was used for background resistance measurement. The electrode was sterilised in 70% ethanol for 15 minutes before measurements commenced, then rinsed in media. For measurement of resistance, the short tip of the electrode had to be inside the insert, while the longer tip was immersed in the media bellow the insert, at 90° angle. Resistance was recorded in all wells, including the empty insert, and then background resistance was substracted from all samples to measure true tissue resistance.

5.2.8 Western Blotting

Confluent HMEC-1 or HUVEC cells were treated or not with 10μM S1P, overnight, then lysed and protein measured with BCA protein assay (see chapter 2.6). Samples with loading buffer were loaded on SDS-PAGE together with a protein ladder; same amount of protein was loaded per sample. Gel was run at 30mA for about an hour. Western blotting "sandwich" was prepared, with sponges, whattman paper, the gel and a piece of PVDF membrane, and run in transfer buffer. Membrane was blocked and stained with primary antibody anti- phospho VE-cadherin Y658 (Abcam) overnight. After washing the membrane, appropriate secondary anti-mouse antibody, HRP conjugated, was added and incubated for 1 hour. Finally, membrane was washed, incubated with chemiluminescent substrate and developed in X-ray film. Membrane was then stripped and stained again with control antibody for total VE-cadherin (Abcam).

5.2.9 Flow cytometry

Flow cytometry was performed mainly as described in chapter 2.3. After treatment, adherent cells were detached from substrate using 10mM EDTA in PBS or cell dissociation buffer enzyme-free PBS-based (GIBCO, Invitrogen), since an enzymatic dissociation (ie. trypsin), could digest surface proteins of interest. Moreover, 1mM EDTA was added in the FACS Buffer (2% FBS in PBS) to avoid cell clumping. Cells were washed, counted and added in flow cytometry tubes. Fc receptor blocking was

performed and cells were incubated with directly conjugated primary antibodies ICAM-1 and VCAM-1 (BioLegend) for 45 minutes at 4 °C. After washing and resuspending in FACS buffer, acquisition was performed in a FACS Canto II (BD Biosciences) instrument. Analysis of results was carried out using FlowJo 7.6 (Treestar) software.

5.2.10 Cellix flow-based adhesion assays with endothelial cells

For adhesion assays with endothelial cells, the VenaEC biochips were used (see chapter 2.7). Substrates were sterilized by UV light for 30 minutes, then HMEC-1 or HUVEC endothelial cells were cultured on top of them, inside 6-well plates. When almost confluent, they were treated overnight with 10μM S1P or 100ng/ml TNF-α or both, or left untreated, and then the biochip was assembled and connected to Cellix device. Cellix device and *VenaFlux* software were switched on and initialised. After washing the pump connections, the biochip was mounted on the microscope stage and the channel to be used was briefly washed. Neutrophils were stained with CFSE after isolation before being added in the Cellix biochip channel. After neutrophils were added in one end of the channel (about 200,000 cells), flow was initiated at a shear stress of 0.5 dyne/cm^2. Several representative fields of view were captured along the channel's length, in both optical and fluorescent view. This was repeated for all the channels used in the experiment. For each image captured, adherent cells were measured and results analysed using Prism3 software (GraphPad Software Inc).

5.3 Results

5.3.1 Chemokine expression induction by S1P

5.3.1.1 Optimization using A549 cell line

There have been studies where S1P has been shown to induce various kinds of cells to produce chemokines. More specifically, the chemokine CXCL8 (IL-8), a major neutrophil chemoattractant, has been shown to be produced by several diverse cell types when treated with S1P, including alveolar epithelial cells (Milara *et al.*, 2009). The A549 cell line is an alveolar epithelial cell line that was used to show IL-8 induction by S1P (Milara *et al.*, 2009). Before moving on to investigating endothelial cells, A549 cells were used to optimize the techniques to measure chemokine induction by S1P. A549 cells (see chapter 2.1) were chosen as they are very easy to grow and maintain, compared to endothelial cells (especially primary cells), and have been studied before in regards to IL-8 production after S1P stimulation (Milara *et al.*, 2009).

A549 cells were treated with different concentrations of S1P and then the supernatants were used in an IL-8 ELISA assay. It appears that 1, 5 or 10μM of S1P all cause a significant increase in IL-8 secretion after 24 hours treatment, with 10μM being the most potent. The S1P analogs SEW2871 and FTY720P did not have an effect in IL-8 secretion (figure 5.1). After 24 hours of S1P treatment there was a significant increase in secreted IL-8; the same appeared to be when the cells were treated with S1P for 48 hours. After 72 hours though, the effect was reduced (figure 5.2). At less than 24 hours treatment, as little as 4 hours were sufficient to cause a significant increase in IL-8 production compared with untreated cells, and 8 hours also caused IL-8 secretion. The S1PR1 agonist CYM-5442 appeared to also effectively increase IL-8 secretion, especially after 8 hours treatment, revealing S1PR1 as an important receptor for the effect on IL-8 production that S1P has (figure 5.3).

To further investigate any correlations of IL-8 induction by S1P on A549 cells, with possible effects on endothelial cells, a comparative real-time PCR was performed to measure mRNA expression of S1P receptor genes on A549 and endothelial cell lines HMEC-1 and Eahy926. It was found that S1PR1, although expressed at high levels in all three cells, it was 100-250 fold higher in the endothelial cell lines. On the other hand, S1PR3 was expressed by A549 cells, but barely expressed by the endothelial cells. S1PR4 was expressed at very low levels by all three cells, whereas S1PR5 was

expressed at relatively high levels by A549 and HMEC-1 cells, but barely expressed by Eahy926 (figure 5.4).

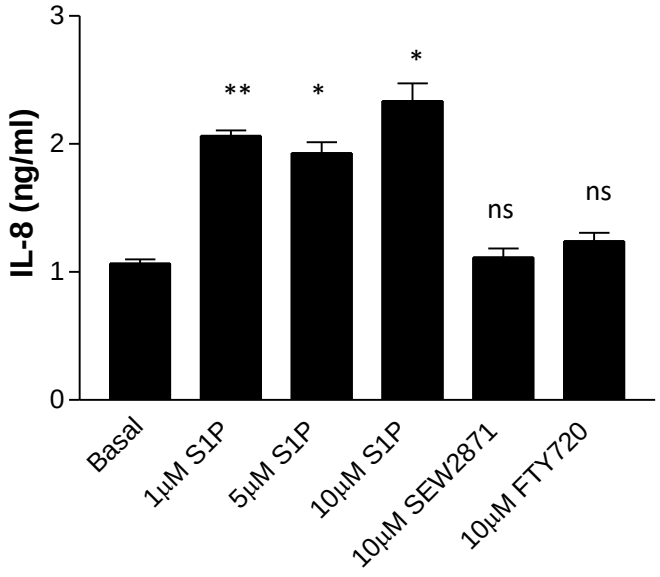


Figure 5.1. IL-8 production by A549, after stimulation with S1P and analogs. A549 cells in 6-well plates were treated with different concentrations of S1P, 10μM SEW2871, 10μM FTY720 or medium only (basal) for 24 hours. Supernatants were collected and used at an IL-8 ELISA experiment. Bars represent means ± SEM. Data representative of 3 independent experiments. * $p<0.05$, ** $p<0.01$, ns $p>0.05$ compared with basal.

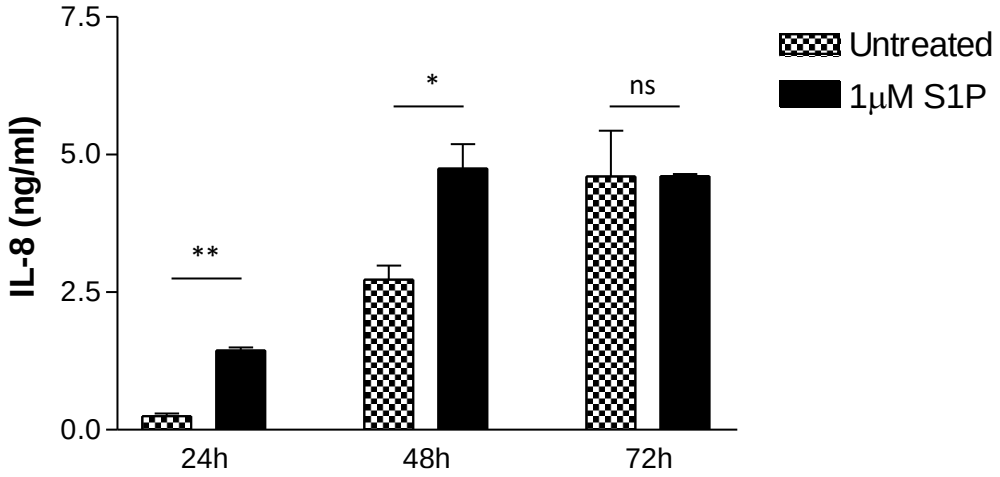


Figure 5.2. IL-8 production by A549, time course. A549 cells in 6-well plates were either treated with 1μM S1P or left untreated, for 24 - 72 hours. Supernatants were collected and used at an IL-8 ELISA experiment. Bars represent means ± SEM. Data representative of 3 independent experiments. * $p\leq0.05$, ** $p<0.01$, ns $p>0.05$

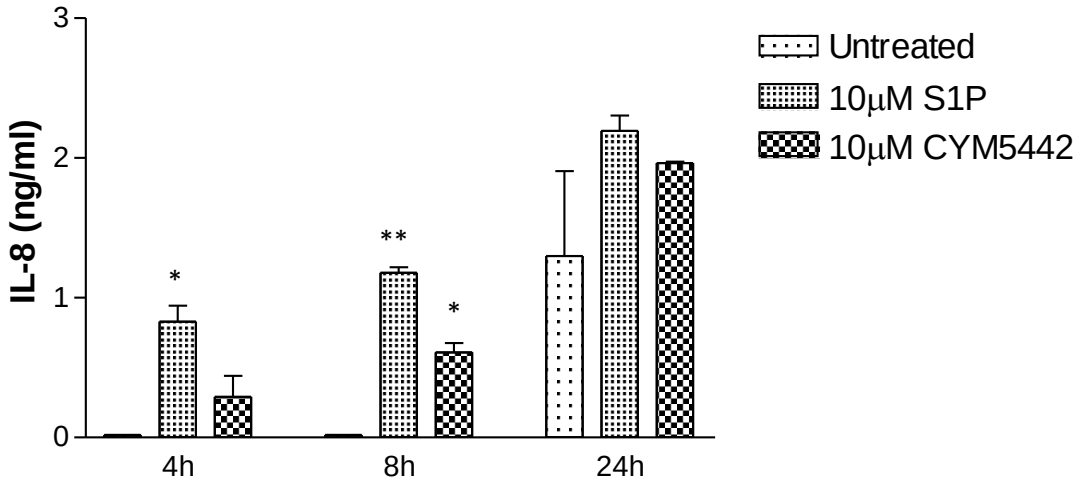


Figure 5.3. IL-8 production by A549, time course under 24 hours. A549 cells in 6-well plates were either treated with 10μM S1P, 10μM CYM5442 or left untreated, for 4 - 24 hours. Supernatants were collected and used at an IL-8 ELISA experiment. Bars represent means ± SEM. Data representative of 3 independent experiments. * $p \leq 0.05$, ** $p < 0.01$, ns $p > 0.05$ compared with untreated.

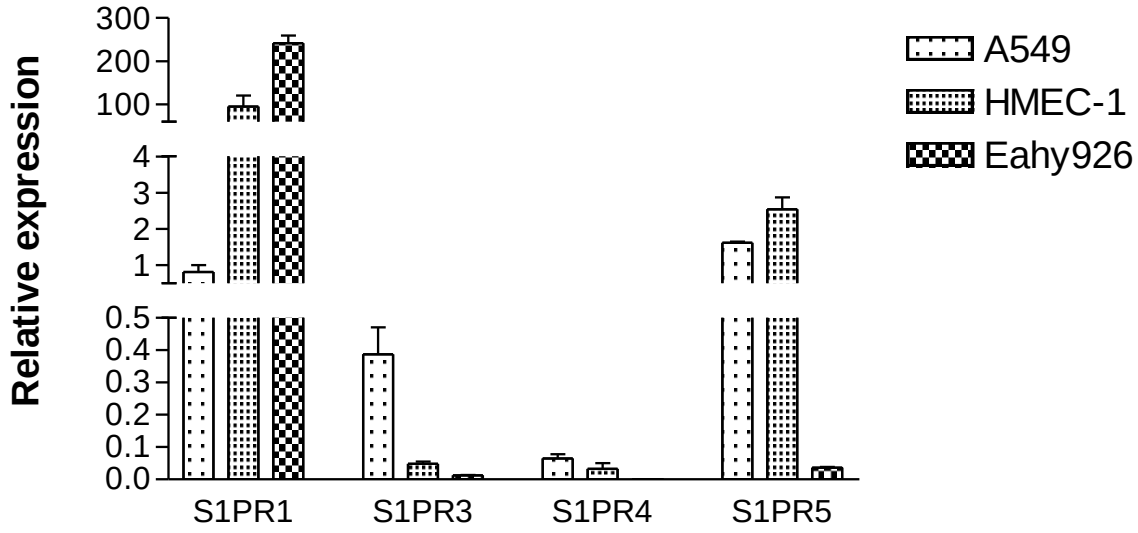


Figure 5.4. Comparison of S1P receptor mRNA expression between A549 and endothelial cell lines. S1P receptor mRNA expression by A549, HMEC-1 and Eahy926 cell lines was measured by real-time PCR. Data were normalized for the housekeeping gene GAPDH and presented as relative expression compared with the expression of S1PR1 by A549. Bars represent means ± SEM.

5.3.1.2 IL-8 expression induction

To determine whether S1P can induce IL-8 expression by endothelial cells, the endothelial cell line HMEC-1 was used first. When S1P concentrations of 1-10μM were used, it was found that 5 or 10μM S1P could cause increased IL-8 secretion by HMEC-1 cells, as was determined by IL-8 ELISA assay. Compared with the positive control TNF-α, this induction was much lower (figure 5.5). When HMEC-1 cells were treated with 10μM S1P for 4, 8 or 24 hours, there was an increase in IL-8 secretion, significant only after 24 hours treatment. Treatment with the S1PR1 agonist CYM5442 though caused significant increase in IL-8 secretion at 4 hours treatment as well as 24 hours (figure 5.6).

To verify these results, a real-time qPCR was performed using mRNA from HMEC-1 cells treated with 10μM S1P or CYM5442 for 4, 8 or 24 hours, to determine whether mRNA expression of *IL-8* correlated with levels of secreted chemokine. It was found that at only 4 hours treatment with either molecule the expression of *IL-8* had increased significantly, but at 8 and 24 hours it had declined to normal levels again (figure 5.7). This can be understood, since there is a delay between the increase in mRNA expression and the increase in protein synthesis and eventually protein secretion.

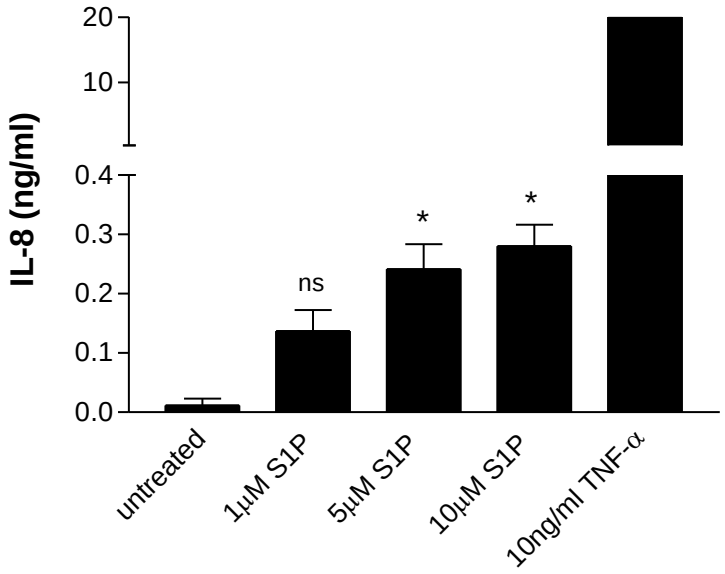


Figure 5.5. IL-8 production by HMEC-1 after S1P stimulation. HMEC-1 endothelial cells in 6-well plates were treated for 24 hours with different concentrations of S1P, or 10ng/ml TNF-a as a positive control. Supernatants were collected and used at an IL-8 ELISA experiment. Bars represent means ± SEM. Data representative of 3 independent experiments. * $p<0.05$, ns $p>0.05$ compared with untreated.

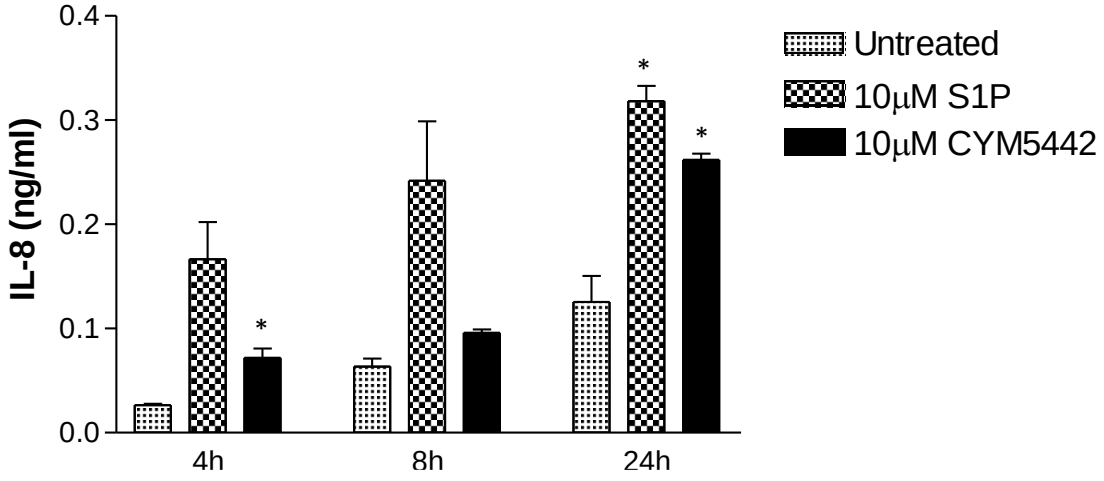


Figure 5.6. IL-8 production by HMEC-1, time course. HMEC-1 cells in 6-well plates were either treated with 10μM S1P, 10μM CYM5442 or left untreated, for 4 - 24 hours. Supernatants were collected and used at an IL-8 ELISA experiment. Bars represent means ± SEM. Data representative of 2 independent experiments. * $p\leq0.05$, ns $p>0.05$ compared with untreated.

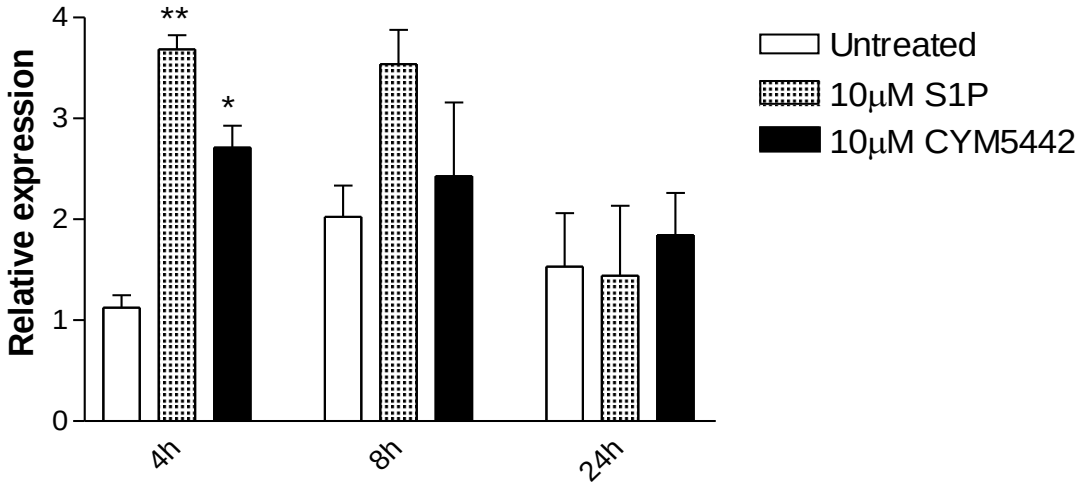


Figure 5.7. IL-8 mRNA expression by HMEC-1, time course. HMEC-1 cells in 6-well plates were either treated with 10μM S1P, or 10μM CYM5442 or left untreated, for 4 - 24 hours. IL-8 mRNA expression was measured by real-time PCR. Data were normalized for the housekeeping gene GAPDH. Bars represent means ± SEM. Data representative of 2 independent experiments. * $p \leq 0.05$, ** $p < 0.01$, ns $p > 0.05$ compared with untreated.

To further investigate effects of S1P on IL-8 expression by endothelial cells, primary endothelial cells were used next, specifically HUVEC cells (see chapter 2.1). First, a comparison of S1P receptor mRNA expression was made between HUVEC and HMEC-1 cells, by real time qPCR. Although both cells expressed high levels of *S1PR1*, HUVEC expressed 3-fold more than HMEC-1. On the other hand, *S1PR2* was highly expressed by HMEC-1 cells, in even higher levels than *S1PR1* in HUVEC was expressed, whereas HUVEC expressed it in much lower levels. Similarly, *S1PR5* was expressed in relatively high levels by HMEC-1, but barely expressed by HUVEC. The opposite occurs with *S1PR4*, which was expressed by HUVEC, but barely expressed by HMEC-1. As for *S1PR3*, both cells only expressed it at minimal levels (figure 5.8).

Next, HUVEC cells were used in ELISA assays for IL-8. As a further control, after supernatants were collected to be used in ELISA, the cells were then lysed and protein concentration was measured by BCA assay, to show that all treatments resulted in similar amount of total protein, with no significant variation, as was shown by ANOVA analysis (figure 5.9). Cells were treated with 10μM S1P, the S1PR1 agonist CYM5442, or the S1PR3 agonist CYM5541. After 24 hours treatment, all molecules caused significant increase in IL-8 secretion. S1P and CYM5442 also increased IL-8 secretion at 4 and 8 hours treatment as well (figure 5.10). It appears that both S1PR1 and S1PR3 are important for IL-8 expression by endothelial cells, but probably more than one receptor has to be induced for optimal results.

These results were partly verified by real time qPCR for IL-8 mRNA expression. When HUVEC were treated with S1P for 24 hours, there was a significant increase in IL-8 expession. CYM5442 treatment did not seem to have an effect (figure 5.11). Perhaps, as was the case in HMEC-1, treatment for less amount of time would result in increased mRNA expression which was then ablated after 24 hours treatment.

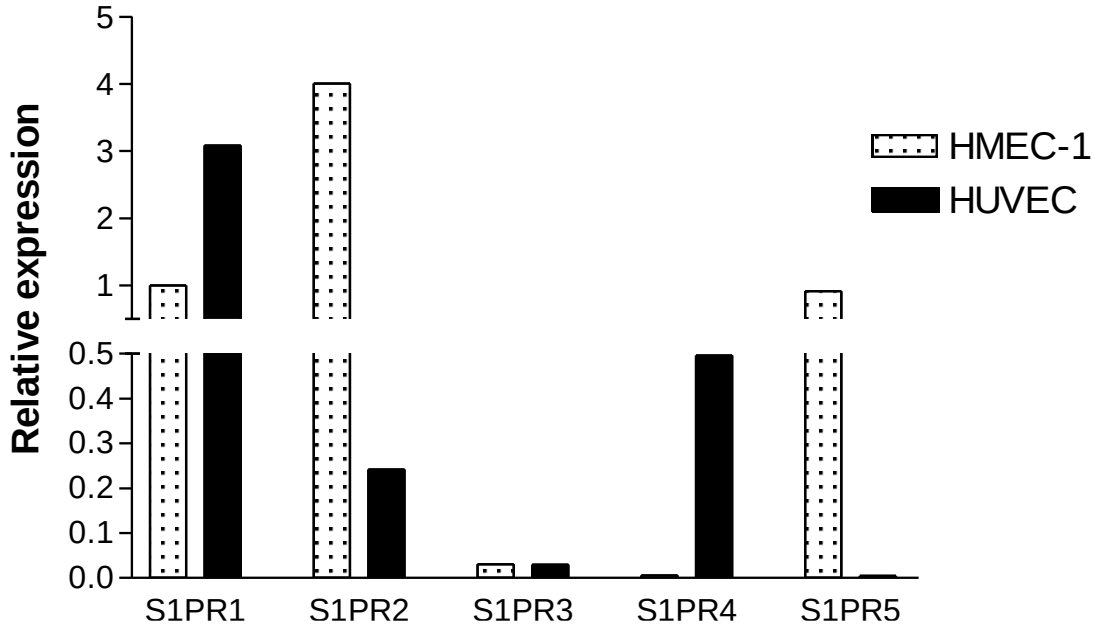


Figure 5.8. Comparison of S1P receptor mRNA expression between HMEC-1 cell line and HUVEC primary endothelial cells. S1P receptor mRNA expression by HMEC-1 cell line and HUVEC cells was measured by real-time PCR. Data were normalized for the housekeeping gene GAPDH and presented as relative expression compared with the expression of S1PR1 by HMEC-1.

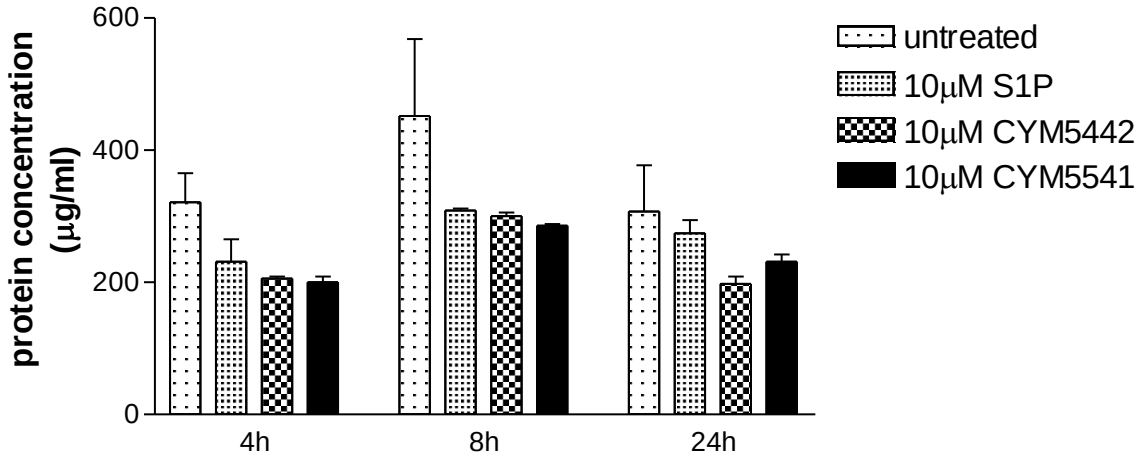


Figure 5.9. Protein concentration of HUVEC cells after ELISA experiment. HUVEC cells in 6-well plates were treated with S1P or analogs, or left untreated, for 4 - 24 hours. Supernatants were collected and used at an IL-8 ELISA experiment. The cells were then lysed and protein concentration was measured using a BCA assay.

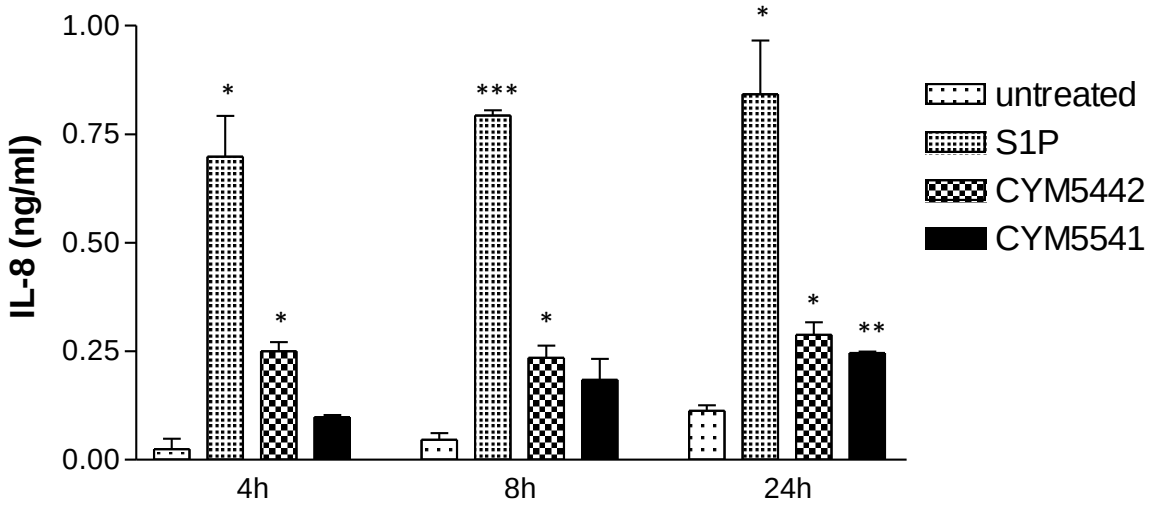


Figure 5.10. IL-8 production by HUVEC, time course. HUVEC cells in 6-well plates were either treated with 10µM S1P, 10µM CYM5442, or 10µM CYM5541, or left untreated, for 4 - 24 hours. Supernatants were collected and used at an IL-8 ELISA experiment. Bars represent means ± SEM. Data representative of 3 independent experiments. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns $p > 0.05$ compared with untreated.

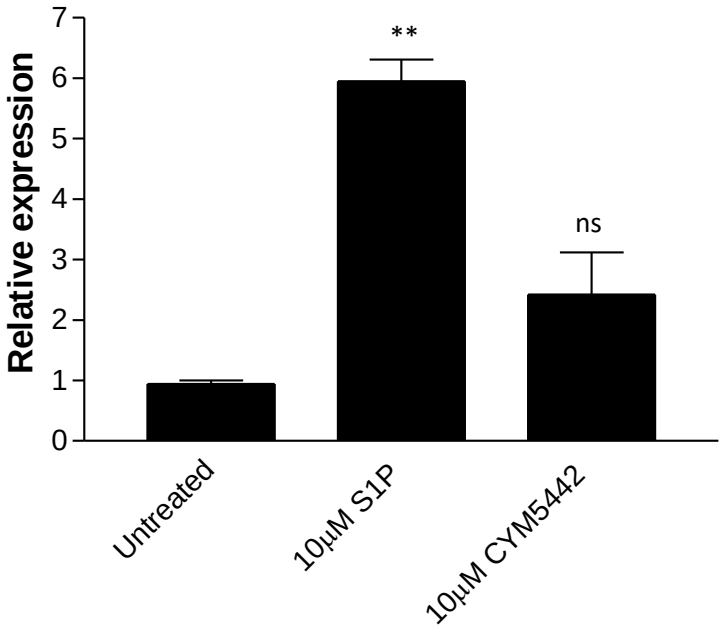


Figure 5.11. IL-8 mRNA expression by HUVEC. HUVEC cells in 6-well plates were either treated with 10µM S1P, or 10µM CYM5442 or left untreated, for 24 hours. IL-8 mRNA expression was measured by real-time PCR. Data were normalized for the housekeeping gene GAPDH. Bars represent means ± SEM. ** $p < 0.01$, ns $p > 0.05$ compared with untreated.

5.3.1.3 Chemokine array

After determining the effects S1P treatment has on IL-8 chemokine secretion by endothelial cells, the next step was to investigate possible effects on other chemokines. So a chemokine array was used, with supernatants from HUVEC cells untreated or treated with 10μM S1P for 24 hours (figure 5.12). The two chemokines that were most highly expressed after S1P treatment were CXCL8 and CCL2. As was mentioned before CXCL8 (or IL-8) is a major neutrophil chemoattractant. On the other hand, CCL2 (or MCP-1) is a chemoattractant for monocytes mainly, but also T cells, dendritic cells and other inflammatory cells, but not neutrophils. Other chemokines induced by S1P included CXCL1 (or GROα), another neutrophil chemoattractant, CX3CL1 (or fractalkine), a chemoattractant for T cells and monocytes, and CCL7 (or MCP-3), another monocyte chemoattractant. CXCL12 (or SDF-1), a lymphocyte chemoattractant, was secreted at very low levels after S1P treatment. However, compared with the untreated that did not secrete any of the above mentioned chemokines, this is significant ($p= 0.0171$). On the other hand, the chemokine CXCL10 (or IP-10), a chemoattractant for many cells including monocytes/macrophages, T cells, and dendritic cells, appeared to be secreted at very low levels by untreated cells and increase slightly after S1P treatment. However this increase was marginally not significant ($p=0.0528$). The non-chemokine molecules midkine and fibrinogen were included in this array as positive controls, as they were expressed by both untreated and S1P treated cells, and although there was a trend of midkine secretion decreasing and fibrinogen expression increasing by S1P treatment, these effects were not significant ($p= 0.0986$ and $p= 0.0907$ respectively) (figure 5.12).

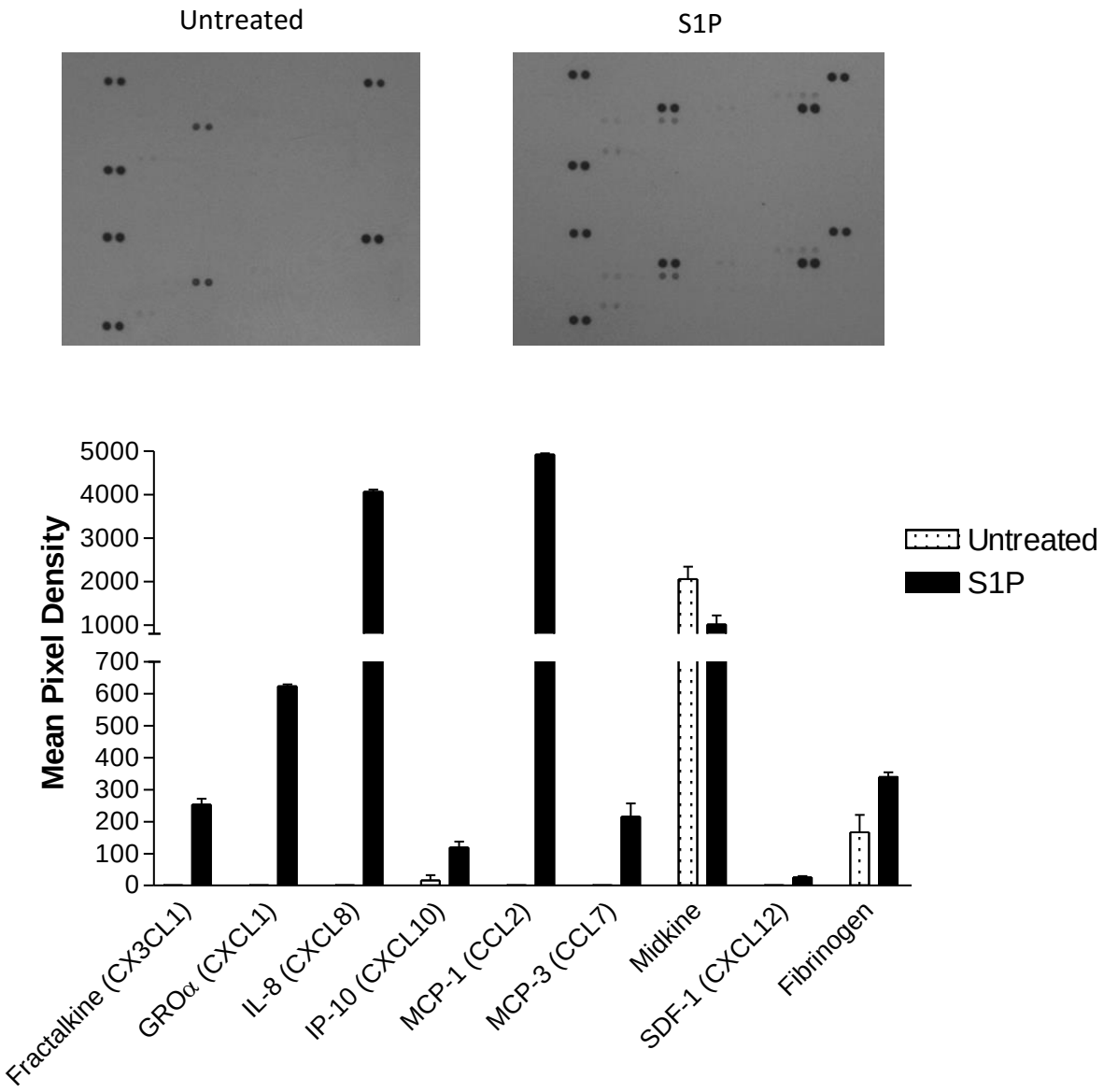


Figure 5.12. Chemokine array with HUVEC endothelial cells. HUVEC cells in 6-well plates were untreated or treated with 10μM S1P for 24 hours. Supernatants were then collected and used in a chemokine array. Top: images of the developed membrane arrays; duplicate membranes for untreated and S1P treated samples. Bottom: Diagram showing mean pixel densities for different chemokines in the array; bars represent means ± SEM.

5.3.2 Effects of S1P on endothelial barrier function

5.3.2.1 Neutrophil trans-endothelial migration

In order to further evaluate whether S1P can affect neutrophil migration through the endothelial cells, neutrophil trans-endothelial chemotaxis experiments were performed. HMEC-1 endothelial cells were grown on top of chemotaxis filters to form a monolayer, and then they were treated overnight with different concentrations of S1P, before a chemotaxis experiment with neutrophils at the top chamber and medium with 10ng/ml IL-8 at the bottom was performed. Concentrations of 1-10μM S1P were enough to cause significant decrease in neutrophil chemotaxis, whereas 0.1μM S1P was not (figure 5.13). As 5 and 10μM S1P appeared to be more potent, 10μM were chosen to be used in a similar experimental set up, with endothelial cells being treated with 10μM S1P, the S1PR1 agonist CYM5442, the S1PR3 agonist CYM5541 or the S1PR1, 3, 4 and 5 agonist FTY720P. S1P, CYM5442 and FTY720P all had similar inhibitory effects on neutrophil migration. On the other hand, CYM5541 appeared to have no effect. This result indicates S1PR1 but not S1PR3 as being responsible for inhibiting neutrophil migration through treated endothelial cells (figure 5.14).

Furthermore, these experiments were repeated with HUVEC primary endothelial cells. The results were similar, with all S1P concentrations apart from 5μM causing significant decrease in neutrophil chemotaxis through the treated HUVEC cells, and 10μM being the most potent (figure 5.15). Again, when the different S1P receptor agonists were used, S1P, CYM5442 and FTY720P caused a significant decrease in neutrophil chemotaxis, whereas CYM5541 did not have a significant effect, indicating S1PR1 as the major receptor responsible for inhibiting migration as was the case with HMEC-1 cells (figure 5.16). The effects S1P had on neutrophil migration through treated endothelial cells suggest S1P can enhance endothelial barrier function.

In order to observe the interactions between neutrophils and endothelial cells, HMEC-1 and HUVEC cells were set up in filters as for trans-endothelial chemotaxis assays, neutrophils were added on top and left to migrate towards IL-8. Then the membranes with the cells were fixed and prepared for observation by Transmission Electron Microscopy (TEM). Neutrophils can be observed in contact with endothelial cells (HUVEC) as they adhere to the endothelium and form pseudopodia in order to crawl on the endothelial surface seeking for appropriate regions to migrate through the endothelial monolayer (figure 5.17).

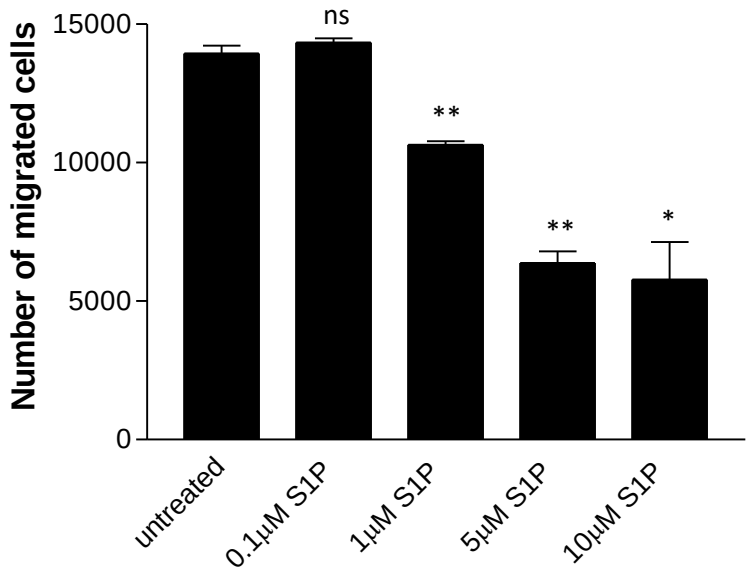


Figure 5.13. Neutrophil transendothelial migration through HMEC-1 treated with S1P concentrations. HMEC-1 endothelial cells on chemotaxis filters were treated overnight with different concentrations of S1P. Neutrophils were then added on top, and left to migrate for 120 minutes towards 10ng/ml IL-8. Cells that passed into the wells were measured by flow cytometry. Bars represent means ± SEM. Data representative of 4 independent experiments. * $p \leq 0.05$, ** $p < 0.01$, ns $p > 0.05$ compared with untreated.

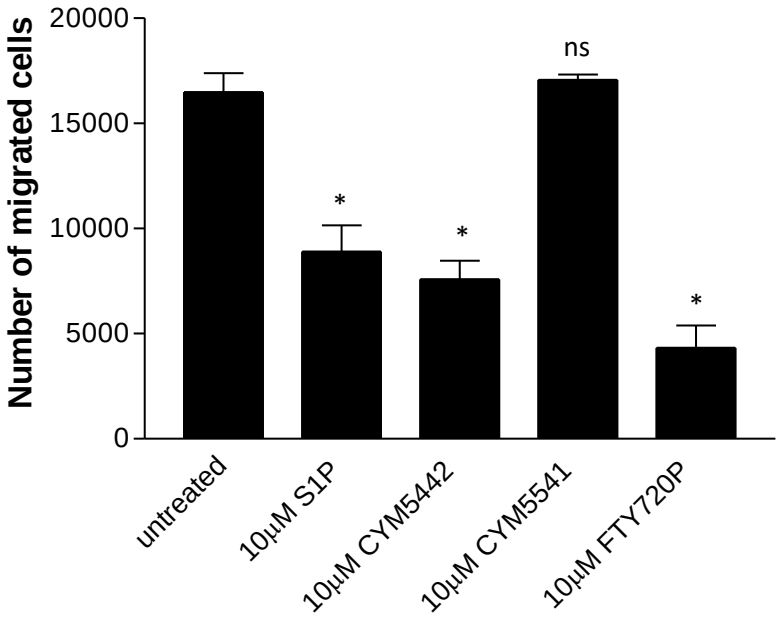


Figure 5.14. Neutrophil transendothelial migration through HMEC-1 treated with S1P analogs. HMEC-1 endothelial cells on chemotaxis filters were treated overnight with 10μM S1P, 10μM CYM5442, 10μM CYM5541, or 10μM FTY720P. Neutrophils were then added on top, and left to migrate for 120 minutes towards 10ng/ml IL-8. Cells that passed into the wells were measured by flow cytometry. Bars represent means ± SEM. Data representative of 4 independent experiments. * $p \leq 0.05$, ns $p > 0.05$ compared with untreated.

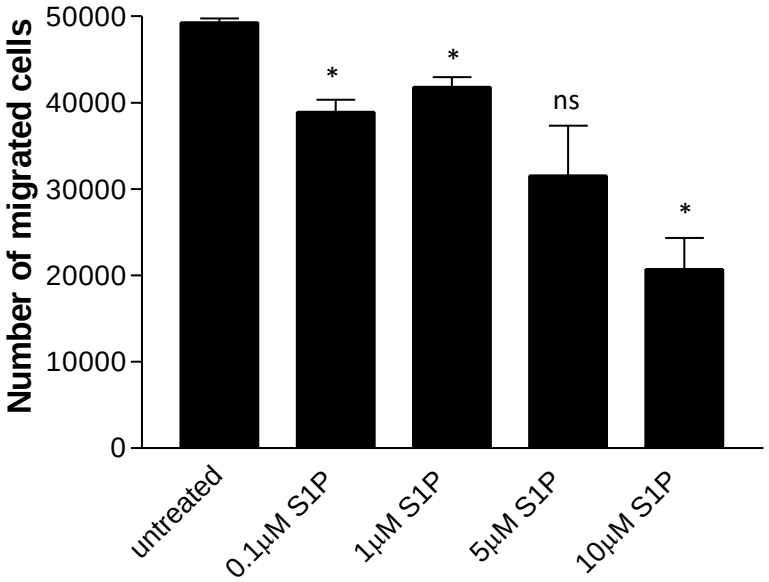


Figure 5.15. Neutrophil transendothelial migration through HUVEC treated with S1P concentrations. HUVEC cells on chemotaxis filters were treated overnight with different concentrations of S1P. Neutrophils were then added on top, and left to migrate for 120 minutes towards 10ng/ml IL-8. Cells that passed into the wells were measured by flow cytometry. Bars represent means ± SEM. Data representative of 4 independent experiments. * $p \leq 0.05$, ns $p > 0.05$ compared with untreated.

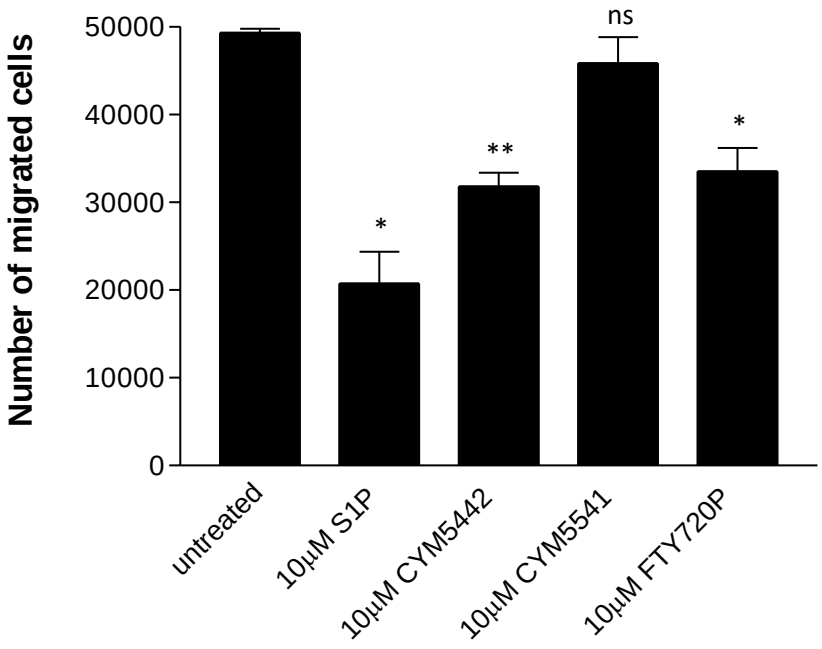


Figure 5.16. Neutrophil transendothelial migration through HUVEC treated with S1P analogs. HUVEC cells on chemotaxis filters were treated overnight with 10µM S1P, 10µM CYM5442, 10µM CYM5541, or 10µM FTY720P. Neutrophils were then added on top, and left to migrate for 120 minutes towards 10ng/ml IL-8. Cells that passed into the wells were measured by flow cytometry. Bars represent means ± SEM. Data representative of 4 independent experiments. * $p \leq 0.05$, ** $p < 0.01$, ns $p > 0.05$ compared with untreated.

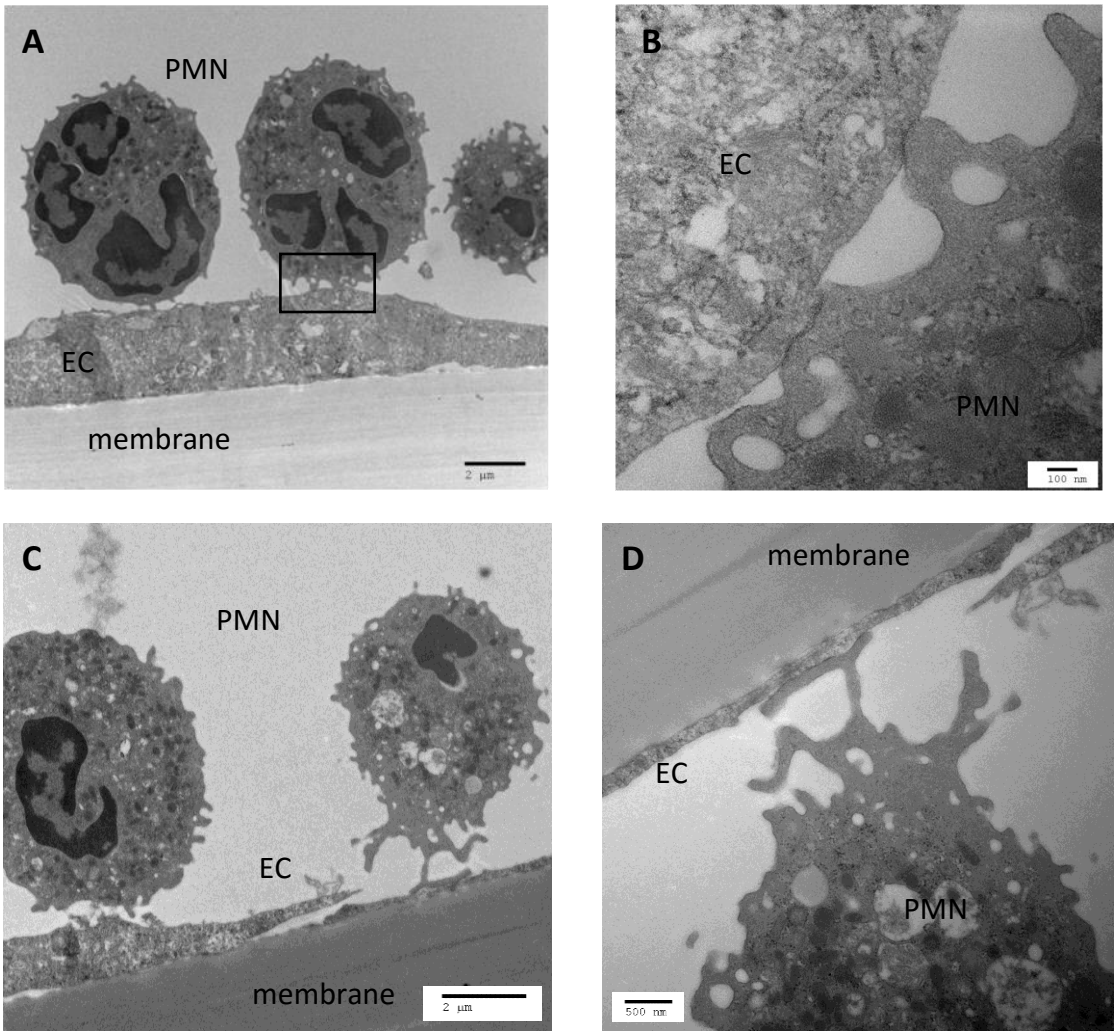


Figure 5.17. Electron Microscopy of neutrophil – endothelial cell interactions. Endothelial cells were set up on 3µm pore filters and neutrophils were added on top and left to migrate towards 10ng/ml IL-8 for 2 hours as in trans-endothelial chemotaxis assays. (A) Transmission Electron Microscopy (TEM) image of neutrophils interacting with HUVEC endothelial cell. (B) Higher magnification of the area in the square on B, showing the contact regions of a neutrophil to an endothelial cell. (C) TEM image of neutrophils forming pseudopodia to interact with HUVEC cells. (D) Higher magnification of C where the contact of the neutrophil to the endothelial cell by pseudopodia can be seen. PMN: neutrophils, EC: endothelial cells.

5.3.2.2 Endothelial permeability assays

As a way of verifying that S1P can indeed affect endothelial barrier functions, endothelial permeability assays using FITC conjugated bovine serum albumin, were performed. In these assays, HMEC-1 or HUVEC cells were again grown on top of perforated membranes to form monolayers, then treated overnight with S1P or S1P receptor agonists, before FITC-Albumin was added on the top chamber and left to diffuse to the bottom chamber. Amount of albumin that had diffused to the bottom chamber was measured by fluorescence at 485nm / 528nm, using a standard curve method to calculate concentration. The results showed a trend for decreased permeability after treatment for most agonists, although it was not significant. This effect was observed for both HMEC-1 and HUVEC endothelial cells (figures 5.18, 5.19 respectively).

Another method to assess endothelial barrier integrity is by measuring electrical resistance of the confluent endothelial cell monolayer. This was tried, before and after treatment with S1P and analogues, for HMEC-1 and HUVEC endothelial cells. However, the results were inconclusive due to very low overall measurements of resistance (data not shown). The low resistance measurements can be expected, since endothelial cells rarely form tight junctions *in vitro* (Burns *et al.*, 1997a), leading to gaps on the endothelial layer that decrease layer resistance.

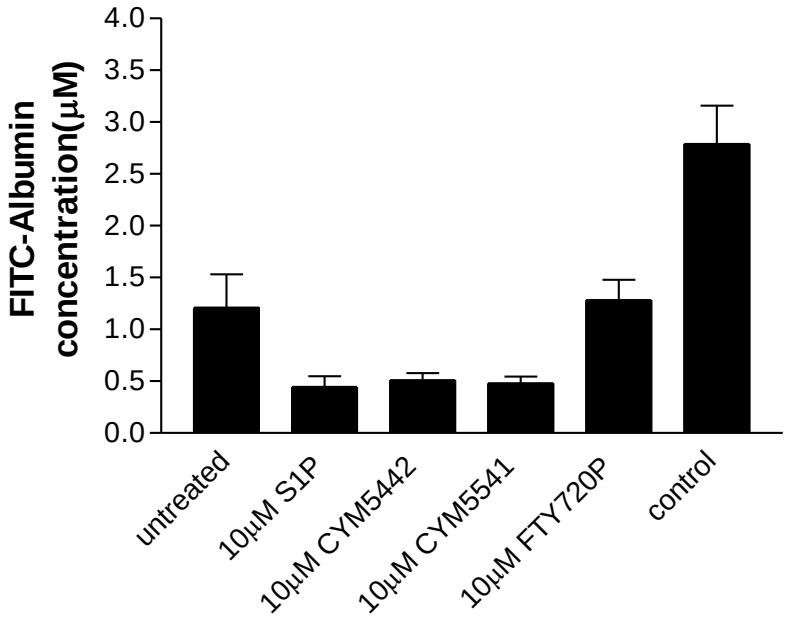


Figure 5.18. HMEC-1 FITC-Albumin permeability assay. HMEC-1 cells on filters were treated overnight with 10μM S1P, 10μM CYM5442, 10μM CYM5541, or 10μM FTY720P. FITC-Albumin was then added on top, and left to diffuse for 120 minutes. Fluorescence was then measured on the wells and FITC-Albumin concentration was calculated. There was also a control filter without any cells. Bars represent means ± SEM. Data representative of 4 independent experiments.

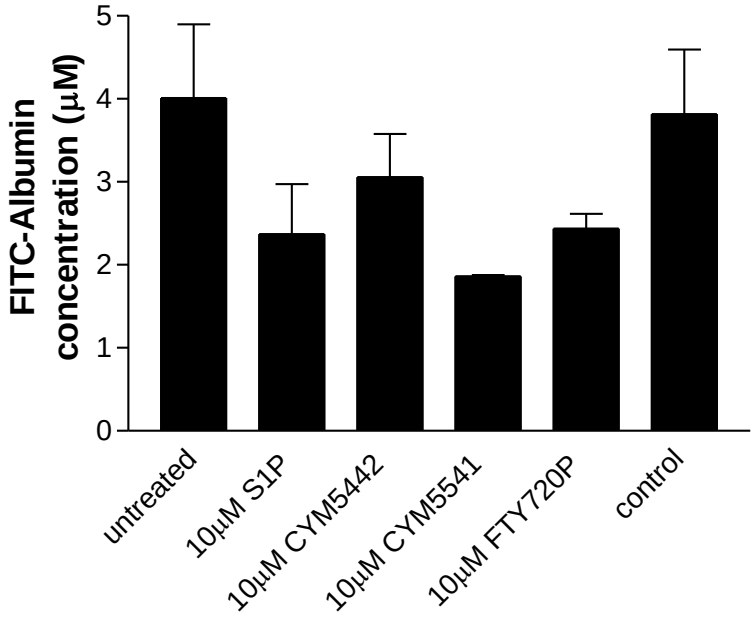


Figure 5.19. HUVEC FITC-Albumin permeability assay. HUVEC cells on filters were treated overnight with 10μM S1P, 10μM CYM5442, 10μM CYM5541, or 10μM FTY720P. FITC-Albumin was then added on top, and left to diffuse for 120 minutes. Fluorescence was then measured on the wells and FITC-Albumin concentration was calculated. There was also a control filter without any cells. Bars represent means ± SEM. Data representative of 3 independent experiments.

5.3.2.3 VE-cadherin phosphorylation

In an attempt to further look into the mechanisms behind the effects S1P has on endothelial barrier function, the endothelial adherens junction molecule VE-cadherin was investigated. VE-cadherin (or CD144) is a member of the cadherin superfamily of cell-cell junction molecules, expressed exclusively by endothelial cells, and is important in the regulation of endothelium permeability and barrier functions (see chapter 1). VE-cadherin can be phosphorylated at several sites, and most of these phosphorylations are linked with an increase in vascular permeability and / or increased leukocyte migration. The tyrosine 658 (Y658) phosphorylation site of VE-cadherin was chosen to investigate the effect S1P treatment has on endothelial barrier function, as it has been examined in regards to neutrophil migration in the past (Allingham *et al.*, 2007). Specifically, HMEC-1 or HUVEC cells were treated overnight with 10μM S1P and then lysed and used in a western blot experiment for phosphorylated VE-cadherin (Y658), with total VE-cadherin as control. Both untreated HMEC-1 and HUVEC appear to constitutively express phosphorylated VE-cadherin, but when treated with S1P, expression of phosphorylated VE-cadherin decreases (figure 5.20).

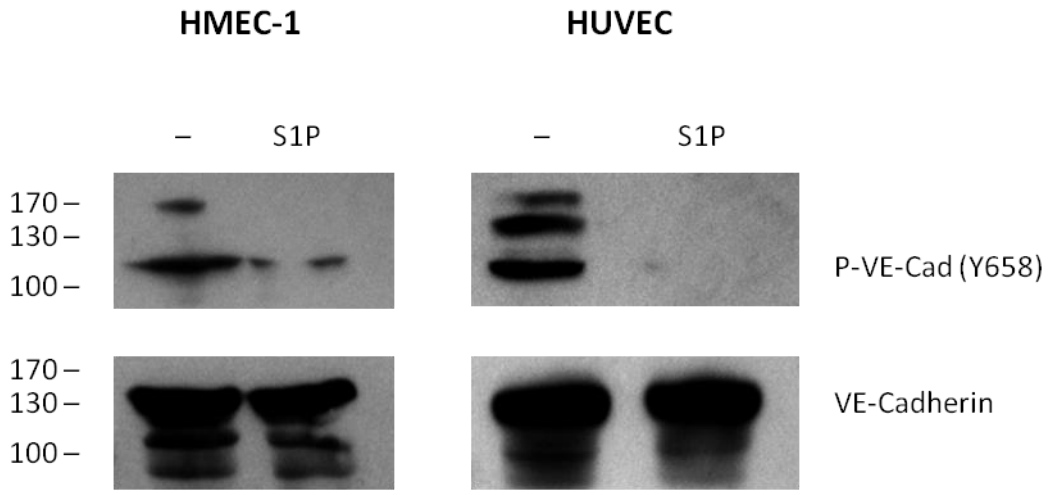


Figure 5.20. Expression of phospho-VE-Cadherin (Y658) after S1P treatment. HMEC-1 or HUVEC endothelial cells were treated overnight with 10μM S1P or left untreated. Lysates were then prepared and used in western blotting experiments with antibody for phosphorylated VE-Cadherin (Y658), as well as total VE-Cadherin as a loading control. Data representative of 2-3 independent experiments.

5.3.3 Effects of S1P on adhesion molecule expression

S1P could affect neutrophil migration indirectly by having an effect on endothelial cells' adhesion molecule expression. To investigate this, HMEC-1 endothelial cells were treated with S1P or TNF-α as positive control, as well as TNF-α with S1P together, and then used in flow cytometry experiment with antibodies for the adhesion molecules ICAM-1 and VCAM-1. All untreated cells expressed ICAM-1, but only around 15% expressed VCAM-1. S1P treatment seemed to increase both molecules' expression at a small degree. TNF-α on the other hand, caused major increase in expression of both ICAM-1 and VCAM-1, with the latter now being expressed by all cells. Interestingly, when cells were treated with TNF-α and S1P together, VCAM-1 expression appeared to decrease compared to cells treated with TNF-α alone. (figure 5.21, table 5.1)

HUVEC primary endothelial cells were also used in the same experimental set up, to measure expression of adhesion molecules ICAM-1 and VCAM-1. Around 75% of untreated HUVEC expressed ICAM-1, whereas VCAM-1 was expressed by only 17% of the cells. S1P treatment caused increased expression of both molecules, with ICAM-1 being expressed by all cells and VCAM-1 expressed by 78% of the cells. Again, TNF-α caused an even greater increase on ICAM-1 and VCAM-1 expression, with all cells expressing both molecules. Similar with HMEC-1, adding S1P together with TNF-α, caused a different expression profile, with VCAM-1 expression decreased compared to treatment with TNF-α alone. (figure 5.22, table 5.1)

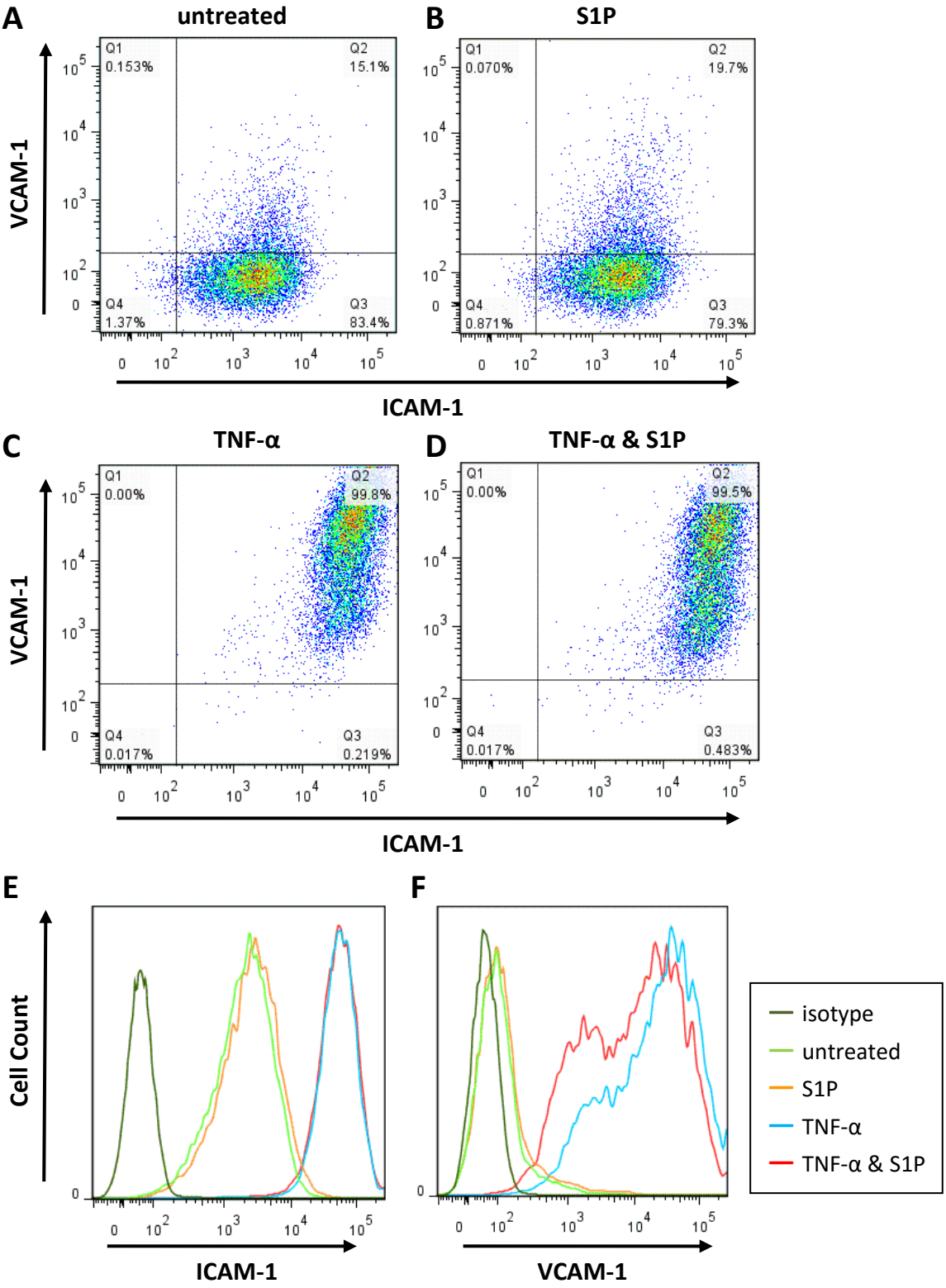


Figure 5.21. Effects of S1P on HMEC-1 adhesion molecule expression. HMEC-1 endothelial cells were treated overnight with 10μM S1P, 100ng/ml TNF-α, or 100ng/ml TNF-a & 10μM S1P or left untreated. They were then stained with directly conjugated monoclonal antibodies for ICAM-1 and VCAM-1 adhesion molecules or appropriate isotype controls and analysed by flow cytometry. (A-D) Dot plots of ICAM-1 and VCAM-1 expression simultaneously in untreated, treated with S1P, TNF-α, or TNF-α & S1P cells respectively. (E-F) Histograms presenting ICAM-1 or VCAM-1 expression respectively after different treatments. **Dark green**: isotype control, **light green**: untreated, **orange**: S1P, **blue**: TNF-α, **red**: TNF-α & S1P. Data representative of 3 independent experiments.

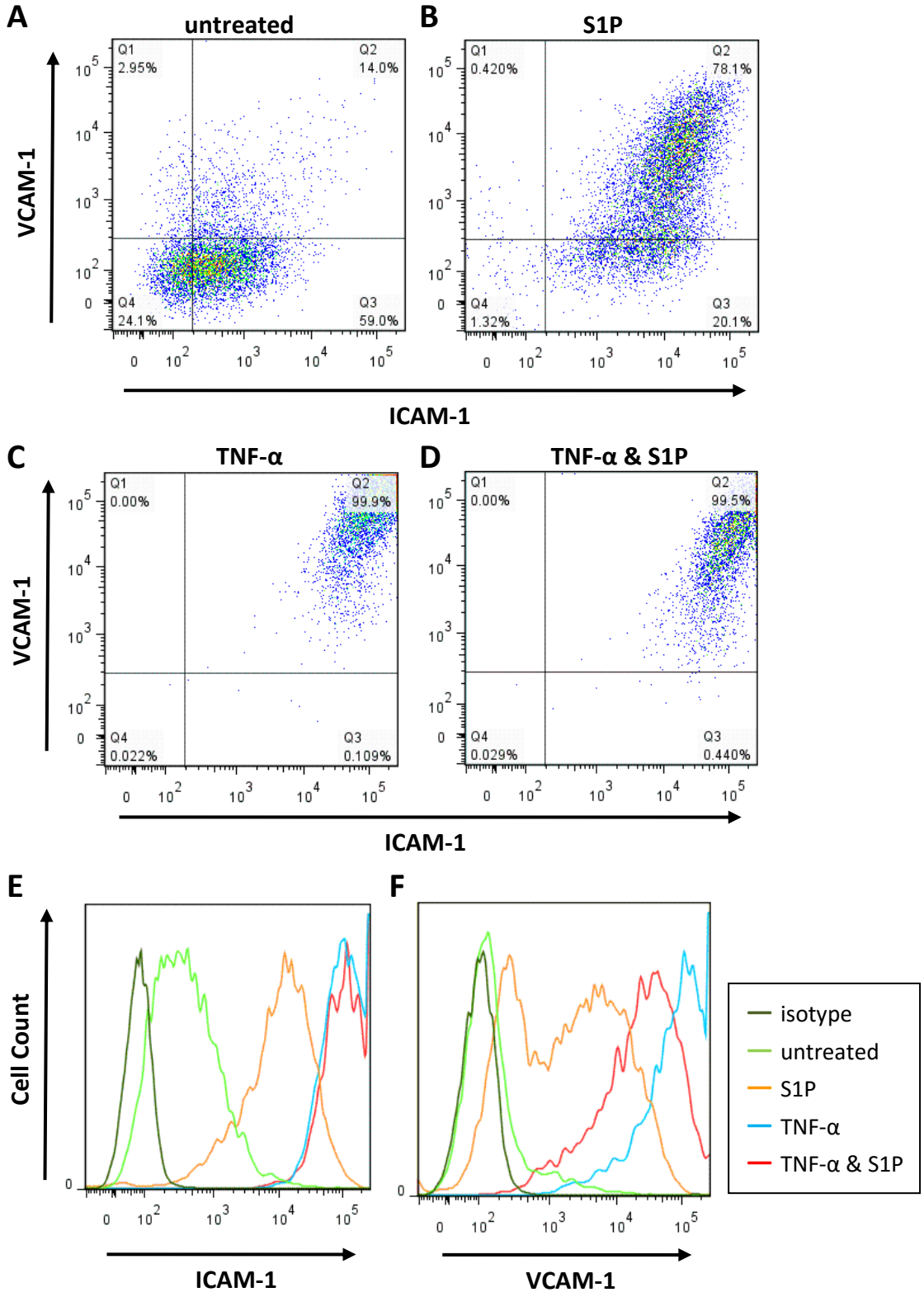


Figure 5.22. Effects of S1P on HUVEC adhesion molecule expression. HUVEC endothelial cells were treated overnight with 10μM S1P, 100ng/ml TNF-α, or 100ng/ml TNF-a & 10μM S1P or left untreated. They were then stained with directly conjugated monoclonal antibodies for ICAM-1 and VCAM-1 adhesion molecules or appropriate isotype controls and analysed by flow cytometry. (A-D) Dot plots of ICAM-1 and VCAM-1 expression simultaneously, in untreated, treated with S1P, TNF-α, or TNF-α & S1P cells respectively. (E-F) Histograms presenting ICAM-1 or VCAM-1 expression respectively after different treatments. **Dark green**: isotype control, **light green**: untreated, **orange**: S1P, **blue**: TNF-α, **red**: TNF-α & S1P. Data representative of 5 independent experiments.

	MFI			
	HMEC-1		HUVEC	
	ICAM-1	VCAM-1	ICAM-1	VCAM-1
untreated	1980	85.5	348	128
S1P	2401	93.7	10600	1782
TNF-α	51400	23200	102000	85400
TNF-α & S1P	50700	10100	99300	24800

Table 5.1. Median Fluorescence intensity of ICAM-1 and VCAM-1. HMEC-1 or HUVEC cells were treated overnight with 10μM S1P, 100ng/ml TNF-α, or 100ng/ml TNF-a & 10μM S1P or left untreated. They were then stained with directly conjugated monoclonal antibodies for ICAM-1 and VCAM-1 adhesion molecules or appropriate isotype controls and analysed by flow cytometry. The table depicts the median fluorescence intensity values (MFI) of ICAM-1 and VCAM-1 for a representative experiment (see figures 5.20, 5.21).

5.3.4 Neutrophil flow-based adhesion assays with endothelial cells

It appears that S1P can affect adhesion molecule expression by endothelial cells. To investigate whether this translates to an effect on neutrophil adhesion to endothelial cells, Cellix flow based adhesion assays with endothelial cells were performed. In these assays, HMEC-1 or HUVEC endothelial cells were cultured on biochips to form channels that simulate blood vessels. Before the experiment, they were treated with S1P or TNF-α or TNF-α with S1P overnight. Freshly isolated neutrophils were then added to flow through the channels, images were captured, and cells that adhered on the endothelial surface were measured. To make the process of calculating the cells that adhered easier, neutrophils were stained with CFSE just before being added to the assay and fluorescent microscopy was used to visualize them. This made it easier to distinguish neutrophils from endothelial cells and debris (figure 5.23).

Neutrophils did not seem to adhere to untreated HMEC-1 or HUVEC at all. Treatment with S1P, although showed some small adherence in certain instances, did not cause a significant difference. When endothelial cells were treated with TNF-α though, there was a significant increase in adhered cells. This increase was much higher for primary HUVEC cells, with around 85 cells on average adhering compared to 3 cells for HMEC-1 (figures 5.24, 5.25). Some HUVEC cells were also treated with both TNF-α and S1P, and this caused an interesting decrease in adherence compared with treatment with TNF-α alone (figure 5.25). This observation agrees with previous findings of TNF-α with S1P treatment of endothelial cells decreasing adhesion molecule expression compared with treatment with TNF-α alone (figures 5.21, 5.22, table 5.1).

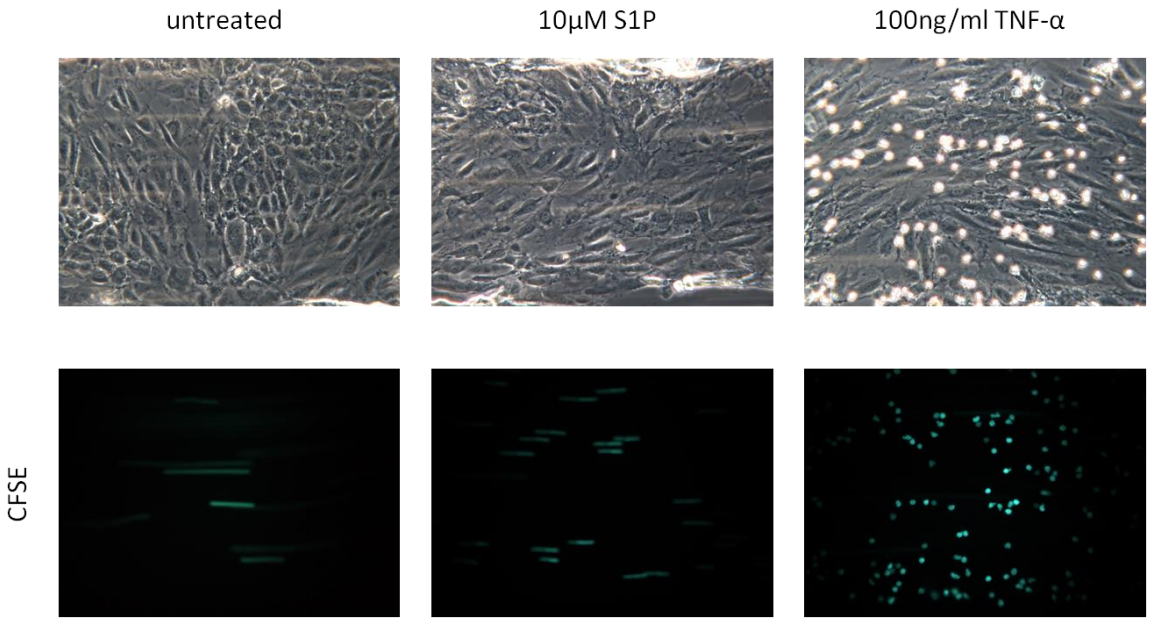


Figure 5.23. Neutrophil flow based adhesion to endothelial cells. Representative images of flow based adhesion assays where HUVEC cells, untreated or treated with 10µM S1P or 100ng/ml TNF-α, were coating biochip channels, and neutrophils flowed through and adhered on their surface. Neutrophils were stained with CFSE to distinguish from other cells and debris by fluorescence. Top row: optical images, bottom row: fluorescent images. Cells that are adhered are clearly defined in both types of images, non-adhered cells are blurred.

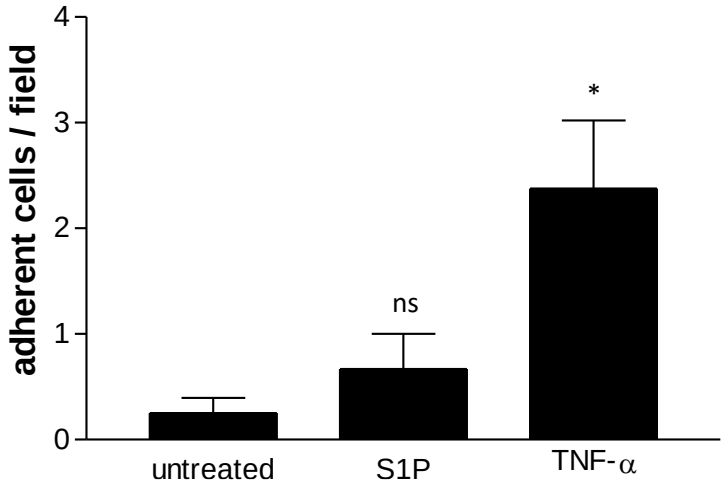


Figure 5.24. Neutrophil flow-based adhesion to HMEC-1. Isolated neutrophils stained with CFSE, were used in a flow- based adhesion assay to HMEC-1 endothelial cells untreated or treated with 10μM S1P or 100ng/ml TNF-α. Bars show means ± SEM from 2 independent channels in the same experiment. Data representative of 3 independent experiments. * $p<0.05$, ns $p\geq0.05$ compared with untreated.

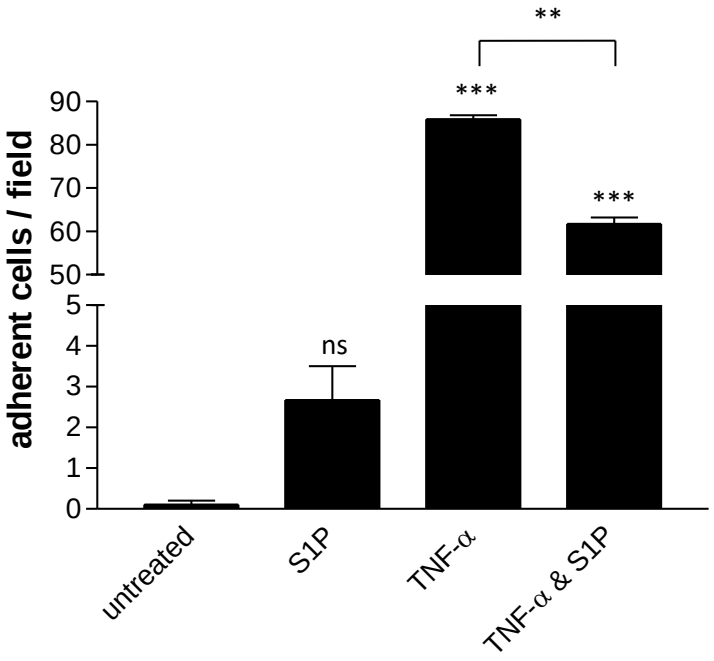


Figure 5.25. Neutrophil flow-based adhesion to HUVEC. Isolated neutrophils stained with CFSE, were used in a flow- based adhesion assay to HUVEC endothelial cells untreated or treated with 10μM S1P or 100ng/ml TNF-α or both. Bars show means ± SEM from 2 independent channels in the same experiment. Data representative of 3 independent experiments. ** $p<0.01$, *** $p<0.001$, ns $p\geq0.05$ compared with untreated, or as shown.

5.4 Discussion

S1P can affect multiple endothelial cell functions that indirectly have an effect on neutrophil migration. First of all, S1P can induce endothelial cells to upregulate mRNA expression and protein secretion of the potent neutrophil chemoattractant cytokine CXCL8 (IL-8). This result partly agrees with the findings of Lin et al., who found S1P can induce mRNA and protein expression of IL-8 in HUVEC cells in a time and concentration dependent manner (Lin *et al.*, 2006). They found 16 hours was the optimal time of S1P treatment for mRNA expression of IL-8 by HUVEC, whereas in the current study, 4 hours appeared optimal for increased IL-8 mRNA expression by HMEC-1, and after 8 hours it seemed to be attenuated compared with untreated cells (Lin *et al.*, 2006).

S1PR1 and S1PR3 appear to be important for increased IL-8 production, as the use of the agonists CYM5442 and CYM5541 showed, although each one caused lower increase in IL-8 compared with S1P, revealing a possible additive effect. This observation agrees with a study where siRNA was used to knock out S1PR1 or S1PR3 from HUVEC cells, resulting in inhibition of S1P induced IL-8 mRNA expression (Lin *et al.*, 2007). Other chemokines were also upregulated by S1P, especially CCL2 (MCP-1), expressed at comparative levels with IL-8, a finding shared with a previous study (Lin *et al.*, 2006). Importantly, among the chemokines that were affected by S1P is CXCL1, which is another neutrophil chemoattractant.

On the other hand, S1P treatment of endothelial cells was found to inhibit neutrophil migration through them. One could argue that since S1P induces IL-8 production of endothelial cells, the IL-8 presented by the endothelial cells to the neutrophils might interfere with the IL-8 gradient, causing the neutrophils to adhere to the endothelial layer instead of passing through. However, the IL-8 produced by endothelial cells was at very low concentrations making it unlikely that this was the reason of reduced neutrophil migration. More probably, this inhibitory effect indicates an enhancement of endothelial barrier integrity by S1P, that correlates with decreased permeability, as has been reported in the past by several studies (Garcia *et al.*, 2001; Singleton *et al.*, 2005; Itagaki *et al.*, 2007). The S1PR1 agonist CYM5442 appeared to also inhibit neutrophil trans-endothelial migration, whereas CYM5541, an S1PR3 agonist, did not have an effect. It appears then that S1PR1 is the receptor responsible for maintaining endothelial barrier integrity, as was also verified by Singleton and colleagues (Singleton *et al.*,

2005). Indeed, S1PR3 and S1PR2 have actually been found to have barrier disrupting effects, causing increased vascular permeability (Singleton *et al.*, 2006; Sanchez *et al.*, 2007).

As for a mechanism for the barrier enhancing effects S1P exhibits, the current study focused on VE-cadherin, which is an important molecule of adherens junctions, which control permeability and transcellular cell migration. It has been found in the past that S1P can increase localization of VE-Cadherin and other adherens junction molecules such as β-catenin at cell-cell contact regions, assisting in assembly of adherens junctions, without affecting total VE-cadherin protein levels (Lee *et al.*, 1999; Mehta *et al.*, 2005). Total VE-cadherin levels were not altered in our study either. Both S1PR1 and 3 had been found to be important for the effect of increased localization at cell junctions (Lee *et al.*, 1999). According to another study though, VE-cadherin is not required for S1P to cause transient increase in endothelial barrier function measured by trans-endothelial electrical resistance, although it is vital for maintained increase (Xu *et al.*, 2007). Cell spreading to close intercellular gaps, mediated by actin filament rearrangement is the reason for the increase in endothelial barrier integrity according to the same study, but also proposed by other studies in the past (Garcia *et al.*, 2001; Singleton *et al.*, 2005; Xu *et al.*, 2007). This project however focused on VE-cadherin phosphorylation that has been shown to affect endothelial permeability and leukocyte migration through the endothelium (Allingham *et al.*, 2007; Turowski *et al.*, 2008; Wessel *et al.*, 2014).

VE-cadherin can be phosphorylated at several sites, usually causing disassembly of adherens junctions, but can sometimes have junction tightening effects (Gavard and Gutkind, 2006; Allingham *et al.*, 2007; Turowski *et al.*, 2008). Opinions vary on which sites are responsible for which effect, but one site that is generally agreed to affect junction assemply negatively, assisting in leukocyte migration and endothelial permeability, is tyrosine 658 (Potter *et al.*, 2005; Allingham *et al.*, 2007). In the current study, it was found that although S1P did not affect total VE-cadherin protein amounts, it could reduce or completely abolish phosphorylation of VE-cadherin at tyrosine 658 for both HMEC-1 and HUVEC cells. This result is reminiscent of the finding of another study that S1P can reduce phosphorylation of N-cadherin, a molecule of the cadherin superfamily, important for cell-cell adhesion between endothelial cells and mural cells, thus stabilizing blood vessels (Paik *et al.*, 2004). Other VE-cadherin phosphorylation sites as well as phosphorylation of other junction molecules could be considered as a

continuation of this work in investigating the mechanisms of S1P - mediated endothelial barrier enhancement.

Furthermore, S1P can affect adhesion molecule expression by endothelial cells. It was found that both ICAM-1 and VCAM-1 adhesion molecules surface expression was increased by S1P. What was interesting though is that S1P can inhibit TNF-α induced surface expression of VCAM-1. This means S1P has both stimulatory and inhibitory effects on adhesion molecule expression, probably involving different S1P receptors. This translated to effects on neutrophil adhesion to endothelial cells under flow conditions, where although S1P was not enough to induce adhesion by itself, it did reduce TNF-α induced adhesion. Similarly, Theilmeier *et al.* had shown that S1P can reduce adhesion under flow of murine macrophages to TNF-α activated endothelium (Theilmeier *et al.*, 2006). Several studies have shown S1P can induce mRNA and/or protein expression of ICAM-1 or VCAM-1 adhesion molecules on endothelial cells, which agrees with the findings on surface expression in our study (Shimamura *et al.*, 2004; Kimura *et al.*, 2006; Lin *et al.*, 2007). The data on S1P inhibiting TNF-α induced expression of VCAM-1 agree with the results of another study, where they found similar results for both VCAM-1 and ICAM-1, with S1PR3 being responsible for this effect (Kimura *et al.*, 2006). On the other hand, a study showed TNF-α induced VCAM-1 expression and other endothelial activation functions, were mediated by sphingosine kinase, since its inactivation greatly reduced TNF-α effects on endothelium (Xia *et al.*, 1998).

In conclusion, it appears S1P can affect neutrophil trans-endothelial migration indirectly in different ways. On one hand, it induces endothelial cells and other types of cells to produce IL-8 and other neutrophil chemoattractants, and increases adhesion molecule expression of endothelial cells, indirectly assisting neutrophil adhesion and subsequent migration through the endothelium. On the other hand, it can inhibit neutrophil trans-endothelial migration by enhancing endothelial barrier integrity. Before S1P can be used as a therapeutic agent, both of these contradicting effects have to be taken into consideration, and further investigation is needed to establish the responsible S1P receptors so targeted therapies can be applied. The mechanisms have to be examined in more depth, too.

CHAPTER 6

In vivo effects of S1P

Chapter 6. *In vivo* effects of S1P

6.1 Introduction

From the previous chapter (chapter 5), it became clear that S1P can affect neutrophil migration indirectly, on the one hand by enhancing endothelial barrier integrity, inhibiting neutrophil migration through the endothelium, and on the other hand by inducing IL-8 production and adhesion molecule expression by endothelial cells, possibly enhancing neutrophil trafficking. Since these two effects are contradictory, it would be interesting to discover which effect is more pronounced, if any, *in vivo* and whether S1P administration will eventually enhance or inhibit neutrophil trafficking in an *in vivo* setting.

Several studies have demonstrated effects of S1P or analogues when administered *in vivo*. In a mouse model of myocardial ischemia reperfusion injury, S1P administered intravenously prior to the induction of ischemia could decrease the infarct size and diminish the number of neutrophils attracted to the site (Theilmeier *et al.*, 2006). In another study, S1P caused vasoconstriction of canine basilar arteries when administered in the cerebrospinal fluid (Tosaka *et al.*, 2001). Intravenously administered S1P, and in some cases intraperitoneally administered FTY720, significantly reduced pulmonary and renal leakage and inflammation in a mouse model of acute lung injury (Peng *et al.*, 2004). The S1P analogue FTY720, which is phosphorylated *in vivo* into FTY720-P, has been extensively shown to inhibit T and B cell migration from secondary lymphoid organs, by causing internalization and degradation of S1PR1 (Chiba *et al.*, 1998; Pinschewer *et al.*, 2000; Kahan, 2004). FTY720 and the S1PR1 agonist SEW2871 can also affect dendritic cell trafficking, with *in vivo* administration in mice causing increased numbers of circulating DCs in the blood, and decreased numbers in lymph nodes and spleen (Lan *et al.*, 2005).

There are several animal models for inflammation and cell recruitment. Among the most wide-spread in their use especially in rodents, are the paw oedema model (Winter *et al.*, 1962), the pleural cavity inflammation model (Spector and Willoughby, 1957), the peritoneum cell recruitment (Murch and Papadimitriou, 1981) and the air pouch model (Selye, 1953). However, the paw oedema model does not allow for collection of inflammatory exudate to analyse the cells recruited, only measurement of paw swelling. The other three models, on the other hand, allow recruitment of cells in cavities to be

measured by collection of the cavity fluid. The pleural cavity model though, is difficult to perform in mice and is more widely used in rats. Moreover, it usually works by injection of inflammatory agents such as carrageenan in the pleural cavity causing inflammation and wider cell recruitment (Di Rosa *et al.*, 1971). If focus on one cell type is preferred, and a single pro-inflammatory molecule such as a cytokine or chemokine needs to be investigated, the other two models of cell recruitment are cleaner and better suited. The type of cell that is recruited plays a role in which cell recruitment model would be more suitable, the peritoneal recruitment or the air pouch model. For example, for monocyte recruitment in a mouse air pouch several days would be needed, whereas recruitment of monocytes in a mouse peritoneal cavity would be sufficient after 16-24 hours (Dawson *et al.*, 1991). Neutrophil recruitment however can be produced in similar extent in both the peritoneal cavity and the air pouch model (Perretti *et al.*, 1994).

In the current project both of these mouse models of cell recruitment were used to assess the effects of S1P on neutrophil trafficking *in vivo*. In the air pouch chemotaxis model, an air pouch was formed subcutaneously on the back of the animal by injection of sterile air, and was then filled with liquid containing the neutrophil chemoattractant IL-8, to assess neutrophil migration in the air pouch, with or without S1P administration. In the peritoneal recruitment model, IL-8 was administered intraperitoneally and neutrophil migration to the peritoneal cavity was measured, as well as whether S1P administration had an effect on it. These *in vivo* models could help show whether S1P indeed has an effect on neutrophil trafficking similar to the *in vitro* effects, and whether this effect is stimulatory or inhibitory.

6.2 Materials and Methods

6.2.1 Reagents

For the *in vivo* experiments a different form of S1P was used. It was Huzzah S1P (Avanti Polar Lipids, USA), a Human Serum Albumin (HSA) / S1P complex. Huzzah S1P consists of S1P conjugated with HSA, a physiologically relevant carrier protein, at a 2:1 ratio. This makes S1P easily soluble in aqueous solutes without the need of an organic solute that could affect viability and phenotype of cells or in this case have adverse effects when injected in animals. Huzzah S1P was reconstituted in sterile PBS in appropriate S1P concentration (usually 500μM), and injected in mice immediately, or kept overnight at 4 ^{o}C before being injected in mice. Huzzah Control (Avanti Polar Lipids, USA) was utilized as vehicle for the experiments that used Huzzah S1P, and it was essentially HSA of the same grade as in Huzzah S1P, that was also reconstituted in sterile PBS, at the same concentration used for S1P.

The recombinant human CXCL8 (Peprotech) was used for these experiments. This was reconstituted in sterile PBS at appropriate concentration for each experiment.

6.2.2 Animals and procedures

Female BALB/c mice bought from Charles River UK were used for all animal experiments. They were maintained under appropriate pathogen free conditions and used at ages 7 - 10 weeks. All animal procedures were done in accordance with UK Home Office and EU Institutional Guidelines and within the parameters of current personal and project licences. Work with live mice was performed by trained staff at the Comparative Biology Centre (Medical School, Newcastle University, UK).

6.2.2.1 Air pouch cell recruitment

For the air pouch experiment (see figure 6.1), 3ml of sterile air were injected subcutaneously into the back of each animal under general anesthesia (day 1). The next day (day 2), as well as days 4 and 5, another 1 ml of air was injected in the same air pouch without anesthesia. On day 5, the animals were also injected intravenously (i.v.) with 100μl S1P 500μM or vehicle. After 24 hours, 1ml of PBS containing 5μg IL-8, or PBS only was injected into the air pouch. Mice were euthanized by cervical dislocation 4 hours later, and air pouch was lavaged with 1ml of 1mM EDTA in PBS twice to collect air pouch fluid. The air pouch fluid was then centrifuged at 500x g to pellet cells,

which were then resuspended in PBS with 2% FBS, counted by heamocytometer and separated into tubes to be stained for flow cytometry or used for bead counting (see chapter 2.4.1). For two of the samples that had excess of cells, a cytospin on glass slides was performed, slides were fixed in methanol and stained with Diff-Quick stain to differentiate different cell types under the microscope.

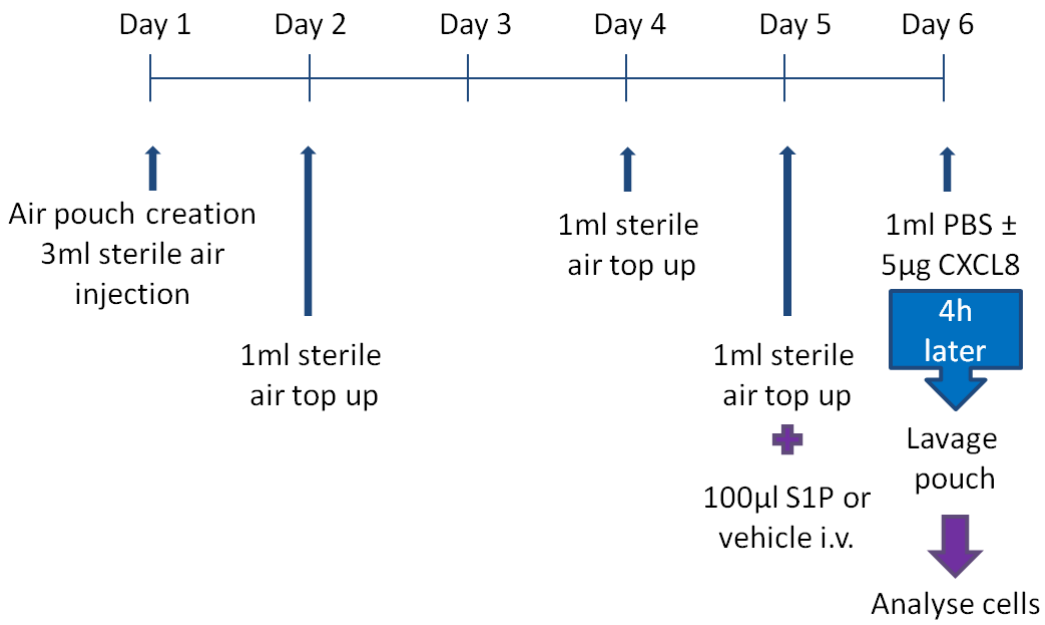


Figure 6.1. Air pouch cell recruitment mouse model. Diagram depicting air pouch creation steps for cell recruitment experiment.

6.2.2.2 Peritoneal cell recruitment

For the peritoneal recruitment experiments, mice were injected intraperitoneally (i.p.) with 5µg IL-8 in 200µl, or with 200µl PBS and 4 hours later euthanized by cervical dislocation or carbon dioxide asphyxiation. In some experiments mice were injected i.p. with 200µl S1P (Huzzah S1P) 500µM or vehicle (Huzzah Control) 24 hours before the IL-8 injections. Peritoneal fluid was collected as soon as possible after death, by injecting 5ml PBS with 2% FBS and 1mM EDTA in the peritoneal cavity, massaging to dislodge attached cells, and then collecting liquid back with syringe and Pasteur pipette. Fluid was centrifuged at 500 x g to pellet cells that were then resuspended in PBS with 2% FBS and 1mM EDTA, counted with a heamocytometer and separated into tubes for flow cytometry and bead counting (see chapter 2.4.1).

6.2.3 Multicolour flow cytometry

Cells derived from peritoneal or air pouch fluid were stained in the same tube, with appropriate directly conjugated primary antibodies. An antibody mix with appropriate antibody concentrations was created first and then added in the flow tubes. Staining proceeded as described in chapter 2.3. Compensation is necessary in multicolour flow cytometry as the fluorescent emission spectra of many fluorochromes can overlap in a certain degree and be detected by the same PMT. Compensation allows the subtraction of the part of a fluorescent molecule's emission spectra that can be detected by other than its primary detector. Single stains were used as compensation controls for the multicolour flow cytometry, as well as "Fluorescence Minus One" (FMO) controls, which contained all antibodies expect one each, to properly place negative gates. Antibodies used were anti - mouse CD3ε, CD19, CD11b, CD11c, Ly6G and F4/80 (Biolegend, UK; see chapter 2.3 for full list). The viability dye Zombie Aqua Fixable Viability Kit (Biolegend, UK) was used to distinguish alive from dead cells. Flow cytometry was performed on FACSCanto II instrument (BD Biosciences) and data were analysed using FlowJo 7.6 software (Treestar).

6.3 Results

6.3.1 Air pouch model of cell recruitment

To determine the effects S1P can have on neutrophil recruitment *in vivo*, the air pouch mouse model of cell chemotaxis was employed. In this model, mice are injected subcutaneously with sterile air a number of times until an air pouch is formed. This pouch can then be filled with chemokine and cell recruitment in the pouch is measured by collecting the air pouch fluid. For the purposes of this study, mice were injected intravenously (i.v.) with 100µl S1P 600µM or vehicle (PBS with Human Serum Albumin) after the air pouch had been formed, and 24 hours later they were injected with 5µg CXCL8 (IL-8) in 1ml PBS or PBS only into the air pouch. This resulted in 3 groups of mice, a control group injected with S1P and then PBS, a group injected with vehicle then IL-8 and a group injected with S1P then IL-8. Mice were euthanized 4 hours later and air pouch fluid was collected. Cells collected this way were then stained with multiple antibodies and viability dye for flow cytometry and were counted using counting beads. The antibodies used were CD19 and CD3 for B and T cells respectively, Ly6G and CD11b for neutrophils, F4/80 for macrophages and CD11c for dendritic cells. The viability dye Zombie Aqua (Biolegend) was used to distinguish alive and dead cells.

Two of the samples were also cytospinned and stained with Diff-Quick stain to be observed under the microscope. The first sample was from an animal treated with S1P only with no IL-8 afterwards, as a control. The second sample was from an animal treated with vehicle then IL-8. In the first sample, various kinds of cells can be seen, whereas in the second sample the cells are almost only neutrophils (figure 6.2).

For the flow cytometric analysis of the cells infiltrating the airpouch, gating was first done on forward scatter (FSC)/side scatter (SSC) axis, followed by FSC-A/FSC-H to exclude any doublets. Zombie aqua negative cells were then chosen as live cell population and gated on CD3/CD19 axis. CD3 negative CD19 negative ($CD3^-$ $CD19^-$) cells were chosen to exclude B cells and T cells. These were further analyzed on Ly6G/CD11b axis to gate for neutrophils as a distinct Ly6G high CD11b high ($Ly6G^{hi}$ $CD11b^{hi}$) cell population (figure 6.3). Due to the variability of the data no statistically significant differences between the three groups were observed (figure 6.4). Total cell counts were varied within samples from the same group. Total neutrophil count and

percentage of neutrophils in total cells were measured, but no effect could be determined. Moreover, when comparing with the control, that received no IL-8, there was not a significant increase in cell recruitment in the other two groups. This indicates some technical fault with the experiment, probably due to blood contamination (figure 6.4). Higher numbers of mice samples might be required to identify any significant effects.

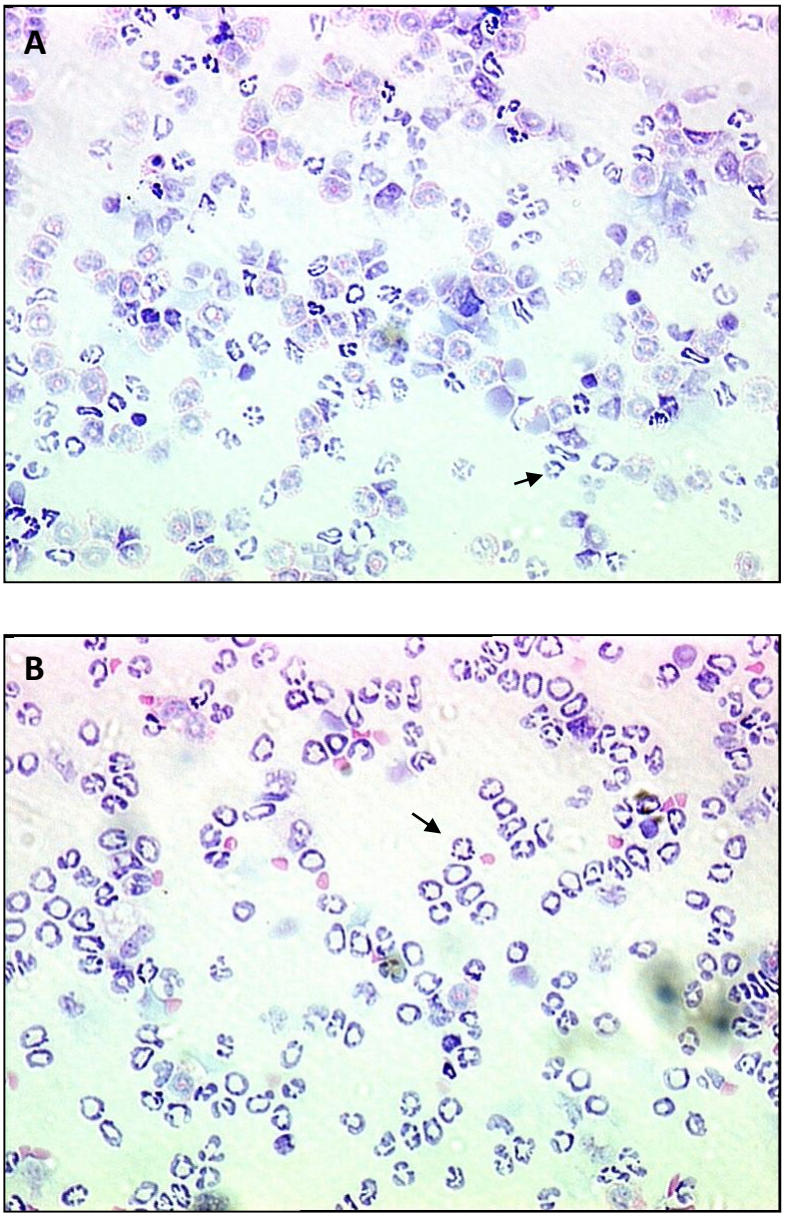


Figure 6.2. Images of cells recruited in air pouches. Cells from air pouch of an animal treated with S1P then PBS (A) and an animal treated with vehicle then IL-8 (B). Neutrophils (arrow) are visible in both samples, but in B there are much more compared with other types of cells.

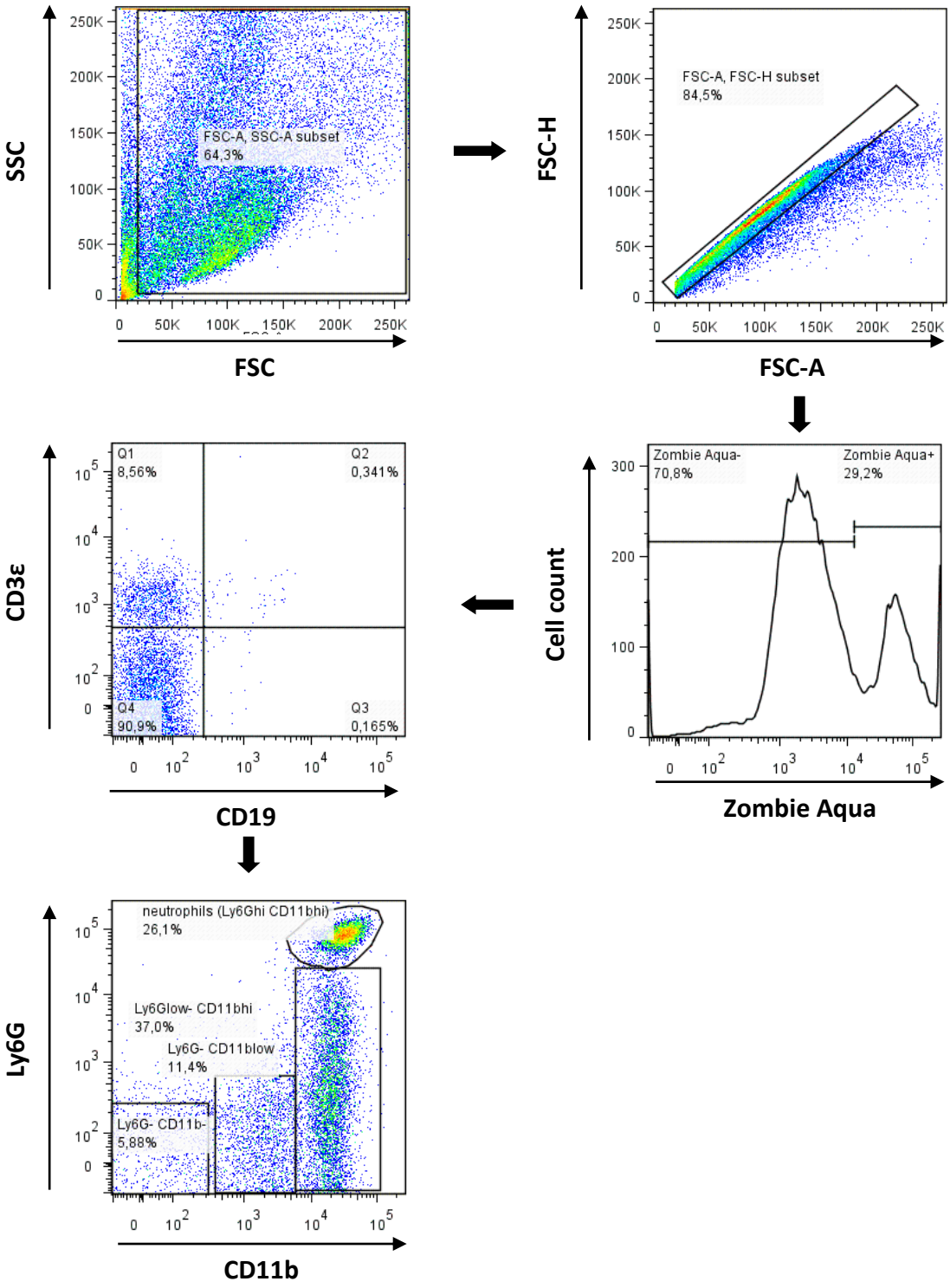


Figure 6.3. Gating on neutrophils for cell recruitment in air pouch. The whole cell population of the air pouch is gated first on the FSC/SSC axis. Then doublets are removed by gating on the FSC-A/FCS-H axis. Alive cells are selected by gating on Zombie Aqua (viability dye) negative events. Gating on CD3/CD19 axis allows selection of $CD3^-$ $CD19^-$ cells, excluding B and T lymphocytes. These cells are then gated on Ly6G (neutrophil marker)/CD11b axis to select neutrophils, which are $Ly6G^{hi}$ $CD11b^{hi}$.

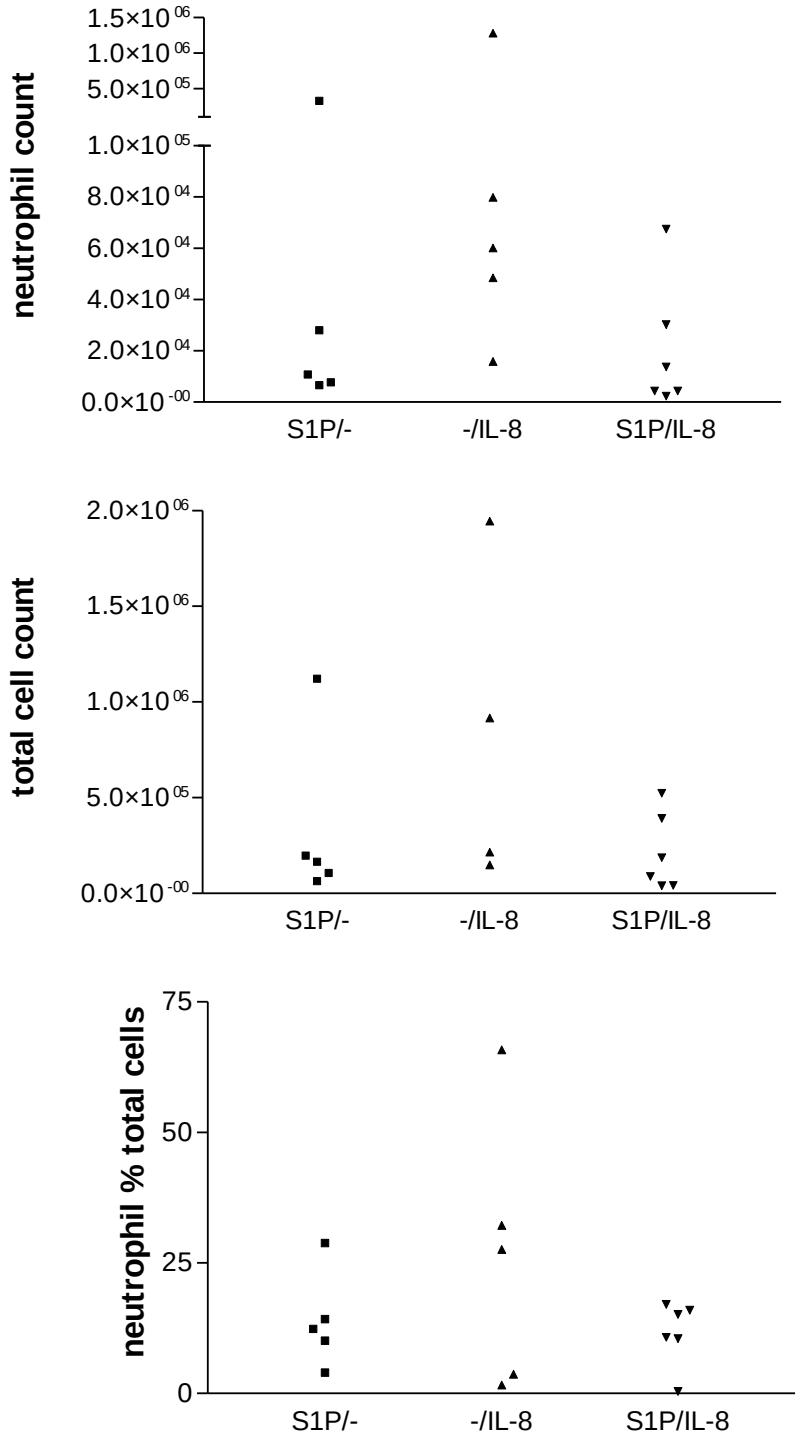


Figure 6.4. Effects of S1P on air pouch cell recruitment. Air pouches were formed on mice that were then injected i.v. with 100µl S1P 500µM and 24 hours later 5µg IL-8 in 1 ml PBS were injected into the air pouch. Mice were euthanized 4 hours later and fluid was collected from air pouches. Cells were stained for flow cytometry and counted using beads. Neutrophils counts, total cell counts and percentage of neutrophils in total cells were measured.

6.3.2 Optimization of peritoneal cell recruitment by IL-8

In order to further investigate the effects S1P might have *in vivo* a model of cell recruitment in the peritoneum was used. In this model, the chemokine IL-8 is injected in the peritoneum of mice, and after 2 – 8 hours there is a recruitment of cells in the peritoneal cavity, and especially neutrophils. Before moving on to add S1P and observe if it can affect IL-8 induced cell recruitment, an optimization experiment was performed, to make sure there is enough recruitment achieved by IL-8 alone. In this experiment, mice were injected intraperitoneally (i.p.) with 5μg IL-8 in 200μl PBS, or with PBS alone as vehicle. The mice were euthanized 4 hours later and peritoneal fluid was collected. The cells were then stained with multiple antibodies for flow cytometry in order to determine the different cell populations. CD19 and CD3 were used to gate on B cells and T cells respectively, Ly6G and CD11b to distinguish neutrophils, F4/80 for macrophages and CD11c for dendritic cells. A viability dye was also used to distinguish alive and dead cells (Zombie Aqua, Biolegend). Moreover, counting beads were utilized, to measure cell counts accurately.

After dead cells had been excluded, $CD3^{-}$ $CD19^{-}$ cells were gated. These were then analyzed on a Ly6G/CD11b axis, and neutrophils were determined to be a distinct population that was $Ly6G^{hi}$ $CD11b^{hi}$. Interestingly, there was a considerable amount of neutrophils on both IL-8 treated and vehicle treated mice (figures 6.5-6.6). This was not the case with test mice that were not injected at all, which had almost no neutrophils in their peritoneal cavity (data not shown). This probably means that the act of injecting itself causes some inflammation and leads to the recruitment of neutrophils. However, mice injected i.p. with IL-8 had significantly more neutrophils than mice injected with vehicle alone. Moreover, the total cell count was significantly higher, too (figure 6.7).

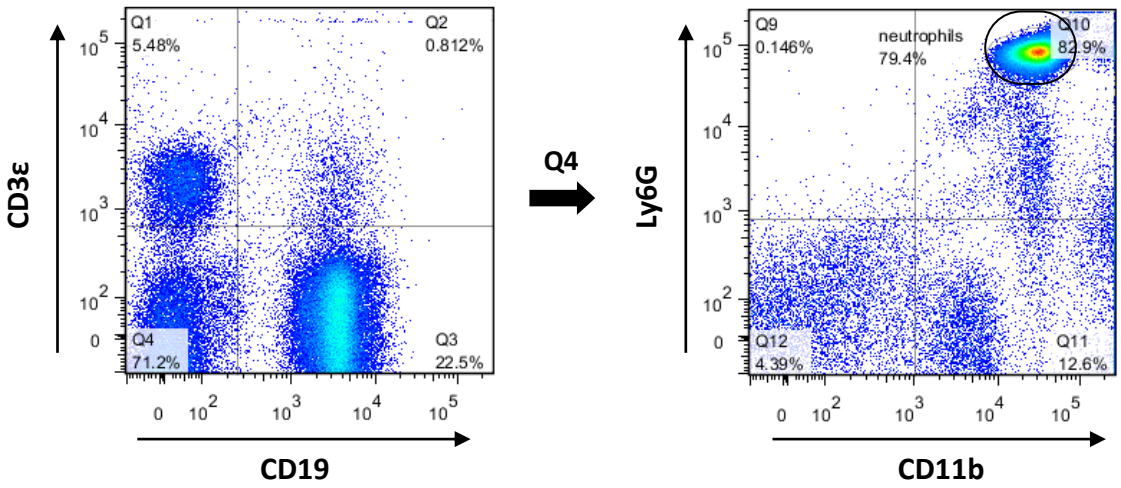


Figure 6.5. Neutrophils in peritoneum after IL-8 injection. Alive cells were gated on CD3/CD19 axis to exclude T and B lymphocytes. The $CD3^{-}$ $CD19^{-}$ population was further gated on Ly6G/CD11b axis, where neutrophils were gated as $Ly6G^{hi}$ $CD11b^{hi}$ population. Data representative of one mouse sample out of 3 that were treated with 5µg IL-8 i.p.

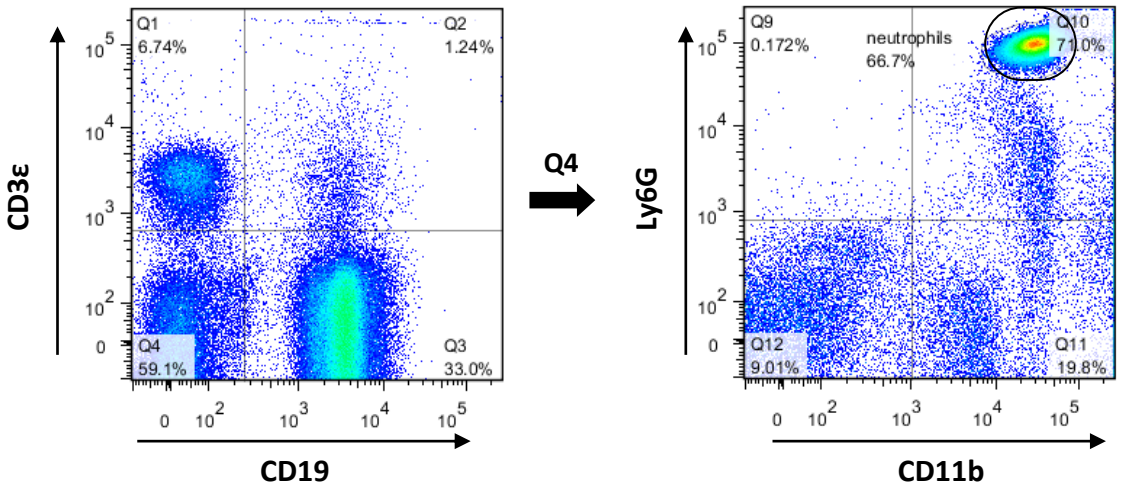


Figure 6.6. Neutrophils in peritoneum after vehicle injection. Alive cells were gated on CD3/CD19 axis to exclude T and B lymphocytes. The $CD3^{-}$ $CD19^{-}$ population was further gated on Ly6G/CD11b axis, where neutrophils were gated as $Ly6G^{hi}$ $CD11b^{hi}$ population. Data representative of one mouse sample out of 3 that were treated with vehicle (PBS) i.p.

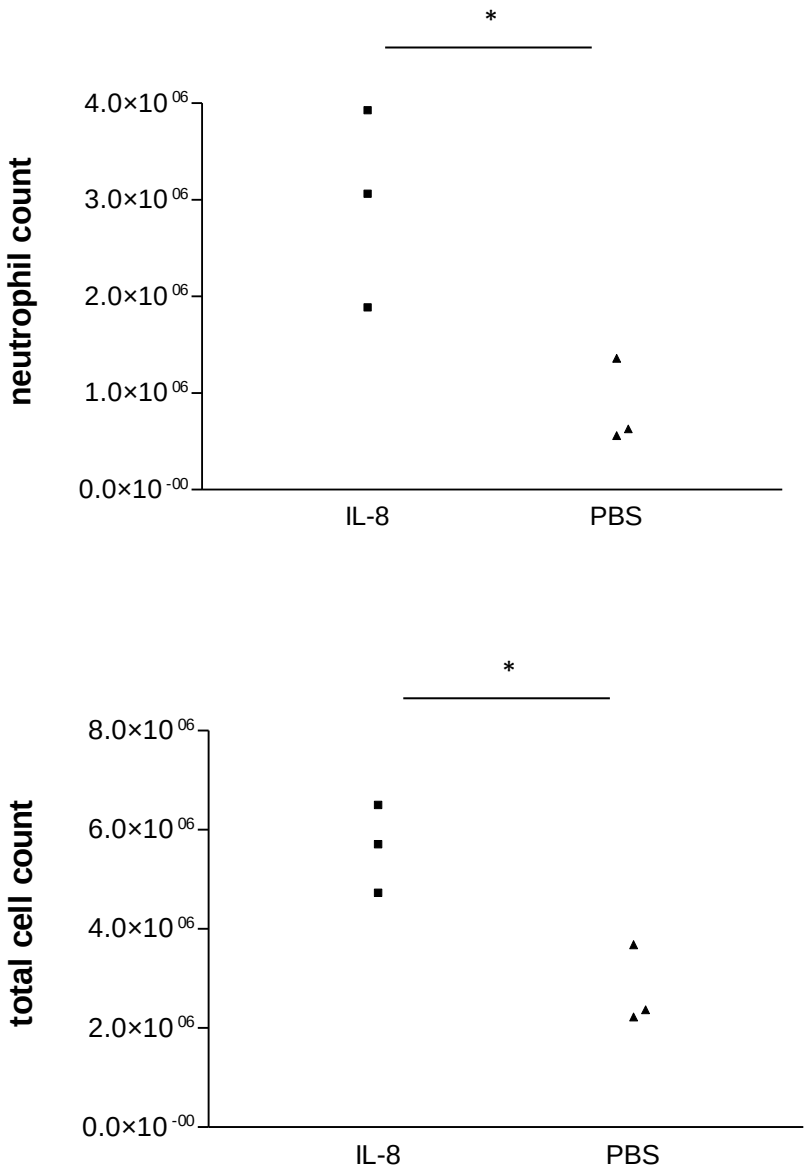


Figure 6.7. Neutrophil and total cell count in the peritoneum after IL-8 recruitment. Mice were injected i.p. with 5µg IL-8 in 200µl PBS or with 200µl PBS only. 4 hours later mice were euthanized and peritoneal fluid collected. Cells were stained for flow cytometry and counted using beads to measure total cell count and neutrophil population count. * $p<0.05$

6.3.3 Effects of S1P on peritoneal cell recruitment by IL-8

The next step was to include S1P treatment in the peritoneal cell recruitment experiment in order to examine what effects this would cause. Mice were separated in 4 groups this time. One group was injected intraperitoneally with 200μl S1P 500μM and 24 hours later further injected with 5μg IL-8 in 200μl as before; a second group was injected with vehicle and then IL-8, the third group was injected with S1P and then PBS as vehicle for IL-8, and finally the last group was injected with vehicle twice. Like before, mice were euthanized 4 hours after the second injection and peritoneal fluid was collected. The cells were again stained with multiple antibodies and viability dye, and beads were also used for counting. Although originally there were 5 mice per group, the presence of blood in some of the samples lead to their exclusion from further analysis.

Gating on peritoneal cells was first done on FSC/SSC axis, followed by FSC-A/FSC-H to exclude any doublets. As with previous experiments, neutrophils were determined to be the distinct $Ly6G^{hi}$ $CD11b^{hi}$ population of the $CD3^{-}$ $CD19^{-}$ proportion of the live cell population (figure 6.8). Compared with the control groups, that received no IL-8, the total cell count and neutrophil recruitment was significantly higher in the other two groups that received IL-8 in the second injection (figure 6.9). This was in accordance with the optimization experiment (figure 6.7). Moreover, the group that received S1P 24 hours before the IL-8 injection had decreased neutrophil count in comparison to the group that received vehicle and then IL-8. The total cell counts for these two groups were not significantly different (figure 6.9). It appears then, that S1P inhibited neutrophil recruitment in the peritoneum induced by IL-8.

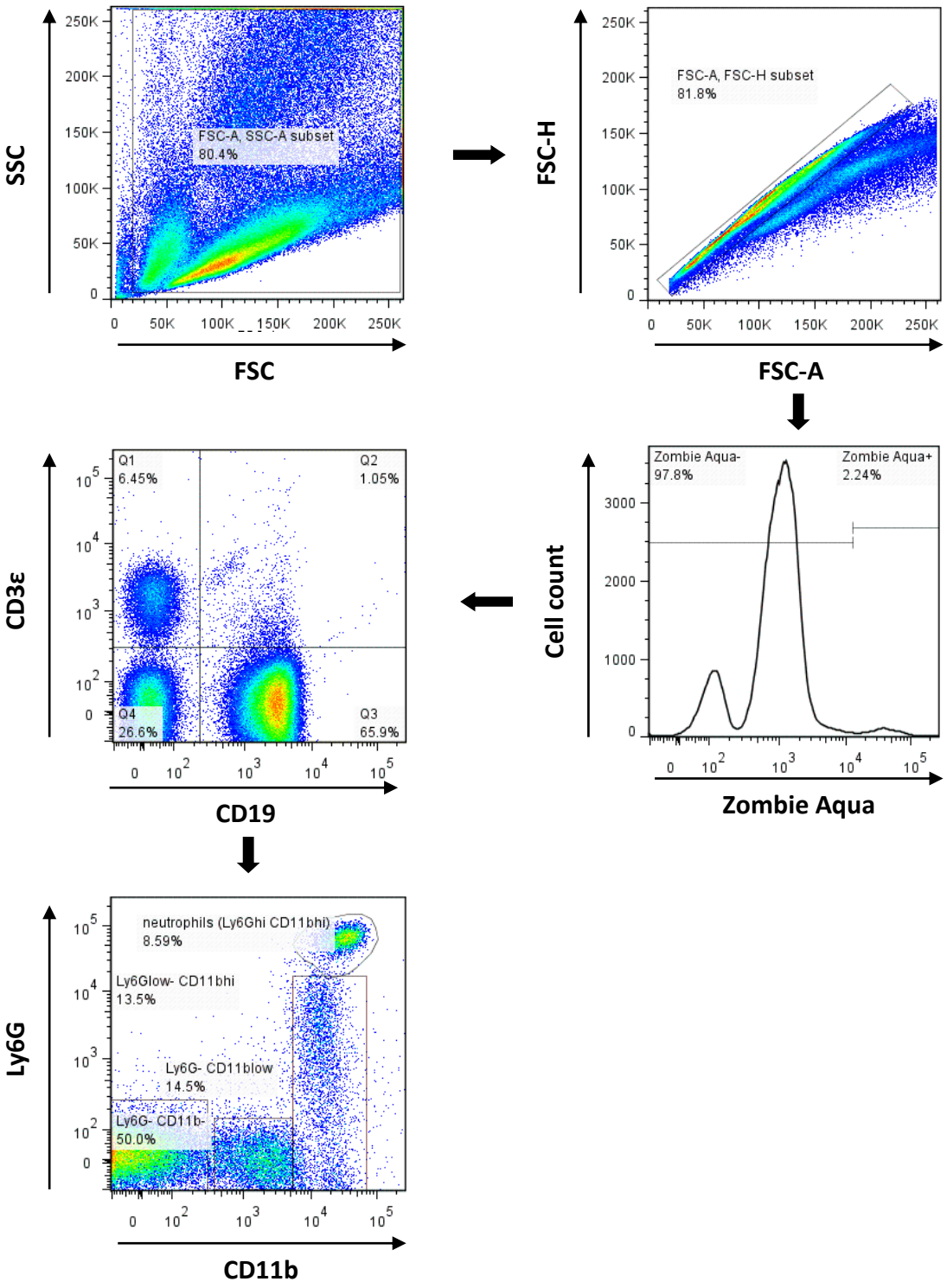


Figure 6.8. Gating on neutrophils for cell recruitment in peritoneum. The whole cell population of the mouse peritoneum is gated first on the FSC/SSC axis. Then doublets are removed by gating on the FSC-A/FCS-H axis. Alive cells are selected by gating on Zombie Aqua (viability dye) negative events. Gating on CD3/CD19 axis allows selection of $CD3^-$ $CD19^-$ cells, excluding B and T lymphocytes. These cells are then gated on Ly6G (neutrophil marker)/CD11b axis to select neutrophils, which are $Ly6G^{hi}$ $CD11b^{hi}$.

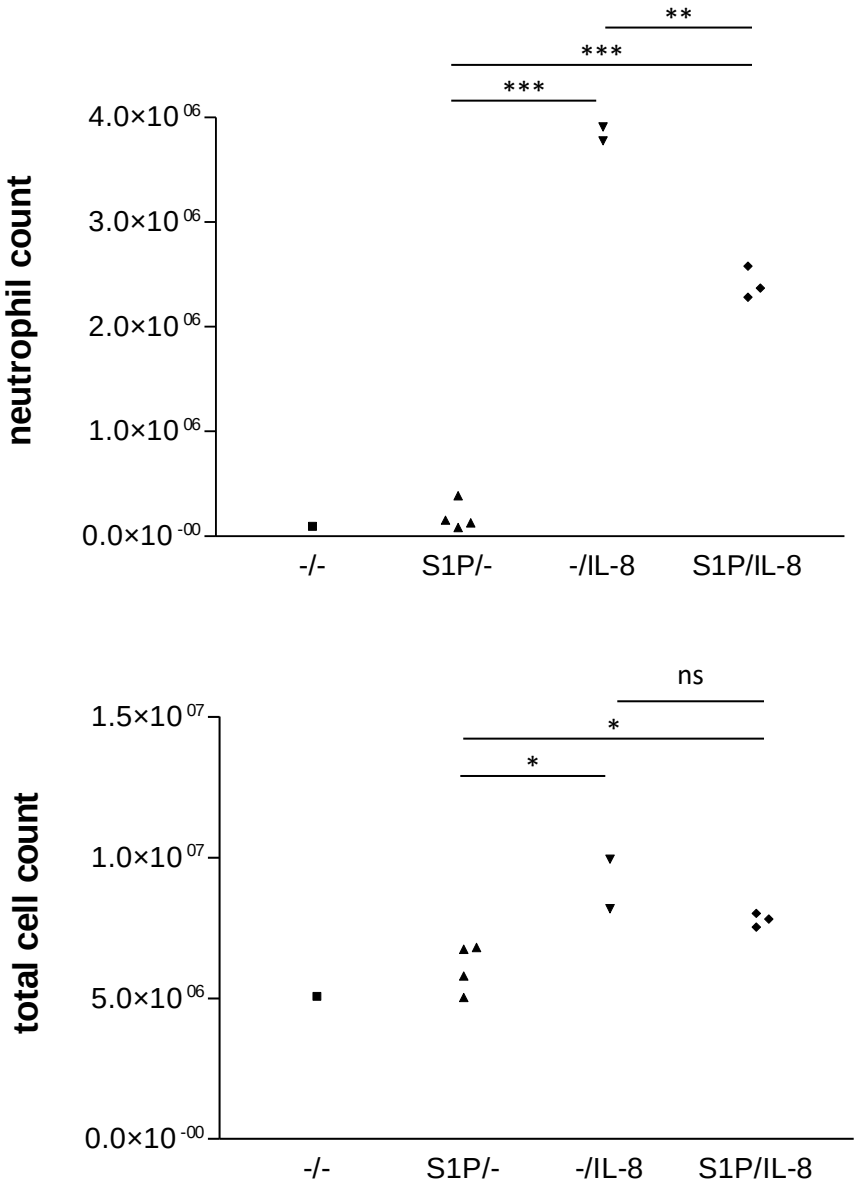


Figure 6.9. Effects of S1P on IL-8 cell recruitment in the peritoneum. Mice were injected i.p. with 200µl S1P 500µM or vehicle (-), then 24 hours later injected with 5µg IL-8 in 200µl or vehicle (-). After 4 hours mice were euthanized and peritoneal fluid collected. Cells were stained for flow cytometry and counted using beads. Total cell count and neutrophil count ($Ly6G^{hi}$ $CD11b^{hi}$) were measured. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns $p\geq0.05$. Originally n=5 per group, but samples with blood were excluded from analysis.

6.4 Discussion

Although CXCL8 (IL-8) is a human chemokine that is not expressed in mice, the human chemokine receptors CXCR1 and CXCR2, which bind IL-8, have homologues in mice, that can recognize human IL-8 (Fan *et al.*, 2007). For this reason, mice can be used successfully as an *in vivo* model system to measure neutrophil recruitment mediated by IL-8, as has been demonstrated by several studies (Singer and Sansonetti, 2004; Proost *et al.*, 2008; Gangavarapu *et al.*, 2012). The most common system used is peritoneal recruitment, with IL-8 injected i.p. causing rapid recruitment of neutrophils in the peritoneal cavity of the mouse. In the current project, this model was used to successfully recruit neutrophils. Moreover, S1P injection 24 hours before the administration of IL-8 showed significant inhibition of neutrophil recruitment in the peritoneum. This result is in agreement with previous *in vitro* data that showed S1P treatment of endothelial cells inhibited neutrophil migration through them, by enhancing endothelial barrier function (see chapter 5). It appears then that *in vivo* S1P also enhances endothelial barriers, preventing neutrophils from migrating towards the peritoneal cavity by following IL-8 gradients. Although other *in vitro* data showed that S1P can also induce chemokine production and adhesion molecule expression by endothelial cells (see chapter 5), this effect appeared secondary *in vivo*, with the inhibitory effect being more pronounced. However, a higher number of animals, and use of S1P receptor agonists would help verify and expand these results in the future.

Another well established mouse model for cell recruitment is the air pouch model (Edwards *et al.*, 1981; Sin *et al.*, 1986; Romano *et al.*, 1997a). In this model, IL-8 is injected in an air pouch created on the back of the animal, causing neutrophil recruitment similar with the peritoneal recruitment model (Perretti *et al.*, 1994; Romano *et al.*, 1997b). For this project however, when this model was used to assess the effects of S1P on neutrophil recruitment induced by IL-8, the results were too variable to reach a conclusion. Generally, cell recruitment was not optimal, since IL-8 did not appear to cause increased recruitment over vehicle. It is possible that the use of more animals, with optimization experiments for neutrophil recruitment beforehand, would help provide a clear result. Cell recruitment should be measured at different time points following the administration of a range of IL-8 concentrations, leading to the identification of optimal conditions for maximised neutrophil recruitment. Then, using an optimized IL-8 concentration and time, S1P effects on neutrophil recruitment could be better assessed.

To sum up, mice can be used as *in vivo* models for IL-8 induced cell recruitment. S1P appears to have an inhibitory effect in neutrophil recruitment *in vivo* according to a mouse model used, pointing towards an effect in endothelial barrier function *in vivo* as was shown *in vitro*. However, these *in vivo* results were pilot experiments and further examination using more animals, and different S1P receptor agonists would help expand and provide more information on the mechanisms of action by S1P *in vivo*.

CHAPTER 7

Discussion

Chapter 7. Discussion

Neutrophils are major mediators of many inflammatory conditions including ischemia-reperfusion injury. They migrate through the blood stream to the sites of inflammation by following chemokine gradients and passing through the endothelial barrier according to a firmly regulated procedure of rolling, firm adhesion, endothelial crawling and diapedesis. Theurapeutic approaches to many conditions could target neutrophils, and specifically their migration, by affecting any of these specific processes. The main objective of this study was to investigate whether the lipid mediator S1P or any of its analogs and agonists could be used to target neutrophil migration to the sites of inflammation. S1P could affect neutrophil migration directly by induction of S1P receptor signalling on neutrophils and crosslinking with chemokine receptors, causing inhibition in chemokine mediated migration or adhesion to endothelium; or it could affect neutrophil migration indirectly, by signalling on the endothelium, causing enhancement of the endothelial barrier and disrupting neutrophil diapedesis through it, or affecting endothelial cell chemokine production and adhesion molecule expression, that would guide neutrophils to migrate to the inflammation site.

This study showed direct signalling of S1P on neutrophils exists but does not result in an effect in migration or adhesion. On the other hand, S1P signalling on endothelial cells was found to have conflicting effects on neutrophil migration. It can enhance endothelial barrier integrity leading to inhibition of neutrophil diapedesis through the endothelium. However, S1P signalling also results in chemokine production by endothelial cells, which would attract more neutrophils to the site, leading to an increase in neutrophil trafficking. Furthermore, S1P also stimulates adhesion molecule expression on endothelial cells that would also assist neutrophil migration. The question raised was which effect would eventually prevail in an *in vivo* environment, so that a decision can be made on whether S1P could be used as a therapeutic approach in the future. An *in vivo* model gave a provisional answer to this question, by showing that S1P administered *in vivo* can inhibit neutrophil recruitment to the site of chemokine injection. Further work is required though, to investigate whether that is indeed the case, which S1P receptors are involved, and the mechanisms of action, before proceeding to human trials.

7.1 Use of cell lines vs. primary cells

An interesting point raised during the course of this study is the use of cell lines as models for primary cells *in vitro*. The first cell line was created on 1951 with the HeLa cells, derived from cervical cancer cells that were isolated and cultured in the lab (Scherer *et al.*, 1953; Lucey *et al.*, 2009). Cell lines are either cancer derived or otherwise immortalized cells that can be kept in culture almost indefinitely. That is their greatest advantage, they can be cultured for as long as you need them in order to perform your experiments, unlike primary cells that need to be used immediately and have to be isolated every time you need to perform an experiment with them. Another advantage of cell lines is the fact that all cells are basically identical, derived from the same donor, divided from the initial cell culture. This allows researchers to perform standardized, easily repeatable experiments, unlike using primary cells that may differ from donor to donor. On the other hand, the main disadvantage of using cell lines instead of primary cells is the fact that they are phenotypically different from their primary counterparts. The simple event of immortalisation, whether that was because they are of cancer origin or they were immortalised in the process of creating the cell line, can change their characteristics dramatically, and all these divisions can change them even further by the accumulation of mutations. So, every researcher should have that in mind when using a cell line as a model for primary cells, and not expect results to be exactly the same as when primary cells are used. Sometimes though, the differences might be subtle enough that results can be adequately accepted as a valid model for the cells of interest.

In this project, both cell lines and primary cells were used. HL60 cell line was tried as a human neutrophil model, before moving on to using primary human neutrophils. However, when differentiated HL60 were compared with primary neutrophils, many differences arose, that lead to the conclusion that they were not suitable to be used in this project further. First of all, their IL-8 signalling was severely impaired, with an important IL-8 receptor, CXCR1, missing from their surface. This lead to deficient migratory function towards IL-8, that was in contrast to the migratory capabilities of primary cells. Other functions of IL-8 signalling were also missing, like the stimulatory effects of IL-8 to neutrophil adhesion. In terms of S1P signalling that was the focus of this project, HL60 completely lacked an important S1P receptor that was highly expressed by primary neutrophils, S1PR1. This severely limited their value as a model to assess the effects of S1P on neutrophils. A few other differences, such as the

expression of CD69 and abnormal adhesion patterns contributed in eventually moving on to using only primary neutrophils for the rest of this study. Of course the use of the cell line was still useful in terms of optimization of experiments, before moving on to repeat them with the harder to obtain and more demanding primary neutrophils.

Endothelial cell lines were also used in this project. HMEC-1 and Eahy926 were tried before moving on to HUVEC primary endothelial cells. Eahy926 were abandoned early on, as they did not appear to behave like endothelial cells enough (eg. not forming uniform monolayers). This might be because they are actually a hybrid cell line of endothelial and epithelial origin (Edgell *et al.*, 1983). HMEC-1 cells however were found to behave similarly with how primary endothelial cells would be expected to behave. Both HMEC-1 and HUVEC provided similar results when used in trans-endothelial chemotaxis assays. Both cells also appeared to be able to express IL-8 after stimulation with S1P, although primary HUVEC could produce considerably higher amounts compared with HMEC-1 IL-8 production. This means that some functionality was impaired but that was not so significant for the current project. In terms of S1P receptor expression, both cells express S1PR1, although HUVEC express more than HMEC-1; both express S1PR2, although HMEC-1 express much more than HUVEC. As for S1PR4 and S1PR5, HUVEC express the former, whereas HMEC-1 express the later; S1PR3 is barely expressed by both cells. S1P could also induce adhesion molecule expression in both cells, although the expression pattern was slightly different. So there are some differences between the cell line and the primary cells, but in this case at least they could be set aside, since the similarities were more important. It should also be noted, that there are many different types of endothelial cells, and the fact that HMEC-1 are derived from human microvascular endothelial cells, whereas HUVEC are macrovascular cells from human umbilical vein, might be the reason for the observed differences.

7.2 Direct effects of S1P on neutrophils

There are a number of studies that have somehow linked S1P signalling and neutrophils. However, not all of the effects observed are necessarily direct effects on neutrophils. For example, S1P lyase deficiency, that would lead to S1P accumulation, causes impaired neutrophil trafficking (Allende *et al.*, 2011). However, it is not completely clear whether S1P affects neutrophils directly or the observed outcome is the result of S1P affecting other types of cells, i.e. endothelial cells. There are nevertheless a couple of *in vitro* studies, using *ex vivo* human neutrophils that have indeed shown some direct effect of S1P on neutrophil migration. Specifically, they both showed S1P can inhibit neutrophil migration *in vitro* (Kawa *et al*., 1997; Rahaman *et al*., 2006).

In this study, it was investigated whether S1P can indeed affect neutrophils directly. Neutrophils were found to express mainly S1PR1 and S1PR4 as had been reported by previous research (Rahaman *et al.*, 2006), and the main signalling pathway activated by S1P through these receptors was the ERK1/2 pathway. This signalling pathway has been reported in the past to be involved in neutrophil functions such as adhesion, degranulation, oxidative burst, formation of neutrophil extracellular traps (NETs), and IL-8 production (Sue *et al.*, 1997; Capodici *et al.*, 1998; Alvarez *et al.*, 2006; Hakkim *et al.*, 2011). Moreover, S1P signalling was found to be able to enhance IL-8 induced signalling, indicating some crosslinking of S1P receptors with IL-8 receptors. Rahaman *et al.* had reported something similar, when they observed a crosslinking between S1PR4 and CXRCR1 that seemed to result in the observed inhibitory effect of S1P on neutrophil migration towards IL-8 (Rahaman *et al.*, 2006). Nevertheless, and despite what was previously reported, there was no significant effect of S1P on neutrophil chemotaxis towards IL-8 observed in the current study. This result could be explained by the diversity of neutrophils from different donors, allowing for small scale effects to be rendered insignificant; the sensitivity of different chemotaxis counting methods could also play a role, as well as the range of IL-8 concentrations being used – maybe the effect would be more noticeable if sub-optimal concentrations of IL-8 had been used.

In a similar way as with neutrophil migration, neutrophil adhesion to ICAM-1 and VCAM-1 adhesion molecules was not found to be affected by S1P. Neither was the IL-8 induced adhesion to these molecules affected in any way, although IL-8 induced ERK1/2 signalling was enhanced by S1P, and it has been shown in the past that

neutrophil adhesion can be governed by this signalling pathway. However, Kawa *et al.* did not find any effects of S1P on neutrophil adhesion either, although their data was for adhesion to endothelial cells (Kawa *et al*., 1997). On the other hand, Florey and Haskard had shown that S1P can affect immune complex mediated neutrophil adhesion under flow conditions, among other things, although S1P did not have a significant effect by itself (Florey and Haskard, 2009b).

Another aspect that was investigated in the chapter about direct effects of S1P on neutrophils was the role of activation molecule CD69 and whether there was any crosslinking with S1P receptors that affected neutrophil migration in any way. CD69 has been found to be important in lymphocyte trafficking through lymphoid organs, by association with the S1PR1 that is necessary for the egress of these cells. It was therefore hypothesized that CD69 might have a similar role in neutrophils trafficking. However, neutrophils do not constitutively express CD69 on their surface; it needs to be induced by different compounds. There is a theory for the existence of an intracellular storage of CD69 that can be rapidly relocated on the cell surface after proper stimulation (Gavioli *et al.*, 1992; Noble *et al.*, 1999). In this study, although there was evidence of such storage in the form of CD69 mRNA expression, surface expession could not be induced that way. On the other hand, longer stimulation with GM-CSF resulted in CD69 expression on the cell surface, as had been shown before (Atzeni *et al.*, 2002). It was observed that when neutrophils were treated with GM-CSF to induce CD69 expression, it resulted in a considerable decrease in S1PR1 transcription, indicating a link between CD69 and S1P signalling. Moreover, although neutrophils left in media overnight could be stimulated by S1P to increase their impaired chemotaxis when compared to freshly isolated neutrophils, GM-CSF induced CD69 expression resulted in no such effect being observed. Again this could be another indication of CD69 crosslinking with S1P receptors in neutrophils.

7.3 Effects of S1P on endothelium

According to the current study, it appears that S1P cannot affect neutrophil migration significantly by acting directly on neutrophils. However, neutrophil migration can be regulated in indirect ways, through effects on other cells that would lead to altered chemokine production, adhesion molecule expression, etc.; especially if these cells are endothelial cells. Since neutrophils need to pass through the endothelium in order to be recruited to the inflammation sites, any change in endothelium status could affect their trafficking. The most important endothelium aspect that would affect neutrophil trans-endothelial migration is the endothelial barrier, which regulates endothelial permeability. There are several reports in the literature that S1P can enhance endothelial barrier integrity (Garcia *et al.*, 2001; Singleton *et al.*, 2005; Itagaki *et al.*, 2007). This was confirmed in the current study, where S1P was found to inhibit neutrophil migration through treated endothelial cells, as well as decrease endothelial permeability. The S1P receptor that is responsible for this effect appears to be S1PR1, a finding shared with Singleton *et al.*, although a novel S1PR1 agonist was used to show this in our study (Singleton *et al.*, 2005).

The mechanism through which S1P signalling enhances endothelial barrier integrity is not thoroughly understood yet. Some studies report S1P effects on cytoskeleton, and specifically actin rearrangement, that leads to cell spreading to close intercellular gaps (Garcia *et al.*, 2001; Singleton *et al.*, 2005; Xu *et al.*, 2007). Whether that is enough to inhibit neutrophil migration through the endothelium though is still a question. Endothelial cells interact with each other and keep in close contact through intercellular junctions. One of the most important types of these junctions is adherens junctions, which consist of several molecules forming a complex that links with respective complexes from the adjacent cell.VE-cadherin is a major molecule of endothelial adherens junctions, that has been found to be increasingly localized in cell-cell contact regions by S1P without affecting its total protein levels (Lee *et al.*, 1999; Mehta *et al.*, 2005). This localization could assist in adherens junction assembly, leading to increased endothelial barrier functions and decrease in permeability. However, phosphorylation of VE-cadherin can cause junction instability and prevent proper assembly, which would have the opposite effect in permeability and endothelial barrier integrity. So it is important to investigate whether the VE-cadherin affected by S1P is phosphorylated or not, before we can make any conclusions. As was shown in chapter 5, S1P can actually decrease VE-cadherin phosphorylation on tyrosine 658 (Y658), an important residue for

junction assembly, leading to better junction stabilization and eventually enhancement of endothelial barrier integrity.

Neutrophils migrate through the endothelium by following chemokine gradients and adhering to the endothelial cells through adhesion molecules before finding a gap to pass through to the inflamed tissue. So, any effects on chemokine production or endothelial cells adhesion molecule expression would also affect neutrophil trans-endothelial migration. S1P can induce chemokine production by endothelial cells, more notably the chemokine IL-8 which is important for neutrophil migration. The chemokine CXCL1, which is another chemoattractant for neutrophils, is also affected. In the literature it has been reported that S1P induces IL-8 expression not only by endothelial cells (Lin *et al.*, 2006), but also several other types of cells, including alveolar epithelial cells (Milara *et al.*, 2009), immature dendritic cells (Oz-Arslan *et al.*, 2006) and human retinal pigment epithelial cells (Qiao *et al.*, 2012). Increased IL-8 production by S1P would lead to increased neutrophil recruitment, theoretically. Moreover, S1P appears to be able to induce adhesion molecules ICAM-1 and VCAM-1 surface expression by endothelial cells, which would increase neutrophil adhesion to the endothelium, theoretically assisting neutrophil migration. In flow based adhesion assays though, the increase in adhesion molecules did not appear to be enough to increase adhesion of neutrophils to endothelial cells. On the other hand, S1P can inhibit TNF-α induced VCAM-1 expression and neutrophil adhesion to endothelium. Therefore, in an inflammatory state that would cause TNF-α production, S1P could be able to inhibit neutrophil adhesion and subsequently migration to the site of inflammation.

The diagram on figure 7.1 sums up the basic effects S1P has on endothelial cells that could indirectly affect neutrophil migration. On the one hand it could inhibit migration by enhancing endothelial barrier integrity; on the other hand it could assist neutrophil migration by inducing the production of IL-8 and endothelial adhesion molecules ICAM-1 and VCAM-1 (figure 7.1). Since these two are contradicting effects, it would be interesting to find out which effect persists in an *in vivo* environment. Using a mouse model of peritoneal cell recruitment by IL-8, it was discovered that S1P, when administered *in vivo*, could inhibit neutrophil recruitment, concluding that S1P enhancement of endothelial barrier is the more pronounced effect *in vivo*.

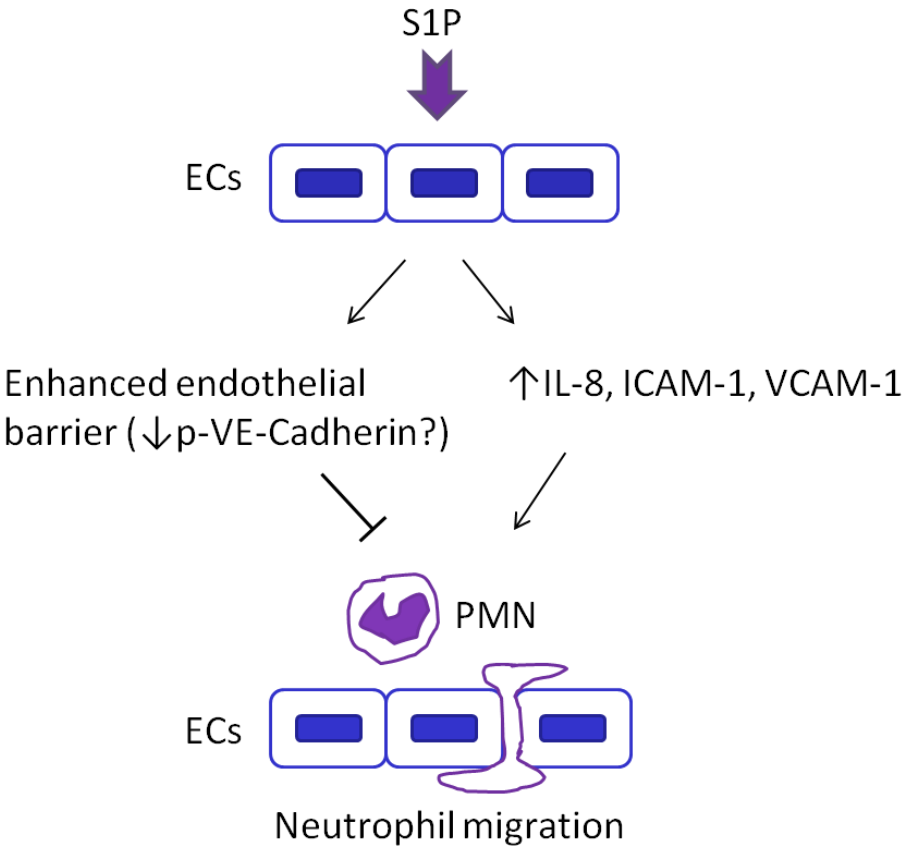


Figure 7.1. Effects of S1P on endothelial cells. Schematic diagram summarising the diverse effects S1P has on endothelial cells and how these could affect neutrophil migration. ECs: endothelial cells, p-VE-Cadherin: phosphorylated VE-cadherin, PMN: neutrophils

7.4 Future work

There are a lot of further investigations that could arise from the work performed in this study. Before moving on to using S1P therapeutically to inhibit neutrophil recruitment in inflammatory conditions, further work is required. The chapter on the direct effects of S1P on neutrophils showed some results conflicting with previous reports. Before reaching to a conclusion as to which result is true, it would be useful to repeat some of these experiments by changing certain parameters that would maybe lead to a different outcome. For example, repeating the experiment on the effects of S1P on neutrophil chemotaxis towards IL-8, using lower, sub-optimal concentrations of IL-8, might produce different results. Even if that is not the case and S1P indeed does not have a direct effect on neutrophil chemotaxis, S1P signalling on neutrophils should produce some effect, since downstream ERK1/2 signalling was observed. Other neutrophil functions, such as neutrophil degranulation, oxidative burst, and NET formation could be investigated to determine which of them are affected by S1P. In regards to any connection of CD69 with S1P signalling on neutrophils, although there was some evidence towards that in this study, it was far from conclusive. There appeared to be a cross-linking between CD69 and S1PR1 expression. This could be further investigated by using immunoprecipitation techniques, to determine if the two molecules are physically linked on the cell surface. Cross-talk between the chemokine receptors CXCR1 or CXCR2 and S1P receptors on neutrophils might also exist and could be investigated in the future using the same technique.

It was found that S1P can inhibit neutrophil trans-endothelial migration *in vitro* by enhancing endothelial barrier integrity. This result could be expanded, by using siRNA to knock out specific S1P receptors on endothelial cells to better determine the S1P receptors responsible for this effect, and confirm the results shown by using S1P receptor agonists. Another method for that would be to use S1P receptor antagonists, together with S1P, that would block the binding of S1P to a specific S1P receptor, observing the outcome to neutrophil chemotaxis. Furthermore, the mechanisms behind the barrier enhancing effects of S1P could be further investigated. It was found that S1P can decrease VE-cadherin phosphorylation at Y658, potentially leading to increased junction stability and barrier enhancement. Other candidate residues that could be investigated are tyrosine 685 and 731, also reported to have an effect on junction assembly. Moreover, other junction molecules like β-catenin can also be phosphorylated leading to increased endothelial permeability. These could be further examined as well

as part of the mechanism of action of S1P on the endothelial barrier. A different method to examine general phosphorylation of junction molecules, other than western blotting using specific phospho-antibodies, would be immunoprecipitation with phospho-tyrosine antibody. This result could be expanded further by looking into tyrosine kinases Src and Pyk2 expression to determine whether S1P signalling leads to inhibition of these molecules, which have been reported in the past to be responsible for VE-cadherin phosphorylation (Allingham *et al.*, 2007).

In order to examine whether S1P signalling is a viable therapeutic target for the manipulation of neutrophil migration under various inflammatory conditions, *in vivo* animal models should be utilized first. The *in vivo* models used in this project could be expanded, by adding higher numbers of animals to result in a more significant outcome. The peritoneal recruitment model appeared to be more promising, but some further optimization of the air pouch model could lead in cleaner results. Moreover, the use of different S1P receptor agonists and antagonists would allow to better focus on the desirable S1P effects, eliminating any unwanted diverse effects. After researching the agonists *in vitro*, to find the most suitable, they could be used *in vivo* to expand the animal models. Another approach that could be followed is the production of knock out animals for different S1P receptors, to investigate whether neutrophil recruitment is impaired or enhanced. However, this might prove difficult for some receptors like S1PR1, which are vital and their absence would cause serious developmental problems, including embryonic lethality. In that case, a conditional or inducible knockout animal model, which would result in the selective inactivation of the receptor on specific types of cells, at the desired time point, would be an alternative approach.

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Publications arising from this study

Published:

Giannoudaki, E., Swan, D.J., Kirby, J.A., Ali, S. (2012) 'Sphingosine-1-phosphate signaling as a therapeutic target', *Cell Health and Cytoskeleton*, 4, pp. 63-72 (**review**)

Giannoudaki, E., Kirby, J.A., Ali, S. (2013) 'Sphingosine 1-phosphate in ischemia-reperfusion injury: effects on trans-endothelial migration of neutrophils', *Immunology*, 140 (Suppl. 1), pp. 39-184 (**abstract**)

Under preparation:

Giannoudaki, E., Kirby, J.A., Ali, S. Sphingosine 1- phosphate: effects on neutrophil trans-migration (working title). Manuscript under preparation for submission to the *Journal of Cell Science*

Oral and poster presentations:

FASEB Science Research Conferences 2014: 'Phospholipid Cell Signalling and Metabolism in Inflammation and Cancer', Niagara Falls, NY, USA, June 2014 (**poster**)

British Society for Immunology Congress 2013, Liverpool, UK, December 2013 (**poster**)

Institute of Cellular Medicine seminar series, Newcastle University, Newcastle upon Tyne, UK, November 2013 (**oral presentation**)

Newcastle University Cardiovascular Research Group Away Day, Newcastle upon Tyne, UK, November 2013 (**poster**)

North East Postgraduate Conference 2013, Newcastle upon Tyne, UK, October 2013 (**oral presentation**)