INVESTIGATING THE REVERSE TRANSMIGRATION OF NEUTROPHILS IN HUMAN AND MURINE *IN VITRO* MODELS OF INFLAMMATION

By

Frantzeska-Maria Diapouli BSc (Hons)

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ABSTRACT

The aim of this project was the study of neutrophil recruitment and reverse transmigration using murine and human *in vitro* models of inflammation. Murine *in vitro* models of inflammation were developed using an immortalised microvascular cell line (MCEC-1) and primary murine vascular endothelial cells (mEC) isolated from heart and lung. We found that MCEC-1 could recruit murine neutrophils without the requirement of cytokine stimulation, although efficient transmigration did require such a stimulus. Primary cells required cytokine stimulation to recruit mEC. Interestingly, and in contrast to human EC, mEC were relatively insensitive to TNF-α stimulation, although IL-1β was a good stimulus for adhesion and migration. Using the IL-1β driven system we generated reverse migrated murine neutrophils and their phenotype and prolonged survival were assessed. The effect of shear stress and nitric oxide on the regulation of the process of reverse migration was examined. Using adoptive transfer strategies we investigated the fate of mRPMNs *in vivo*. A significant part of this work involved the study of human reverse migrated neutrophils at a proteomic level using two-Dimensional Fluorescence Gel Electrophoresis methodology to identify changes in neutrophils associated with reverse migration process. We found that murine reverse migrated neutrophils had a very similar surface phenotype to human reverse migrated cells. They also showed prolonged survival. However, our preliminary data on trafficking *in vivo* did not give a clear indication about their fate upon adoptive transfer into recipient mice. *In vitro* studies showed that flow generated shear stress and nitric oxide delayed, but did not inhibit, the process of reverse migration. Finally, the proteomics study revealed a number of metabolic, cytoskeletal and regulatory proteins that were differentially expressed in human reverse migrated neutrophils although the functional significance of these changes is yet to be explored.

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LIST OF ABBREVIATIONS:

Ab	Antibody
ATP	Adenosine trisphosphate
ANOVA	Analysis of Variance
APC	Antigen presenting cell
BSA	Bovine serum albumin
BVA	Biological variance analysis
C	Complement protein
Ca2+	Calcium
CAM	Cell adhesion molecule
CD	Cluster of differentiation
cDNA	Complementary DNA
CSF	Cerebrospinal fluid
CXCR	CXC chemokine receptor
DC	Dendritic cell
DEANO	Diethylamine NONOate
DIA	Differential In-gel Analysis
DiOC6	3,3′-dihexyloxacarbocyanine iodide
DMSO	Dimethylsulphoxide
DMEM	Dulbecco's Modified Eagle's Medium
EC	Endothelial cell
ECM	Extracellular matrix
EDA	Extended data analysis
ELAM-1	Endothelial leukocyte adhesion molecule-1

eNOS	Endothelial nitric oxide synthase
E-selectin	Endothelial selectin
EGF	Epidermal growth factor
ESL	E-selectin ligand
EDTA	Ethylenediaminetetraacetic acid
ECM	Extracellular matrix
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal calf serum
fMLP or fMLF	formyl-Met-Leu-Phe
g	Gravitational force
GlyCAM-1	Glycosylated cell adhesion molecule-1
GPI	Glycoylphosphatidylinositol
GM-CSF	Granulocyte-macrophage colony stimulating factor
HEV	High endothelial venule
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
h	Hour
HUVEC	Human umbilical vein endothelial cells
HCl	Hydrochloric acid
HSP	Heat shock protein
Ig	Immunoglobulin
IFN-γ	Interferon-gamma
IL	Interleukin
ICAM-1	Intercellular adhesion molecule-1

JAM	Junctional-adhesion molecule
KDa	Kilodalton
L-NAME	NG-nitro-L-arginine methyl ester
L-selectin	Leukocyte selectin
LTB	Leukotriene
LTB4	Leukotriene B4
LPS	Lipopolysaccharide
LX	Lipoxins
LOX	Lipoxygenase
MACS	Magnetic-activated cell sorting
MCEC	Murine cardiac endothelial cell
M-CSF	Macrophage-colony stimulating factor
Mg2+	Magnesium
MHC	Major histocompatibility complex
MFI	Median fluorescent intensity
mEC	Murine endothelial cell
mRNA	Messenger ribonucleic acid
M199	Culture medium 199
MHEC	Murine heart endothelial cell
MLEC	Murine lung endothelial cell
µg	Micro-gram
µl	Micro-litre
µM	Micro-molar
ml	Milli-litre

mm	Milli-metre
mM	Milli-molar
Min	Minute
M	Molar
MCP-1	Monocyte chemoattractant protein-1
MS	Multiple sclerosis
ng	Nano-gram
nM	Nano-molar
NAPDH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
Pa	Pascal
PCA	Principal component analysis
PRR	Pattern recognition receptor
PBMC	Peripheral blood mononuclear cell
PPAR	Peroxisome proliferator-activated receptor
PMA	Phorbol 12-myristate 13-acetate
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline with bovine serum albumin
PBS2A	Phosphate buffered saline with 2% bovine serum albumin
PAF	Platelet activating factor
P-selectin	Platelet selectin
PECAM-1	Platelet/endothelial cell adhesion molecule
PFA	Paraformaldehyde

PGSL1	P-selectin glycoprotein ligand 1
PMN	Polymorphonuclear leukocyte
RA	Rheumatoid arthritis
ROIs	Reactive oxygen intermediates
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RPMN	Reverse migrated neutrophil
SMC	Smooth muscle cells
SEM	Standard error of the mean
TCR	T-cell receptor
TLR	Toll like receptor
TNF-α	Tumour necrosis factor-alpha
TNFR	Tumour necrosis factor-alpha receptor
U	Unit
VCAM-1	Vascular cell adhesion molecule-1
VE-cadherin	Vascular endothelial cadherin
WT	Wild type
2D-DIGE	Two-dimensional difference in gel electrophoresis

CHAPTER 1

GENERAL INTRODUCTION

1.1 Inflammation

The immune system is a highly organised system, which contains many different organs and cells. Its purpose is to protect the body against external invaders such as pathogens by successfully removing them. It also plays a significant role in tissue repair after infection or trauma. A major function of the immune system is to maintain homeostasis and to do this it has to deliver immune/inflammatory cells when and where they are needed (Gallin *et al*., 1992). Generally, the physiological inflammation operates on an acute response model, where rapid resolution of the inflammatory process terminates potentially damaging cellular activity. However, chronic inflammatory diseases may demonstrate prolonged or inappropriate inflammatory activity and/or an impaired resolution phase, all of which contribute to pathological changes in the effected tissues. There may also be an element of systemic damage in organs and tissues distant from the original inflammatory insult.

From ancient times, several symptoms of inflammation have been reported: heat, swelling, redness and pain. These symptoms are caused by vasodilation at the site of inflammation and endothelial cell damage induces the formation of plasma and enzyme mediators that increase vascular permeability and vasodilation. This causes heat and redness. The increase in vascular permeability causes the swelling leading to oedema and pain. Upon initiation of inflammation, leukocytes, which circulate in large numbers in the blood, are rapidly recruited to the site of inflammation. This process appears to be driven by tissue-resident macrophages receiving signals from tissue damage or invading organisms and initiating the recruitment of a subset of leukocytes; neutrophils. Tissue macrophages achieve this by secreting agents, such as IL-1, IL-6 and TNF-α, which activate vascular endothelial cells responsible for neutrophil recruitment by the multiple-step process of extravasation

(Feldmann *et al*., 1996). Following neutrophils, circulating monocytes are recruited, and after entering the tissue these become tissue resident-macrophages or dendritic cells. After the recruitment of these professional phagocytes to the site of inflammation, they execute their immune functions by removing the external pathogen and facilitating the tissue repair.

Under physiological conditions, inflammation is resolved. Whenever this does not happen, persistent chronic inflammation may follow. In this case, leukocytes continue to infiltrate the tissue and stay for prolonged periods in the tissue where they continue to exhibit their pro-inflammatory functions. In this case, aberrant tissue remodelling occurs, leading to the loss of normal tissue and/or organ function.

1.2 Blood

The blood consists of the plasma and the cells that circulate in it. Plasma accounts for 60% of blood volume. It contains water, glucose, electrolytes, and many proteins, some of which are responsible for transport of other organic compounds such as insoluble lipids. Among the many proteins present in plasma are antibodies, complement and coagulation factors, all of which play an important role in the immune and inflammatory processes. The remaining 40% of blood volume constitutes the cellular elements; erythrocytes, platelets and leukocytes. The most abundant cell population in the blood is the erythrocytes, with a concentration of $5x10^{12}$ cells/L. Their function is to facilitate gas exchange, as they transfer O_2 and CO_2 between the lungs and other respiring tissues (Pallister, 1994). Their elliptic shape also contributes for this function, as they are flexible and small with a large surface area/volume ratio. Platelets, which account for $2.5x10^{11}$/L, are cellular fragments with no

nucleus that derive from megakaryocytes in the bone marrow. Their major role is haemostasis, forming clots to limit blood loss. They are also involved in wound repair (Pallister, 1994), thereby securing the integrity of the cardiovascular system upon trauma. There is also increasing evidence that platelets play a role in the inflammatory process by promoting the recruitment of leukocytes (Lindemann *et al.*, 2007). Leukocytes are further divided into different sub-populations, each one playing a role in the innate or adaptive immune response as will be described further in this text.

1.2.1 Cells of the innate immune response

The innate immune response is the first line of defence against pathogens and can be initiated within minutes. The mechanism of action of the innate system is considered to be non-specific and responsible for rapid destruction and clearance of invading pathogens. There are three sub-sets of leukocytes involved in innate immunity: granulocytes (neutrophils, eosinophils, basophils), mononuclear cells (monocytes, macrophages) and mast cells (Figure 1.1). All three have in common the ability to phagocytose pathogens. This happens due to the existence of various types of receptors such as toll-like receptors (TLRs) or pattern recognition receptors (PRRs). These receptors recognise common repeat motives present in carbohydrates or lipids on the surface of foreign but not in the host organism. Coating of the foreign organism with immunoglobulin or complement can increase the efficiency of receptor- mediated phagocytosis.

1.2.1.1 Granulocytes

Granulocytes are cells that contain intracellular vesicles or granules. Depending on the affinity of the contents of the granules to several dyes, granulocytes are divided in three sub-categories: the neutrophils, the eosinophils and the basophils.

Neutrophils are named after granule staining patterns that appear neutral after the application of dyes. They are the most abundant circulating leukocyte population in the blood. They have a short cell life and can be recruited to the site of inflammation within minutes (Cheretakis *et al*., 2006). Their primary function is the phagocytosis of the microbe invaders. Neutrophils are able to recognise foreign antigen via their Fc receptors FcγRIIa (CD32), FcγRIIIb (CD16) and complement receptor CR1 (CD35), although the major receptor for phagocytic uptake is the C3Rb otherwise known as the β2 integrin Mac-1 (CD11b/CD18). The activation of the neutrophils results in the production of a respiratory burst which releases reactive oxygen species capable of killing pathogens. They have two different types of granules: the azurophilic and the specific (secondary granules). The first contain myeloperoxidase, acid hydrolases and neutral proteases that have an antimicrobial action. The second contain lactoferrin, collagenase, alkaline phosphatase and vitamin B12-binding proteins (Alberts *et al*., 2002). Granules in the cytoplasm fuse with the phagolysosome and release their cytotoxic contents. The pathogen is then destroyed within a controlled environment. Another way for pathogen destruction is through respiratory burst. Respiratory burst results in the production of cytotoxic oxygen metabolites such as singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radicals by the enzyme NADPH oxidase, on the membrane of the phagosomes. NADPH oxidase releases oxygen metabolites into the

phagolysosomes during phagocytosis and facilitates the destruction of the pathogen (Edwards, 1994).

Eosinophils are pro-inflammatory cells with a bilobed nucleus. They have a large number of IgE receptors on their membrane and play an important role in the allergic response. Eosinophils also have granules that contain peroxidase and toxic proteins, able to destroy parasites, such as roundworms. Recent data suggest that eosinophils could act as APC (antigen presenting cells) to promote expansion of Th2 cells *in vitro* and *in vivo* (Shi, 2004). Basophils are the least numerous group of granulocytes. These cells are non-phagocytic and participate in the allergic response, by releasing histamine. They also play a role in the host defence against parasites (Askenase, 1980).

1.2.1.2 Monocytes

Monocytes circulate in the blood for many hours before they migrate to the tissue and remain there as resident cells under non-inflammatory conditions. As resident cells, they differentiate into macrophages and dendritic cells. An example of a specialised tissue resident macrophage are the Kupffer cells in the liver which play important scavenger roles. Langerhan cells in the skin are an example of specialised dendritic cells important for maintaining the integrity of the dermal barrier. Macrophages play an important role, both in innate and adaptive immunity. In the former, their functions are the detection and destruction of pathogens, the secretion of pro-inflammatory cytokines and growth factors, agents that in their own right can activate other leukocyte subsets (Janeway *et al*., 2001). Their participation in tumour killing (Cameron and Churchill, 1975), wound repair (Leibovich and Ross, 1975) and antigen presentation is also reported. Importantly, abnormal macrophage activity is

associated with pathology in chronic inflammatory disease such as atherosclerosis and rheumatoid arthritis. Dendritic cells function mainly as antigen presenting cells and their function will be further explored in the section describing the adaptive immune response.

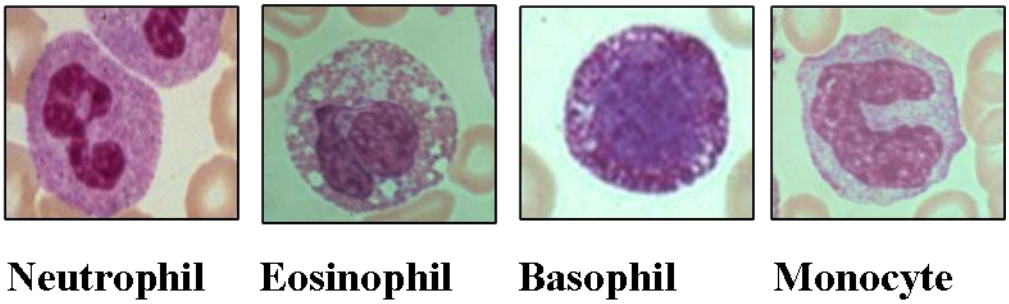


Figure 1.1 Cells of the innate immune system
Each cell type has a morphologically distinct lobular nuclei and cytoplasmic granules. Pictures courtesy of Professor G.B. Nash, Department of Physiology, University of Birmingham.

1.2.2 Cells of the adaptive immune response

Contrary to the innate immune response, the adaptive immune response is characterised by specificity and memory. Upon the first encounter with an invading pathogen, the adaptive immune response starts to function after 4-7 days post-infection. However, subsequent exposure to the antigen requires only 1-2 days for an effective response to occur. The cells of the adaptive immune response are the B and T lymphocytes, the natural killer (NK) cells and the antigen presenting cells (mainly dendritic cells) or APCs. Dendritic cells become activated once they encounter a pathogen and migrate to the lymph nodes via the lymphatic system. In the lymph nodes, they mature and present antigen on their surface. When the antigen is viral, it is presented to $CD8^+$ cells in major complex histocompatibility complex class I. Then $CD8^+$ cells become activated and have the ability to lyse infected cells using perforin and subsequently induce apoptosis (Janeway *et al*., 2001). $CD4^+$ cells once ligated on APCs presenting an antigen in conjunction with MHC class II molecules, become activated and secrete cytokine such interleukin 2 (IL-2) that triggers the proliferation of other $CD4^+$ and $CD8^+$ T cells. Upon ligation of specific antigen (via the T-cell receptor or TCR) on

an antigen presenting cell (often a dendritic cell), T cells become activated. Apart from the between T-cell TCR-CD3 complex and the APC antigen peptide interaction, an additional co-stimulatory signal, provided by T-cell CD28 and APC B7 molecule ligation is required for T-cell activation (Janeway *et al*., 2001). Activated Th2 cells release IL-4, which induces the antigen class switching of B cells. There are also the regulatory T cells (previously named suppressor T cells), which are $CD4^+$ cells that co-express the receptor for IL-2, CD25. Research since the 90's has shown that these cells play an important role in the control of autoreactive T cells *in vivo* (Sakaguchi *et al*., 1995).

B and T cells are derived from common progenitor cells in foetal liver and later in adult life, from the bone marrow. B cells are generated and mature in the bone marrow. In contrast, T cells, after their generation in the bone marrow, migrate to the thymus where they mature and undergo selective deletion of self (auto-) reactive clones. T-cells then migrate to the lymph nodes where they are functional. B cells are responsible for the humoral response. Upon ligation of an antigen presented on a professional antigen presenting cell such as a dendritic cell, B cells undergo clonal expansion and differentiate into plasma cells which subsequently secrete antibodies specific to that antigen (DeFranco, 1987). T-cells are also essential for efficient humoral responses, as co-stimulatory factors derived from antigen stimulated T-cells are essential for B-cell maturation.

There are 5 isotypes of immunoglobulins: the IgA, IgD, IgE, IgG and IgM. Each isotype performs a different function; IgA is in secretions (e.g. mucus), IgE is involved in the mast cell degranulation and allergic and anti-parasite responses, IgG is the major isotype in the blood involved in the opsonisation of pathogens and IgM is the first to be secreted in the

primary immune response via activation of the complement system. Upon activation, B-cells perform class switching, where B cells secrete different isoforms of the antibody specialised to the different phases of the immune response (Janeway *et al*., 2001).

1.3 Vascular endothelium

Endothelial cells (EC) form the inner lining of all the vessels of the vascular tree and, therefore, are in direct contact with blood. This layer is an active barrier between blood and the tissue and regulates a number of important functions, such as the transfer of gases, metabolites and cells (leukocytes) from blood into tissue and *vice versa*. The integrity and the specificity of the barrier function of different vascular beds are regulated by the existence of a system of complex junctions, i.e. the tight junctions, adherens junctions and gap junctions (Rubin, 1992). The EC also secrete an extracellular matrix or basement membrane consisting mainly of laminins, type IV collagen and heparin proteoglycans which is an essential anchorage point for the ECs (Erickson and Couchman, 2000).

Depending on their site in the vascular tree, EC exhibit distinct phenotypic and morphological characteristics. EC in the blood brain barrier have highly organised tight junctions securing the homeostasis of the cerebral environment. But in the sinusoid capillaries of the liver EC the lack of junctions and basement membrane, as well as a highly fenestrated surface, enables the direct movement of macromolecules from the blood to the liver parenchymal cells (Enomoto and Nishiwaka, 2004). Importantly, EC in post capillary venules are involved in the recruitment of leukocytes during inflammation. In response to inflammatory cytokines, they express specific adhesion molecules and chemoattractants

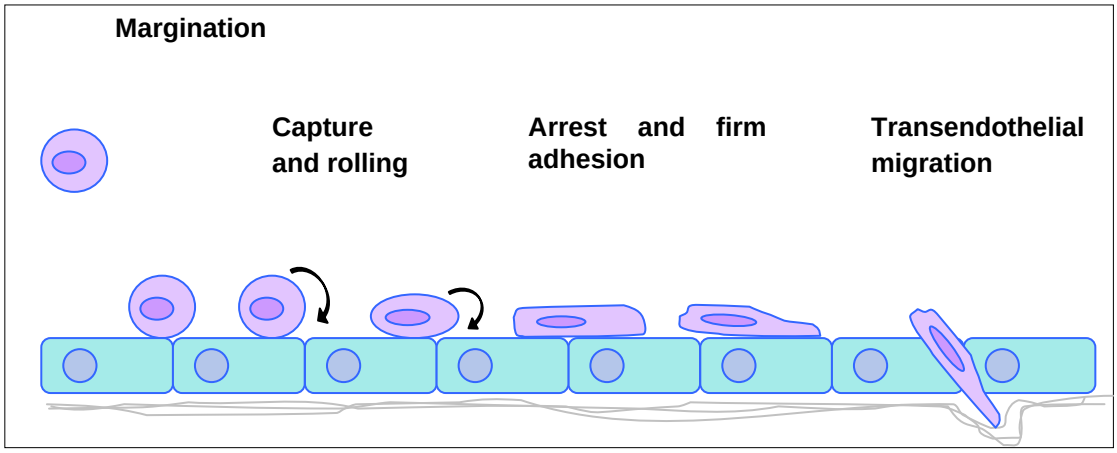


Figure 1.2 The leukocyte adhesion cascade
Leukocytes are captured from flow and roll on specialised adhesion receptors on the endothelium surface. Then they become activated by chemoattractants and arrest on the EC surface. Next, they migrate on the endothelial cells and finally transmigrate through EC junctions/endothelial cells. Following transmigration, they penetrate the basement membrane and finally enter the tissue.

1.4.1 Capture and rolling

The flow of red cells in the blood pushes the leukocytes close to the vessel wall. This rheological phenomenon is called margination and enables the leukocytes to be in contact with the EC of the post-capillary venule. This process is independent of any inflammatory activation of the vasculature. When inflammation does occur, leukocytes are captured from flow via receptors highly expressed on the EC and leukocytes. Selectins are a large family of receptors of which there are three members: endothelial (E)-selectin, leukocyte (L) - and platelet (P)-selectin. All selectins contain a -NH2 terminal C-type lectin domain, a conserved epidermal growth factor (EGF-like) motif, a number (2-9) of short complement regulatory (CR) repeats, a transmembrane domain and a short cytoplasmic tail. Selectin ligands are glycoproteins and glycolipids with sialyl Liews X (SleX) motifs in their structure and bonds formed between selectins and their ligands are calcium dependent. Selectins form strong yet

short-lived bonds, and the continuous breaking of bonds and making of new bonds results in a dynamic form of adhesion resulting in the rolling of the cells in the direction of blood flow.

L-selectin

L-selectin expression is restricted to leukocytes, usually on their microvilli. It is important for leukocyte recruitment in post capillary venules as well as the homing of lymphocytes to the lymph nodes (von Andrian *et al*., 1993). L-selectins can bind to P-selectin glycoprotein ligand-1(PSGL-1) (Spertini *et al*., 1996), CD34 (Oxley and Sackstein, 1994) and glycosylated cell adhesion molecule-1(GlyCAM-1) (Lasky *et al*., 1992). Soon after they have been activated, leukocytes undergo proteolytic cleavage of their L-selectin. The physiological role of this phenomenon is not known. It has been shown that inhibition of this process *in vivo* results in increased leukocyte adhesion and transmigration (Hafezi-Moghadam *et al*., 2001). However, no change in adhesion and transmigration was observed when the shedding was inhibited *in vitro,* in flow and static adhesion assays using tumour necrosis factor-alpha (TNF-α) stimulated EC (Allport *et al*., 1997a).

P-selectin

P- selectin is expressed in EC and platelets. In EC, P-selectin is stored in Weibel-Palade bodies (Bonfanti *et al*., 1989) and it translocates to the membrane within minutes after stimulation with oxygen radicals (Patel *et al*., 1991), histamine (Asako *et al*., 1994) or thrombin (Theoret *et al*., 2006). P-selectin can also be mobilised by inflammatory cytokines such as TNF-α, so that, 2-4 hours after stimulation it is expressed and is detected on the EC surface (Springer, 1994). However, it is unclear whether this expression occurs due to the translocation of the molecules previously in Weibel-Palade bodies or by *de novo* synthesis of

the protein. P-selectin is suggested to play an important role in the early stages of inflammation. When P-selectin was blocked *in vivo* using a function-blocking antibody in the canine cremaster muscle, total leukocyte adhesion was reduced by a 60% (Dore *et al*., 1993). Additionally, in a model of peritoneal cavity inflammation in the P-selectin knockout mouse, there was a delay in neutrophil recruitment to the peritoneal cavity (Mayadas *et al*., 1993). Mice lacking the major P-selectin ligand, the PSGL-1, also exhibited dramatic reduction in neutrophil recruitment to the peritoneal cavity 2 hours after inflammation induction by thioglycollate (Yang *et al*., 1999). Some investigators have suggested that P-selectin ligation on the leukocytes can trigger signal transduction pathways in them which finally lead to integrin up-regulation and activation (Hidari *et al*., 1997). Nevertheless, there is strong *in vitro* evidence that suggest that leukocytes are able to roll indefinitely on P-selectin coated surfaces or P-selectin on platelets (Sheikh and Nash, 1996).

P-selectin in platelets is present in α-granules (Stenberg *et al*., 1985). In activated platelets, granule release results in P-selectin expression on the surface of the platelets. It has been demonstrated that platelets are able to support leukocyte adhesion *in vitro*. Neutrophils could adhere on P-selectin lipid bilayers and activated platelets within physiological shear stresses in the absence of other selectins and IgSF members (Buttrum *et al*., 1993; Diacovo *et al*., 1996; Lalor and Nash, 1995; Lawrence and Springer, 1991; Rainger *et al*., 1998). *In vivo* studies have confirmed the importance of P-selectin as a capture receptor and more recently, it has been shown to have a synergistic role with E-selectin in increasing the capture of neutrophils in postcapillary venules (Smith *et al*., 2004; Springer, 1995; Zimmerman *et al*., 1992). Thus, P-selectin not only plays a major role in thrombus formation but also the recruitment of leukocytes to sites of inflammation.

This platelet-mediated adhesion may play a role in physiological and pathological inflammatory conditions. There is evidence that this occurs for neutrophil recruitment in hypoxia and reperfusion injury (Kogaki *et al*., 1999). Moreover, it has been shown that platelets may contribute to the formation of atherosclerotic plaque in the apolipoprotein-E (apo-E) knockout mice (Manka *et al*., 2004).

E-selectin

E-selectin is expressed in EC after stimulation with interleukin-1 beta (IL-1β), TNF-α, lymphotoxin or bacterial endotoxin, reaching maximal expression 6 hours post-stimulation and returning to initial levels after 24 hours (Bevilacqua *et al*., 1987). E–selectin ligands on leukocytes are PSGL-1 (Moore *et al*., 1994), E-selectin ligand -1(ESL-1) (Steegmaler *et al*., 1995) and CD44 (Dimitroff *et al*., 2001). It has been recently reported that each ligand has a specific function in the rolling process. Thus, using hairpin RNA to knock down ESL-1 in hematopoietic cells deficient in PSGL-1, CD44 or both ligands it was shown that ligation of PSGL-1 induces tethering, ESL-1 mediates steady rolling and CD44 slow rolling. It has been suggested that close collaboration between the three of these ligands might be necessary for efficient recruitment (Hidalgo *et al*., 2007).

An additional mechanism for rolling is mediated by integrins. It has been reported that cell lines expressing α4β7-integrin roll on mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1). Lymphocytes can roll on immobilised VCAM-1 via VLA4 (α4β1 integrin). This mechanism has been described in monocytes and T-cells. In addition, LFA1 (αLβ2

integrin) can support the rolling of lymphocytes interacting with ICAM-1 (Reviewed by Ley *et al*., 2007).

1.4.2 Activation and firm adhesion

Leukocytes can be activated by a number of chemoattractant molecules such as chemokines, bacterial derived N-formyl peptide (fMLP), the complement fragments C5a and C3a, leukotriene B4 (LTB4) and platelet activating factor (PAF) (Gerard and Gerard, 1994; Goldman and Goetzl, 1982; Hanahan, 1986; Schiffmann *et al*., 2008). However, in the context of physiological inflammation, the role of endothelial derived and presented chemokines are thought to be essential for leukocyte activation during the recruitment process.

Chemokines are a family of small, low molecular weight (8-16KDa) chemoattractant cytokines with the ability to recruit different leukocyte populations by signalling through specific G-coupled counter receptors. There are 4 sub-groups of chemokines depending on the position of two highly conserved cysteines in the amino acid sequence: CXC, CX3C, CC and C. CXC chemokines can be further divided into two groups depending on the presence or absence of an ELR motif next to the CXC motif. The ones containing ELR motif are the CXCL1, 2, 3, 5, 6, 7, 8 chemokines and have the ability to activate neutrophils and some other leukocyte subpopulations. In contrast chemokines lacking the ELR motif mainly activate lymphocytes and monocytes (Laing and Secombes, 2004). The activity of CC chemokines is effectively restricted to monocytes. From the C group, two chemokines have been identified, the lymphotaxins-α and β (Gray *et al*., 1984). From the CX3C group fractakline is the only known member (Bazan *et al*., 1997). Chemokines are secreted by EC at

the sites of inflammation that have been activated by cytokines such TNF-α or IL-1. The binding of chemokines to their G-coupled protein receptors (CCR, CXCR, CX3CR) on leukocytes induces leukocyte integrin activation and, as a consequence, the leukocytes adhere firmly to the EC surface. Using *in vitro* models of inflammation it was shown that CXCL8 chemokine activates the integrin CD18/CD11b and thus leads to the firm adhesion of neutrophils on ECM proteins or EC, and in flow, it can cause neutrophil activation in less than 1 second of tethering via P-selectin (Peveri *et al*., 1988; Rainger *et al*., 1997; Yoshimura *et al*., 1987). Chemokines and their receptors show varying degrees of specificity. As an example there are two main chemokine receptors that play a part in neutrophil recruitment to sites of inflammation: CXCR1 and CXCR2. While CXCR1 can bind only CXCL8 with high affinity and CXCL6 with lesser affinity, CXCR2 interacts with all the ELR motif containing chemokines (Murdoch and Finn, 2000).

Table 1.1 Chemokines and their receptors

Group	Systematic name	Common name	Receptor
CXC	CXCL1	Gro-α	CXCR2
	CXCL2	Gro-β	CXCR2
	CXCL3	Gro-γ	CXCR2
	CXCL4	PF4	CXCR2
	CXCL5	ENA-78	CXCR2
	CXCL6	GCP-2	CXCR1,2
	CXCL7	NAP-2	CXCR2
	CXCL8	IL-8	CXCR1,2
	CXCL9	Mig	CXCR3
	CXCL10	IP-10	CXCR3
	CXCL11	I-TAC	CXCR3
	CXCL12	SDF-1	CXCR4
	CXCL13	BCA-1	CXCR5
	CXCL14	BRAK	Unknown
	CXCL16	CXCL16	CXCR6
CC	CCL1	I-309	CCR8
	CCL2	MCP-1	CCR2
	CCL3	MIP-1α	CCR1,5
	CCL4	MIP-1β	CCR1,5
	CCL5	RANTES	CCR1,3,5
	CCL7	MCP-3	CCR2
	CCL8	MCP-2	CCR2
	CCL11	Eotaxin	CCR2
	CCL13	MCP-4	CCR2,3
	CCL14	HCC-1	CCR1
	CCL15	HCC-2	CCR1,3
	CCL16	HCC-4	CCR1
	CCL17	TARC	CCR4
	CCL18	DC-CK1	Unknown
	CCL19	ELC	CCR7
	CCL20	LARC	CCR6
	CCL21	SLC	CCR7
	CCL22	MDC	CCR4
	CCL23	MPIF-1	CCR1
	CCL24	MPIF-2	CCR3
	CCL25	TECK	CCR9
	CCL26	Eotaxin-3	CCR3
	CCL27	C-TACK	CCR10
CX3C	CX3CL1	Fractalkine	CX3CR1
C	XCL1	Lymphotaxin	XCR1
	XCL2	Lymphotaxin	XCR2

Integrins

Integrins are receptors that mediate the attachment of the cell to one another or to the extracellular matrix (ECM) and play an important role in cell migration and signalling. They are formed by two linked heterodimers, α and β chain. Each chain contains a large extracellular domain forming loops, one transmembrane domain and a short cytoplasmic tail. There are 18 α and 8 β chains, with various dimmer combinations giving rise to 24 integrins. The most important integrins for leukocyte recruitment are the β2 integrins which are found on all leukocytes. $\alpha_L\beta_2$ (CD18/CD11a) is expressed on lymphocytes, NK cells, monocytes and macrophages, neutrophils, dendritic cells and eosinophils (Smith, 2008). Its ligands are ICAM-1,-2,-3 and junctional adhesion molecule–A (JAM-A) (Ostermann *et al*., 2002). $\alpha_M\beta_2$ integrin (or Mac-1) contributes to neutrophil adhesion by binding to ICAM-1 or -2 on the EC surface (Smith *et al*., 1989). Blocking of β_2 integrin results in the inhibition of neutrophil adhesion in a static, *in vitro* model of inflammation (Luscinskas *et al*., 1989). Under flow conditions, antibodies against β_2 integrin specifically inhibited the firm adhesion stage but not rolling adhesion (Bahra *et al*., 1998), suggesting that β_2 integrins are required for firm adhesion and transmigration not for rolling adhesion. Interestingly, blocking of CD11a inhibits the process of neutrophil transendothelial migration, even though cells can attach firmly to the endothelial surface.

After firm adhesion, neutrophils migrate over the EC monolayer on their attempt to find the appropriate location to transmigrate in a CD18/CD11b and ICAM-1 dependent manner (Phillipson *et al*., 2006). At this stage a dramatic actin cytoskeleton rearrangement takes place in neutrophils, which enables the dynamic interaction between integrins and their ligands on EC surface. Lamellipodia formation at the front edge of the neutrophils, in parallel

with the detachment of integrin bonds at the rear of the cell, enables the neutrophils to move forward (Moissoglu and Schwartz, 2006).

1.4.3 Transmigration

The leukocyte adhesion cascade is completed by the transmigration of leukocytes through the EC monolayer. This occurs in two ways; either by paracellular (between endothelial cell junctions) or by transcellular transmigration (through endothelial cells). Although it is suggested that paracellular transmigration is the main type of transmigration for neutrophils, transcellular transmigration through EC has been reported to take place *in vitro* and *in vivo* (Carman and Springer, 2004; Feng *et al*., 1998). There is also evidence that transmigration can occur at tricellular junctions after IL-1β stimulation of EC *in vitro* (Burns *et al*., 1997). There are a number of molecules that have been reported to promote integrin mediated transmigration: CD31, CD99, JAM-A,-B,-C and VE-cadherin (reviewed by Ley, 2007).

CD31 (Platelet Endothelial Cell Adhesion Molecule-1 or PECAM-1) is a 130kDa protein, a member of the IgG superfamily, it is expressed on leukocytes and it is present on endothelial cells, being particularly highly expressed at cell-cell junctions. At the EC junction CD31 molecules interact in a homophillic way, i.e. CD31-CD31 interactions occur between same domains of molecules on opposing cellular surfaces. The role of CD31 in transmigration has been widely studied with contradictory results. Blocking of CD31 on neutrophils, monocytes or EC inhibited transmigration through cytokine stimulated EC in an *in vitro* static assay, suggesting that CD31 is required for transmigration (Muller *et al*., 1993). It should be noted that in our own laboratories the blockade of CD31 had no effect on neutrophil migration

(Luu *et al*., 2003). In addition, the recruitment of leukocytes to sites of inflammation in the CD31 knockout mouse show only minor differences from wild-type animals, i.e. a short delay in passage into tissue, suggesting that CD31 is not essential for transmigration (Duncan *et al*., 1999). There is evidence that CD31 also plays a role in the later stage of leukocyte migration through the subendothelial basal laminin (Dangerfield *et al*., 2002). Work from our own laboratories has shown that although CD31 is not essential for transendothelial migration, it does regulate the direction and velocity of neutrophil migration on EC and their migration velocity in the sub-endothelial environment (Luu *et al*., 2003).

CD99 is a 32 kD highly -O-glycosylated molecule which is expressed on the surfaces of most leukocytes and is concentrated at the borders between endothelial cells. Blocking of this molecule on either leukocytes or endothelial cells blocks transmigration of monocytes (Schenkel *et al*., 2002). For example, blockade of CD99 could block transmigration more than 90% in an *in vitro* model (Schenkel *et al*., 2002). There is also evidence that CD99 blockade inhibits neutrophil transmigration across cytokine treated EC, indicating a role of CD99 in neutrophil transmigration (Bixel *et al*., 2007; Lou *et al*., 2007).

JAMs (Junctional Adhesion Molecules) belong to the IgG superfamily and the three main members, JAM-A,-B and –C, are expressed in different cell types. JAM-A is expressed on leukocytes, erythrocytes, platelets, endothelial and epithelial cells, whereas JAM-B expression is restricted to EC and activated T-cell subpopulations (Dejana, 2004; Williams *et al*., 1999). JAM-A is mainly located at cell junctions or concentrated in cell-cell contacts and there is evidence that JAM-A is involved in transmigration *in vitro* and *in vivo* (Dejana, 2004; Williams *et al*., 1999). Using a function-blocking antibody against JAM-A, monocyte

transmigration through cultured EC and infiltration of monocytes into an air pouch in mice was inhibited (Martin-Padura *et al*., 1998).

JAM-B interacts homotypically and heterotypically with JAM-C. JAM-B can bind the integrin $\alpha_4\beta_{1,}$ but this interaction is JAM-C dependent (Cunningham *et al*., 2002). JAM-C is expressed by leukocyte, EC and platelets. It binds JAM-B, but also the neutrophil $\alpha_M\beta_2$ integrin (Santoso *et al*., 2002). JAM-C has been proposed to play a role in leukocyte transmigration. In *in vivo* models of peritonitis and lung inflammation, it has been demonstrated that soluble recombinant JAM-C administration resulted in a reduction in neutrophil recruitment (Chavakis *et al*., 2004). Similar findings were obtained when antibodies against JAM-C were injected into mice (Aurrand-Lions *et al*., 2005). However, blocking JAM-C could not inhibit neutrophil migration across cytokine-stimulated EC in a human flow model (Sircar *et al*., 2007). When monocytes or HUVEC were treated with blocking monoclonal antibodies against JAM-C, there was no effect on monocyte transmigration (Bradfield *et al*., 2007). Direct observation revealed that more monocytes had migrated back from the abluminal to the luminal side of the endothelium in the presence of the JAM-C antibody (Bradfield *et al*., 2007).

VE-cadherin is a member of the cadherin family and is concentrated at adherens junctions between endothelial cells. It is linked to the cytoskeleton via α- and β- catenins, plakoglobin and p120 (Lampugnani *et al*., 1993). Studies with monoclonal antibodies against VE cadherin demonstrated an increase in the permeability of endothelial cell monolayers, suggesting that VE-cadherin can function as a regulator of increase in endothelial cell

monolayer permeability (Allport *et al*., 1997b; Corada *et al*., 1999; Del Maschio *et al*., 1996). In addition, VE-cadherin is redistributed during neutrophil transmigration (Su *et al*., 2002).

1.4 Migration across the basement membrane

After transendothelial cell migration, neutrophils proceed to penetrate the basement membrane. This stage is reported to be supported by β1 integrins (Dangerfield *et al*., 2002). The basement membrane alters the velocity of neutrophil migration and they become slower than during their penetration of the EC monolayer. Using intravital microscopy methodology to visualise the microcirculation of the unstimulated cremaster muscle, it has been demonstrated that areas low in basement membrane constituents such as laminin, coincide with the areas neutrophils choose to cross the barrier. This suggests that leukocyte migration may be focussed to these areas and that the process of leukocyte migration is minimally disruptive to the basement membrane. Prolonged culture of HUVEC increased their sensitivity to cytokines, but the migration velocity of transmigrated neutrophils was lower (Butler *et al*., 2008; Butler *et al*., 2005).

1.5 Retention of leukocytes at the site of inflammation

Following extravasation, activated neutrophils at the inflamed site execute their immune functions by phagocytosing invading microorganisms and cell debris. Their phagosomes fuse with lysosomal granules containing hydrolytic enzymes and neutrophils produce reactive oxygen species (ROS) that have anti-microbial activity. They also release nuclear content as chromatin forming the extracellular traps (NETs) to trap bacteria free in the

extracellular space. In addition, activated neutrophils produce signals that promote the recruitment of additional phagocytes to the site of inflammation for the effective elimination of microbe invasion and cell debris. They release CXCL8 and CXCL1/2/3 to recruit circulating neutrophils (Cassatella, 1999; Scapini *et al*., 2000). Neutrophils also promote monocyte recruitment in a number of ways. Granule proteins released from recruited neutrophils, such as azurocidin, defensins and cathepsin G, activate the formyl-peptide receptors (FPRs) on circulating monocytes and induce their firm adhesion on the EC (Chertov *et al*., 1996; Chertov *et al*., 1997; Yang *et al*., 2000). Furthermore, an additional mechanism that involves IL-6Rα in a process known as IL-6 trans-signalling induces monocyte recruitment (Romano *et al*., 1997; Taga *et al*., 1989). Activation of neutrophils results in the IL-6Rα shedding. Soluble IL-6Rα, subsequently forms complexes with IL-6 released from macrophages and ECs and these act upon ECs (through gp130), resulting in the up-regulation of VCAM-1 and the release of CCL2, thus promoting monocyte recruitment (Hurst *et al*., 2001). Neutrophils at the site of inflammation also produce a number of chemokines that act as chemoattractants to monocytes, such as CXCL8, CCL2 and CCL3/4 (Cassatella, 1999; Scapini *et al*., 2000).

The cross-talk between neutrophils and macrophages is not limited to the co-ordinated recruitment of additional phagocytes to inflamed tissues. Neutrophils and macrophages co-operate for the destruction of microbial pathogens by phagocytosis as previously described. There is evidence that the former enhance the antimicrobial functions of the latter (Silva, 2010). For example, neutrophil granule molecules are taken from macrophages, thus enhancing their antimicrobial capacity (Byrd and Horwitz, 1991; Mathy-Hartert *et al*., 1996). Macrophages also phagocytose neutrophils and acquire their antimicrobial granule molecules

(Silva, 2010). Macrophages not only promote neutrophil influx, but they also produce signals that enable their retention at the site of inflammation. They release G-CSF and GM-CSF that

prolong the survival of recruited neutrophils. Low concentrations of TNF-α, released from macrophages, also have an anti-apoptotic effect on neutrophils (Silva, 2010).

1.6 Resolution of inflammation

Under physiological conditions, inflammation has to be resolved. Resolution of inflammation appears to be an active and highly regulated process which results in the restoration of tissue homeostasis (Levy *et al*., 2001; Serhan, 2004; Serhan *et al*., 2008). In the early stages of resolution a gradual decrease in neutrophil recruitment is accompanied by enhanced clearance of apoptotic phagocytes. As with the early stages of inflammation, lipid mediators also play a key role in resolution. For example, pro-inflammatory cyclooxygenase products such as prostaglandin-E2 and -D2 promote the synthesis of anti-inflammatory and pro-resolving molecules, such as lipoxins, resolvins and protectins, via the lipoxygenase/cyclooxygenase pathways. This process is known as lipid mediator class switching and is a key event to the initiation of resolution stage (Levy *et al*., 2001).

Lipoxins (LX) are a group of eicosanoids produced from arachidonic acid and they play a crucial role in the resolution of inflammation (Serhan *et al*., 2008). Activated neutrophils metabolize AA to LTA4 (via 5-LOX), which is released at the site of inflammation and further metabolised by platelet 12-LOX, producing the lipoxins LXA4 and LXB4 (Lindgren and Edenius, 1993). LXs are anti-inflammatory and pro-resolution mediators. LXA4 has a major impact in neutrophil recruitment as it stops chemotaxis,

adhesion and transmigration (Fierro *et al*., 2003). It also reduces neutrophil activity by downregulating CD11b/CD18 expression and reducing ROS production and pro-inflammatory chemokines synthesis (Filep *et al*., 1997; Jozsef *et al*., 2002). In macrophages, it stimulates the phagocytosis of apoptotic neutrophils (Godson *et al*., 2000). LXA4 therefore appears to play a dual role in the resolution stage, by reducing neutrophil infiltration at inflamed sites, but also by promoting the monocyte chemotaxis and the phagocytosis of apoptotic neutrophils by them.

Resolvins are lipid mediators synthesised from the omega-3 fatty acids eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) and they are named E-resolvins and D-resolvins respectively. They were first described as products generated from EPA by COX-2 using a murine dorsal air pouch model of inflammation (Serhan *et al*., 2000). It has been demonstrated that E-resolvins inhibit neutrophil recruitment in a TNF-α-induced murine dorsal air pouch model and a zymozan- induced peritonitis model (Arita *et al*., 2005; Serhan *et al*., 2000). D-resolvins are generated from DHA by COX in the presence of aspirin and they also inhibit neutrophil transmigration (Serhan *et al*., 2002). Protectins are generated by LOX from DHA. They play an important role in the resolution of inflammation by inducing macrophage phagocytosis of apoptotic neutrophils (Schwab *et al*., 2007). Protectin D1 and resolvin E1 upregulate CCR5 expression on the surface of apoptotic neutrophils resulting in the removal of CCL3 and CCL5 pro-inflammatory chemokines from the inflamed tissue (Ariel *et al*., 2006).

Macrophages regulate the survival of the recruited neutrophils at inflamed tissues, by releasing a number of mediators. At the initial stages of inflammation, macrophages extend

neutrophil survival. At later stages, after neutrophils have executed their immune functions, macrophages promote their apoptosis via high TNF-α concentrations (van den Berg *et al*., 2001). Apoptosis (or programmed cell death) is thought to be the main route for neutrophil clearance from tissue and is characterised by nuclear chromatin condensation and fragmentation of the nucleus into membrane bound “apoptotic bodies”.

Apoptotic signals are produced after the release of the content of neutrophil granules. Annexin A1 is considered to play a key role to the promotion of neutrophil apoptosis (Perretti and D'Acquisto, 2009; Solito *et al*., 2003). Annexin A1 is a 37 kDa protein, a member of annexin superfamily. Neutrophils, monocytes and macrophages in a resting state constituvely contain annexin A1 in their cytoplasm. Upon activation, annexin A1 is translocated to the membrane and secreted (Perretti and Flower, 2004). It has been demonstrated that this protein promotes neutrophil apoptosis, by dephosphorylating the pro-apoptotic protein BCL-2-antagonist of cell death (BAD) (Solito *et al*., 2003). Annexin A1, released from apoptotic neutrophils, promotes their phagocytosis and clearance from macrophages (Perretti and D'Acquisto, 2009). Other anti-inflammatory function of annexin 1 is the inhibition of neutrophil influx. A number of *in vivo* studies have demonstrated this anti-inflammatory effect, after administration of exogenous annexin A1 (Lim *et al*., 1998; Perretti and D'Acquisto, 2009; Perretti and Flower, 2004). Interestingly, annexin A1 -deficient neutrophils exhibit enhanced transmigration *in vivo* (Chatterjee *et al*., 2005). It is proposed that annexin A1 is a glycocorticoid modulator that promotes the suppression of innate and adaptive immune response by two opposing mechanisms (Perretti and D'Acquisto, 2009).

Once apoptosis has been initiated, neutrophil functional activity is reduced and neutrophil apoptosis is controlled by a complex network of signalling pathways that regulate the up-regulation of molecules such as the anti-apoptotic protein myeloid cell leukemia 1 (Mcl-1) and the pro-apoptotic Bcl-2 family member Bax, as well as activation of the caspase family of proteases (Luo and Loison, 2008). Annexin A1 and lactoferrin released from apoptotic neutrophils abrogate further the neutrophil influx (Bournazou *et al*., 2009; Perretti *et al*., 1996). Apoptotic cells are cleared by phagocytosis by macrophages so that they do not trigger a pro-inflammatory response (Savill, 1989). In addition, they induce the production of transforming growth factor-b (TGF-b) and interleukin 10 (IL-10) from macrophages, promoting resolution and wound repair (Byrne and Reen, 2002; McCartney-Francis and Wahl, 1994). However, if neutrophil apoptosis or phagocytic clearance of dead cells by macrophages is impaired, chronic inflammation may be initiated (Haslett *et al*., 1994) (Figure 1.3).

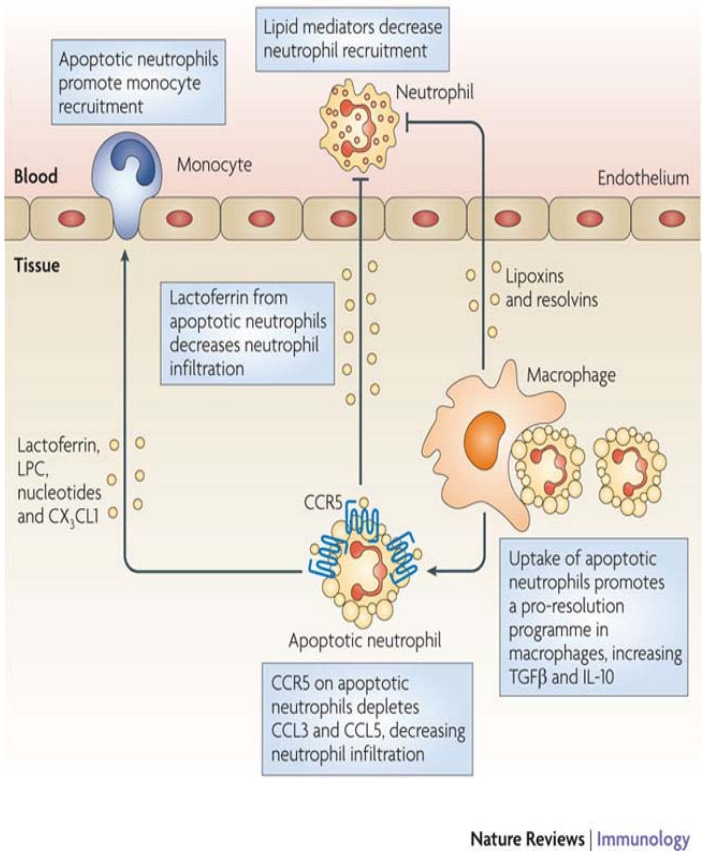


Figure 1.3 Phagocyte interactions during the resolution of inflammation (from Soehnlein and Lindbom, 2010)

When the inflammatory response is completed, macrophages and lymphocytes migrate to the lymphoid organs via the afferent lymphatics. Lymphocytes subsequently recirculate through the blood and lymphatic system. This continuous recirculation via the blood, through tissue into draining lymph and via the thoracic duct, back into the blood, facilitates the immune surveillance and allows immune responses to antigen challenged tissues (Imhof and Dunon, 1995; Mackay, 1993).

1.6.1 Reverse migration

Though it has been reported that leukocyte subpopulations, such as dendritic cells and lymphocytes, have the ability to re-emerge from the tissue via the lymphatic vasculature in order to recirculate in the blood, little is known about the recirculation of other leukocyte subsets (e.g. neutrophils). Furthermore, the general mechanism proposed for neutrophil clearance during resolution of inflammation, suggests clearance of the neutrophils from the tissue by apoptosis (Savill *et al*., 2002). Until recently there was little evidence that neutrophils could exit the tissue (Mathias *et al*., 2006).

A recent report studying the interaction between neutrophils and endothelial cells *in vitro* using a 24h co-culture flow system, has shown that transmigrated neutrophils can reverse migrate back across the endothelial monolayer, suggesting that neutrophils could return back to the blood circulation via the vascular endothelium *in vivo.* In this report more than 90% of migrated neutrophils re-emerged back from the sub-endothelial environment (Buckley *et al*, 2006) and importantly, these cells had a distinctive surface phenotype of $CXCR2^{low}$, CD54 $(ICAM\text{-}1)^{high}$. Interestingly, a small subset (≈0.25%) of human neutrophils in the peripheral blood of healthy donors shared this profile of receptors and the incidence of

'reverse migrated' neutrophils rose to 1-2% in patients with chronic systemic inflammatory diseases such as atherosclerosis or rheumatoid arthritis, implying a connection between inflammation and reverse migration of neutrophils (Buckley *et al*., 2006). This phenomenon challenges the well established perception of neutrophils as short-lived, terminally differentiated, effector cells of the innate immune response that die by apoptosis after having prosecuted their immune functions (Buckley et al., 2006). There are various postulations as to why reverse migration occurs. It has been suggested that it is an additional mechanism for clearance of neutrophils from tissues that is constantly recruiting low numbers of neutrophils during immune surveillance (i.e. gut, lung, peritoneum and other mucosal tissues) (Buckley *et al*., 2006). Indeed, it has been speculated that the increase in ICAM-1 expression on the surface of reverse migrated neutrophils may facilitate their recognition and destruction by the reticuloendothelial cell system after their return to the blood. Another interesting observation in this study was the prolonged longevity of the reverse migrated cells. This prompted speculation that they may be functionally viable and traffic to other tissues (for example the bone marrow). However, what they would do in such an environment remains completely mysterious.

Much less is known about reverse migration in animal models. Quite recently, the work of Mathias *et al*., using a novel transgenic zebrafish model to study the neutrophil recruitment *in vivo*, also showed evidence of reverse migration of neutrophils. With *in vivo* time-lapse imaging they observed retrograde chemotaxis back toward the vasculature, suggesting this as an alternative mechanism of resolution of inflammation (Mathias *et al*., 2006).

Reverse migration is not exclusive to neutrophils. Indeed both monocytes and lymphocytes have been shown to reverse migrate. Thus, blood monocytes co-cultured with endothelium for 2 days differentiated into dendritic cells and macrophages. The DC subset had the ability to migrate back across the endothelium out of the subendothelial environment, whereas macrophages remained in the subendothelial matrix (Randolph *et al*., 1998). Interestingly, blockade of JAM-B/-C interaction resulted in a decrease in the numbers of transmigrated monocytes, through increased reverse migration. *In vivo* studies, confirmed the previous findings. The use of an anti–JAM-C antibody reduced the number of monocytes in the inflammatory site and increased the number of those with a reverse-transmigratory phenotype in the peripheral blood (Bradfield *et al*., 2007). The reverse migration of lymphocytes was observed *in vitro*. Lymphocytes in static cultures with EC migrated back and forth continuously across endothelium (McGettrick *et al*., 2009).

1.7 Murine models of inflammation

Although a lot of research has studied various aspects of neutrophil recruitment in the human system *in vitro*, little has been done using murine models. One of the main reasons is the difficulties in the culture of primary mouse endothelial cells. In addition adequate numbers of isolated neutrophils from whole mouse blood are difficult to obtain due to the small volume of blood circulating in the mouse. This can be overcome by isolation from the bone marrow; however the maturity of these cells is variable. Although mouse endothelial cell lines have been established in order to overcome the difficulties in obtaining primary cells, these do not appear to respond well to inflammatory stimulation. As a consequence, researchers have used a different approach to examine leukocyte recruitment in murine

systems. A lot of research has been done using intravital microscopy in the murine cremaster muscle model, investigating neutrophil transmigration through endothelium and its perivascular basement membrane *in vivo* (Nourshargh and Marelli-Berg, 2005).

Some years ago, researchers established conditionally immortalized growth factor-responsive cardiac endothelial cells from H-2K^{b}-tsA58 mice, and they called this murine cardiac endothelial cell line MCEC-1. They also found that these cells show increased adhesion molecule expression such as CD31, ICAM-1 and E-selectin in response to inflammatory cytokines such as TNF-α and IL-1β (Lidington *et al*., 2002). These cells represent a more realistic opportunity to investigate the inflammatory process *in vitro* using murine tools. Indeed we have conducted a comparison of MCEC-1 and primary murine EC in *in vitro* cytokine driven models of neutrophil migration and reverse migration in this thesis.

1.8 Aims and hypotheses of the project

Overall, the major hypothesis of this project is that reverse migrated neutrophils have a distinct phenotype, are rescued from apoptosis and have a specific protein expression profile. The aim of this project is to study neutrophil recruitment and reverse migration using murine and human *in vitro* models of inflammation.

In Chapter 3, our hypothesis is that murine endothelial cells can respond to different cytokine treatment in a dose dependent manner. For this purpose, we are going to use the MCEC-1 cell line and primary endothelial cells from heart and lung in order to establish flow and static adhesion assays in our *in vitro* models of inflammation.

In Chapter 4, our hypothesis is that murine reverse migrated neutrophils exhibit a specific phenotype and have a prolonged lifespan. We aim to generate murine reverse migrated neutrophils in order to examine their phenotype and survival. Furthermore, we are interested in investigating their fate *in vivo* using an adoptive transfer methodology.

In Chapter 5, our hypothesis is that shear stress inhibits the reverse migration of neutrophils and this mechanism is mediated by NO production. We are going to investigate reverse migration in the presence and absence of continuous flow.

Finally in Chapter 6, our hypothesis is that human reverse migrated neutrophils have a distinct protein expression profile. A significant part of this work will involve the study of human neutrophils at the proteomic level to identify changes in neutrophils that are associated with the process of reverse migration. We are going to use 2-D Fluorescence Difference Gel Electrophoresis methodology (DIGE) to examine the differences in protein expression between human reverse migrated neutrophils and their control groups.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents

All reagents were from Sigma-Aldrich (Poole, Dorset, UK), unless otherwise stated. Reagents included: Collagenase (type 1A), gelatin (2% solution type B from bovine skin), EDTA (Ethylenediaminetetraacetic acid), culture-tested trypsin solution, Histopaque 1119 and Histopaque 1077, phosphate buffered saline (PBS) with/without Ca^{2+}/Mg^{2+}, 10X PBS solution, dimethylsulfoxide hybrid-max (DMSO), albumin bovine fraction (BSA) V solution (7.5%), percoll, fibronectin from human plasma, Hanks' Balanced Salt solution (HBSS) with/without Ca^{2+}/Mg^{2+}, Dulbecco's modified eagle's medium (DMEM), HEPES solution 1M, sodium pyruvate solution 100mM, MEM non-essential amino acid solution (100X), trypan blue, JC-1, dimethylformamide, Tris, glycerol, sodium dodecyl sulfate (SDS), mercaptoethanol, glycine, Tween-20, Triton X-100, *N,N,N′,N′*-Tetramethylethylenediamine (TEMED), ammonium persulfate (APS), acetic acid, N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), diethylamine NONOate (DEANO).

Recombinant human TNF-α, and IL-1β, recombinant murine TNF-α and IL-1β were from R&D Systems, Abingdon, UK. Murine epidermal growth factor (mEGF) was from Peprotech, Inc. (London, UK). Collagenase type 1 was from Worthington Biochemical Corporation (Lakewood, US). Dynabeads® Goat anti-Mouse IgG and ACK lysing buffer were from Invitrogen (Paisley, UK). May Grunwald Giemsa (Diff Quik) was from Dade Behring (Milton Keynes, UK). Depex mounting fluid was from Raymond A Lamb, Laboratory Suppliers (Eastbourne, East Sussex, UK). $DiOC_6$ (3'3'-dihexyloxacarbocyanine iodide) was from Molecular Probes (Cambridge, UK). CD19 Microbeads and MACS columns were from Miltenyi Biotech. (Surrey, UK). Ficoll-Paque, CyDyes, 24cm polyacrylamide gel strips, paraffin oil (Dry strip cover fluid), acrylamide, bis-acrylamide and bindsilane were

obtained from GE Healthcare (Uppsala, Sweden). Complete EDTA-free protease inhibitor cocktail tablets were from Roche (Indianapolis, US). Flamingo fluorescent stain and 10X SDS running buffer were obtained from Biorad (Hercules, US). Human serum albumin (HAS) was obtained from Sanquin (Amsterdam, the Netherlands) and RPMI 1640 medium from Invitrogen (Carlsbad, US). Transwell filters were from BD Biosciences (Oxford, UK). ECL Western Blotting Substrate and Restore western blot stripping buffer were from Thermo Scientific Pierce (Loughborough, UK) and Hyperfilm ECL from GE Healthcare Life Sciences (Amersham, UK). Prestained protein marker, broad range (7-175 kDa) was from New England Biolabs Ltd. (Hitchin, Hertfordshire, UK).

Antibodies

Antibody	Conj.	Clone	Origin	Target	Isotype	Company	Conc.
anti-CD16/32	N/A	93	Rat	Mouse	IgG2a,λ	eBiosciences	10μg/ml
anti-CD31	N/A	YR-131-12	Rat	Human	IgG1	Gift from Prof. C. Buckley, UK	10μg/ml
anti-CD99	N/A	YG32	Rat	Human	not specified	DiNonA, Seoul, Korea	10μg/ml
anti-JAM-C	N/A	H33	Rat	Human	IgG1	Gift from Prof. B. Imhof, Geneva	10μg/ml
anti-ICAM-2	N/A	3C4(mIC2/4)	Rat	Mouse	IgG2a,κ	BD Pharmingen	10μg/ml
anti-VE-cadherin	N/A	11D4.1	Rat	Mouse	IgG2a,κ	BD Pharmingen	10μg/ml
anti-alpha enolase	N/A	Polyclonal	Rabbit	Human	IgG	Lifespan Biosciences	10μg/ml
CAP1	N/A	4A2	Mouse	Human	IgG1	Abnova	2 μg/ml
PP1b	N/A	EP1804Y	Rabbit	Human	IgG	Epitomics	1:2000
RNAse2	N/A	Polyclonal	Mouse	Human	IgG	Abnova	1:2000
B-actin	N/A	AC-15	Mouse	Human	IgG1	Sigma	1μg/ml
HRP anti-mouse IgG	HRP	Polyclonal	Goat	Mouse	IgG	DAKO	1:1000
HRP anti-rabbit IgG	HRP	Polyclonal	Goat	Rabbit	IgG	DAKO	1:2000
anti-ICAM-1	PE	YN1/1.7.4	Rat	Mouse	IgG2b,κ	eBiosciences	0.4μg/ml
anti-CXCR2	APC	242216	Rat	Mouse	IgG2a	R&D Systems	2.5μg/ml
anti-CD31	APC	MEC 13.3	Rat	Mouse	IgG2a,κ	BD Pharmingen	4μg/ml
anti-ICAM-2	FITC	3C4(mIC2/4)	Rat	Mouse	IgG2a,κ	BD Pharmingen	10μg/ml
Rat IgG control	PE	not specified	Rat	Mouse	IgG2b,κ	eBiosciences	0.4μg/ml
Rat IgG control	APC	not specified	Rat	Mouse	IgG2a	eBiosciences	2.5μg/ml
Secondary IgG control	FITC	Polyclonal	Goat	Rat	IgG	R&D Systems	2.5μg/ml
Rat IgG control	N/A	A23-1	Rat	Mouse	IgG2a,κ	BD Pharmingen	10μg/ml

Table 2.1 Antibodies used in this thesis
Antibodies were obtained from eBiosciences, San Diego, US; BD Pharmingen, Oxford, UK; Lifespan Biosciences, Seattle, US; Abnova, Taipei, Taiwan; Epitomics, California, US; Sigma, Poole, Dorset, UK; DAKO, Ely, Cambridgeshire, UK and R&D Systems, Abingdon, UK.

2.2 Cell culture media and solutions

MCEC-1 murine endothelial cell line was cultured in MCEC-1 complete medium: Dulbecco's modified eagle's medium (Sigma) supplemented with 10% heat inactivated fetal calf serum (FCS), 10ng/ml murine epidermal growth factor (mEGF), 2.5µg/ml amphotericin B, 100U/ml penicillin and 100µg/ml streptomycin , 2mM L-glutamin (Sigma,UK) and 10U/ml heparin sodium (CP Pharmaceuticals Ltd, Wrexham,UK).

Murine primary endothelial cells from lung (MLEC) and heart (MHEC) were cultured in mEC complete medium: 80ml base medium [(Dulbecco's modified eagle's medium supplemented with 20% heat inactivated fetal calf serum (FCS), 100U/100µg/ml penicillin/streptomycin, 2.5µg/ml amphotericin B, 2mM L-glutamin and 25mM HEPES)] containing 10U/ml heparin sodium, (CP Pharmaceuticals Ltd, Wrexham,UK), 100ng/ml murine epidermal growth factor (mEGF), 1X nonessential amino acid and 1mM sodium pyruvate.

Human umbilical endothelial cells (HUVEC) were cultured in medium: Medium 199 (M199- Gibco Invitrogen Compounds, Paisley, UK), 20% heat inactivated fetal calf serum (FCS), 10ng/ml epidermal growth factor (EGF), 35µg/ml gentamycin, 100U/100mg/ml penicillin/streptomycin, 1µg/ml hydrocortisone (all from Sigma) and 2.5µg/ml amphotericin B (Gibco Invitrogen Compounds, Paisley, UK).

PBSA: phosphate buffered saline (PBS) containing 1mM Ca^{2+}, 0.5mM Mg^{2+} and 0.15% culture-tested bovine serum albumin.

M199/BSA: Medium 199 containing 0.15% bovine serum albumin.

2D-DIGE and Western blot solutions

Tables 2.2 and 2.3 describe the solutions used for 2D-DIGE electrophoresis and Western Blot.

2D-DIGE solutions	
Sucrose buffer	0.34M sucrose, 1mM EDTA 10mM Tris pH 7.4
Neutrophil lysis buffer	10mM Tris pH 7.4, 10% glycerol, 1% NP40, 50mM NaF, 20mM tetra-Na-pyrophosphate, 1mM DTT, 2mM vanadate, 1mM PMSF 2mM DFP and 1x complete EDTA-free protease inhibitor cocktail tablet
Labelling buffer (10ml)	8M urea, 2M thiourea, 4% CHAPS, 100mM Tris-HCl pH 8.5
IEF buffer (10ml)	8M urea, 2M thiourea, 4% CHAPS, 150mM DTT, 1% Biolyte, 0.002% BFB
12% SDS gel (90ml per gel)	26.4ml H_2O, 22.5ml 1.5M Tris pH 8.8, 26.3 ml 40% acrylamide, 14ml 2% bis-acrylamide, 450µl 20% SDS, 297µl 30% APS, 54µl TEMED
Equilibration buffer (200ml)	6M urea, 2% SDS, 30% glycerol 87%, 75mM Tris-HCL pH 8.8, 0.002% BFB 1%

Table 2.2 Solutions for 2D-DIGE electrophoresis

Western Blotting solutions	
Sample buffer	10 % SDS, 50% glycerol, 10% β-mercaptoethanol, 300 mM TRIS-HCl pH6.8, 0.002% BFB
12% SDS gel 10% (Resolving gel)	5.7ml H_2O, 2.5ml 1.5M Tris pH 8.8, 1.7ml 30% acrylamide/bis-acrylamide, 100µl 10% SDS, 20µl 10% APS, 20µl TEMED
5% SDS gel 5% (Stacking gel)	3.4ml H_2O, 2.5ml 0.5M Tris pH 6.8, 4ml 30% acrylamide/ bis-acrylamide, 100µl 10% SDS, 20µl 10% APS, 20µl TEMED
10XRunning buffer per L	60g Tris 288g Glycine 10g SDS
Transfer buffer (per 2L)	28.8g glycine, 6g Tris, 400ml MeOH
Blocking buffer	5% BSA TBS/0.05% TWEEN-20
Wash buffer per L	TBS/0.05% TWEEN-20

Table 2.3 Solutions for Western Blotting

2.3 Isolation of neutrophils

2.3.1 Isolation of murine bone marrow neutrophils

Wild type C57BL6 mice (20-30g), killed by schedule 1 procedure, were obtained from the Biomedical Services Unit (University of Birmingham, UK). Usually neutrophils isolated from one mouse were used for each experiment.

All legs were immobilized using 21G needles and all the body was sprayed with 70% ethanol. The skin, muscles, and connective tissue were removed and femur, tibia and humerus were isolated from the limbs and kept on ice. The bone marrow was flushed with 10ml of EDTA/HEPES solution (0.01% and 30mM respectively) using a 10ml syringe and a 23G needle, and collected in a 15ml tube. All clumps were gently re-suspended using a 5ml syringe and a 21G needle and the cell suspension was left for 5 minutes, enough time for large pieces of tissue to sediment to the bottom of the tube. The supernatant was then collected and centrifuged at 400g for 5 minutes. The cell pellet was re-suspended in 1ml of flush media. In order to remove red blood cells, a hypertonic lysis step was utilized. Briefly, 4ml of H_2O was added to the cells, and mixed for 30 seconds. Then, 2ml of 4x PBS was added to restore the osmolarity and the cell suspension was centrifuged at 400g for 5 minutes. The pellet was re-suspended in 2ml of flush media and was layered on the top of a 3-step density gradient, made from dilutions of different concentrations of stock solution of Percoll (Sigma) (52, 64 and 72%) and centrifuged at 1500g for 30 minutes at 20^{o}C with no brake (Figure 2.1).

Mature neutrophils were isolated from the layer formed in the interface of the two gradients of highest concentration and washed twice in PBSA, by centrifugation at 400g for 10 minutes (time sufficient to obtain a cell pellet). Neutrophils were counted using a Coulter Multisizer II (Coulter, Leicester, UK) and re-suspended in PBSA at the desired concentration ($2x10^6$ cells/ml), unless otherwise stated.

In order to determine the percentage of mature neutrophils in this heterogenous cell population, 100µl of neutrophil suspension was centrifuged onto a glass slide at 10g for 3 minutes in a Shandon Cytospin II and stained with Diff Quick. They were then air-dried for at

least 5 minutes before mounting a coverslip with Depex mounting fluid. In this way it was possible to calculate the percentage of mature neutrophils, identifying them by their hallo nucleus shape, counting them under a Leitz microscope at x40 magnification.

In some experiments mouse CD19 Microbeads were used for the depletion of contaminating B cells from the bone marrow. The $CD19^+$ cells were magnetically labelled with CD19 Microbeads. Then the cell suspension was loaded onto a MACS column which was placed in the magnetic field of a MACS separator. The magnetically labelled $CD19^+$ cells were retained on the column, whereas the unlabeled cells (PMN) ran through and were collected as $CD19^+$ depleted cells.

2.3.2 Isolation of human neutrophils

Venous blood from healthy donors was collected and immediately transferred into tubes containing K_2EDTA (1.6mg/ml, Sarstedt). Blood was maintained at room temperature and used within 2 hours of venipuncture.

Neutrophils were isolated using a 2-step density gradient method. First, 2.5ml of Histopaque 1119 was placed in the bottom of a 10ml tube and 2.5ml of Histopaque 1077 was layered on the top. Then, 5ml of whole blood was gently layered on the top of the gradient and the tube was centrifuged at 800g for 30 minutes. The neutrophil layer, formed in the interface between the two density gradient media, was collected in a 10ml tube and washed twice by centrifugation in PBSA at 400g for 5 minutes. Neutrophils were then counted using a Counter Multisizer II (Coulter, Leicester, UK) and re-suspended in PBSA at the preferred concentration.

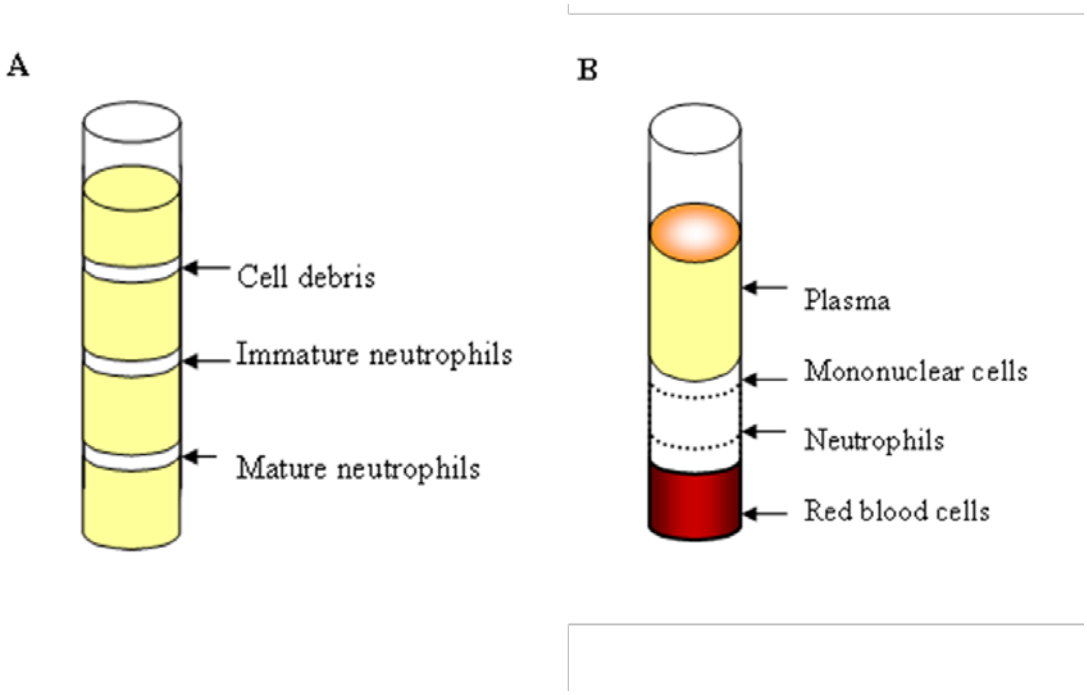


Figure 2.1 Isolation of neutrophils
(A) Mature murine neutrophils are isolated from bone marrow using a 3-step Percoll density gradient. The lower layers contains mainly mature neutrophils. (**B**) Human neutrophils are isolated from whole blood using a 2-step Histopaque density gradient. The neutrophil cell fraction is above red cells.

2.4. Cell culture

2.4.1 Culture of murine endothelial cell line (MCEC-1)

Cryopreserved vials (Nalgene), each containing a 1ml aliquot of MCEC-1 cell line, were removed from liquid nitrogen, and left for 2-3 minutes at room temperature before being transferred into 15ml tubes with 9ml of MCEC-1 medium. They were centrifuged at 400g for 5 minutes. The supernatant was aspirated and the cell pellet was re-suspended in 4ml MCEC-1 medium and transferred into 25cm^2 culture tissue flask previously treated with 1% gelatin.

Cultures in each flask became confluent after 1-2 days. Upon reaching confluence, MCEC-1 cultures were dissociated and passaged onto microslides at a concentration that yielded confluent monolayers after 24 hours. Briefly the monolayers were bathed with 3ml of EDTA (0.02%) for 60 sec. EDTA was removed by aspiration and tryspin (2.5mg/ml)/EDTA was added at a 2:1 ratio for 3-5 minutes. Using phase contrast microscopy, the time point when the cells started to detach from the plastic surface was determined and 6ml of MCEC-1 medium was added to neutralise the trypsin. The cell suspension was transferred into a 15ml tube and centrifuged at 400g for 5 minutes. The pellet was re-suspended in MCEC-1 medium and was transferred either at equal quantities in two 25cm^2 flasks or into one 75cm^2 flask. The cultures were maintained in MCEC-1 medium and, upon reaching confluence, were used in experiments, passaged between 2-4 times (for experiments) or cryopreserved in Dimethylsulfoxide (DMSO).

In order to freeze cells, a cryopreservant medium was used containing: 90% FCS and 10% DMSO. The freezing medium was maintained at all times in ice. Confluent cultures were tryspinized with trypsin/EDTA as previously described. The cell pellet was re-suspended with

the appropriate quantity of freezing medium (for a 75cm^2 flask, 3ml of freezing medium is used) and transferred in 1ml aliquots into ice-cold sterile cryopreservant vials. The cryovials were immediately placed at -80^{o}C for 24 hours and later stored in liquid nitrogen.

2.4.1.1 Culture of MCEC-1 in glass capillaries (microslides)

In order to conduct a flow based adhesion assay, MCEC-1 cells were cultured in glass capillary tubes with a rectangular cross section of 0.3x 3mm and a length of 5 cm (Camlab,Cambridge,UK), previously treated with 3-aminopropyl triethoxysilane (Sigma) (Figure 2.2).

One 25cm^2 flask of MCEC-1 cells was trypsinised as previously described. The pellet after dissociation was re-suspended in 400µl MCEC-1 medium, a quantity sufficient to load up to 6 microslides. Microslides, previously autoclaved and treated with 1% gelatine for 30 minutes, were seeded with endothelial cells incubated for 1 hour at 37^{o}C, 5%, CO_2. The microslides were then double seeded and an additional confluent 25 cm^2 flask was used for this purpose. After 1 hour incubation each slide was loaded with an additional 45µl cell suspension of approximately the same concentration. Then the seeded microslides were placed into a specially constructed glass dish filled with sterile MCEC-1 medium and were connected to the glass ports in the wall of the dish. The glass dish was transferred into an incubator. The other ends of these glass attachments were connected via silicon tubing to an 8 channel intermittent flow pump which allowed perfusion of medium for 60 seconds each hour at a wall shear stress of 0.05 Pa. The microslides formed confluent monolayers after 24 hours.

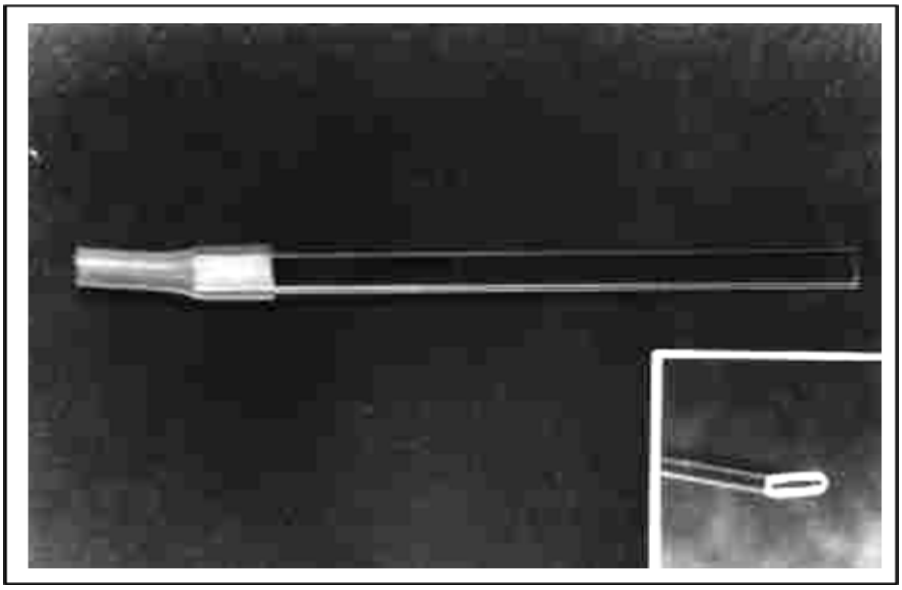


Figure 2.2 Glass microslides
Glass microslides were used as a vessel model. Microslide dimensions were 5 cm X 0.3mm X 3 mm.

2.4.2 Isolation of murine primary endothelial cells from lung (MLEC) and heart (MHEC)

Murine primary endothelial cells (mEC) were isolated from the hearts (MHEC) and lungs (MLEC) of wild type C57BL6 mice, as previously described, with minor modifications (Lim and Luscinskas, 2006). For each isolation, three 7-9 day old mice were used, and obtained from Biomedical Services Unit.

The mice were sacrificed by schedule 1 procedure and their carcasses were immersed in 70% ethanol. They were placed on a polystyrene foam board and their legs were immobilised with 21G needles. The skin in the chest area was removed and the anterior abdomen was cut open. Then the rib cage was removed in order to expose the thoracic cavity. Spraying with 70% ethanol limited bacterial contamination to the minimum.

The hearts and the lungs from all three mice were removed from the thoracic cavity and placed into separate petri dishes. Every lung lobe was cut in two parts and was minced using a scalpel, whereas the hearts, after the removal of any arteries or other large vessels, were minced with a pair of fine forceps. Each minced tissue was transferred into a 50ml tube containing 15ml of pre-warmed type I collagenase solution (200μg/ml). The tubes were subsequently placed in a rotator inside a hybridization oven set at 37^{o}C. The incubation time for heart and lung tissue was 30 and 45 minutes respectively.

After that step, each cellular suspension was forced gently 10 times using a 6 inches long 14G metal needle (Harvard apparatus, Kent, UK) attached to a 20ml syringe and filtered through a 70μm pore strainer (BD Falcon, Oxford, UK). To minimize cell loss, the strainers were further washed with 15ml of base medium, making the final volume of cell suspension up to 30ml. Following a centrifugation at 400g at 4^{o}C for 8 minutes, each cell pellet was re-suspended in the appropriate amount of base medium (1ml for heart and 2ml for lung cells) and transferred to a 5ml tube. Then, 15μl of anti-ICAM-2 coated magnetic beads was added for every millilitre of suspension and the tubes were placed on an end to end rotator for 10 minutes at room temperature. Subsequently, each tube was attached to a vertical magnetic plate for 2 minutes, in order to get unbound and cell bound magnetic beads stuck to the magnet. The supernatant was removed by aspiration and the magnetic beads were re-suspended into 3ml of base medium. This step was repeated twice, thus minimising the selection of contaminating cells. Finally, the anti-ICAM-2 bead coated cells were resuspended in 5ml of complete mEC medium and transferred to a precoated 25cm^{2} culture flask with

10μg/ml fibronectin and 0.1% gelatin. The number of isolated cells and growth rates varied from culture to culture.

2.4.2.1 Culture of MLECs and MHECs

The medium in the flasks was changed every 3 days. Usually the cultures reached confluence after 7-10 days, with the cells showing the classical cobblestone appearance. Upon confluence, the cells were trypsinized and an additional positive selection procedure was performed using anti-ICAM2 beads, as previously described, to ensure the culture's high purity of endothelial cells. After the second positive selection the cells were again transferred to a 25cm^2 flask previously treated with fibronectin and gelatine and cultured to reach confluence. Cells up to fourth passage were used for the following experiments.

2.4.2.2 Culture of primary murine endothelial cells in glass capillaries (microslides)

Third passage murine primary endothelial cells (mECs) were trypsinized and passaged into microslides, as previously described. After dissociation, the cell pellet from 2 confluent flasks was resuspended in 700μl in order to seed 12 microslides, previously autoclaved and treated with fibronectin (10μg/ml) for 30 minutes. Following 1 hour incubation at 37^{o}C, the microslides with MLEC or MHEC were incorporated to a flow culture system. Generally, MLEC monolayers reached confluence after 24 hours, while MHEC monolayers required 2-3 days.

2.4.3 Culture of Human Umbilical Vein Endothelial Cells (HUVEC)

2.4.3.1 Isolation of HUVEC

Primary endothelial cultures were isolated from the veins of human umbilical cords, as previously described (Cooke *et al*., 1993). The cords were obtained from Birmingham Women's Hospital from fully consented donors.

Each cord was cut from the placenta, placed in a steel instrument tray covered in blue roll and sprayed with 70% ethanol. Damaged parts of the cord, such as clamp marks and needle holes were removed. A glass cannula was inserted in one end of the vein and was tied off. The vein was washed with 10-20ml of PBS until clear of blood and finally air was perfused through to remove residual fluid. Another cannula was then placed in the other end of the vein and also tied off.

10ml of collagenase 1A (1mg/ml) was flushed into the vein until the cannuli reservoirs were full (this quantity is sufficient for 4-6 inches of cord). Then, both ends were clipped off and the cord was transferred, in a bag, for a 15-minute incubation at 37^{o}C. At the end of this period, the cord was taken out of the bag, massaged to enable dissociation of the endothelial cells from the wall of the vein and finally flushed with 30ml of PBS followed by air. The cell suspension was collected in a 50 ml tube and centrifuged at 400g for 5 minutes. The pellet was resuspended in 4ml of HUVEC medium and was transferred into a $25cm^2$ culture flask, previously treated with 1% gelatine for 15 minutes. The culture medium was changed after 2 and 24 hours and then every second day. The cultures became confluent after 4-7 days.

2.4.3.2 Culture of HUVEC in glass capillaries (microslides)

First passage HUVEC cultures were trypsinised and passaged into microslides, as previously described. The pellet after dissociation was resuspended in 400µl HUVEC medium, a quantity sufficient to load up to 6 microslides. Microslides, previously autoclaved and treated with 1% gelatine for 30 minutes were seeded once with endothelial cells. After 1 hour incubation at 37^{o}C, 5% CO2, the seeded microslides were incorporated in a flow culture system for 24 hours until becoming confluent.

2.5 Adhesion assays

2.5.1 Flow-based adhesion assays

2.5.1.1 Neutrophil adhesion assays on MCEC-1

Microslides containing confluent MCEC-1 monolayers were detached from the flow culture system and were stimulated with various cytokines (TNF-α: 100U/ml and/ or IL-1β: 0.5ng/ml) for 4 hours. The medium in each microslide was changed using a pipetor every hour.

After the 4-hour incubation, the microslides were glued onto microscope slides, mounted onto a microscope stage and observed by phase contrast videomicroscopy. The microscope was enclosed in a perpex heating box in order to maintain a temperature of 37^{o}C. One end of each microslide was attached via silicon rubber tubing to a Harvard syringe pump, allowing wall shear stress to be set by applying the appropriate flow rate. The other end was attached to an electronical valve (Lee products, Gerards Cross, UK), which could switch between the neutrophil suspension and the wash buffer (Figure 2.3). All flow adhesion assays

were performed at a wall shear stress of 0.05 Pa. Flow rate of the medium was calculated using the following equation assuming a parabolic flow distribution with a Newtonian fluid:

$$Q = \frac{wh^2 T}{6\eta} = \frac{0.27 \times 0.05}{0.042} = \frac{0.0135}{0.042} = \mathbf{3.21\ \mu l/s^{-1}}$$

T = Wall shear stress (Pa)

η = Viscosity of the medium (assumed to be 0.7×10^{-3} Pa.s for aqueous buffers)

Q = Flow rate of the medium ($\mu l/s^{-1}$)

w = Width of the flow channel (mm)

h = Height of the flow channel (mm)

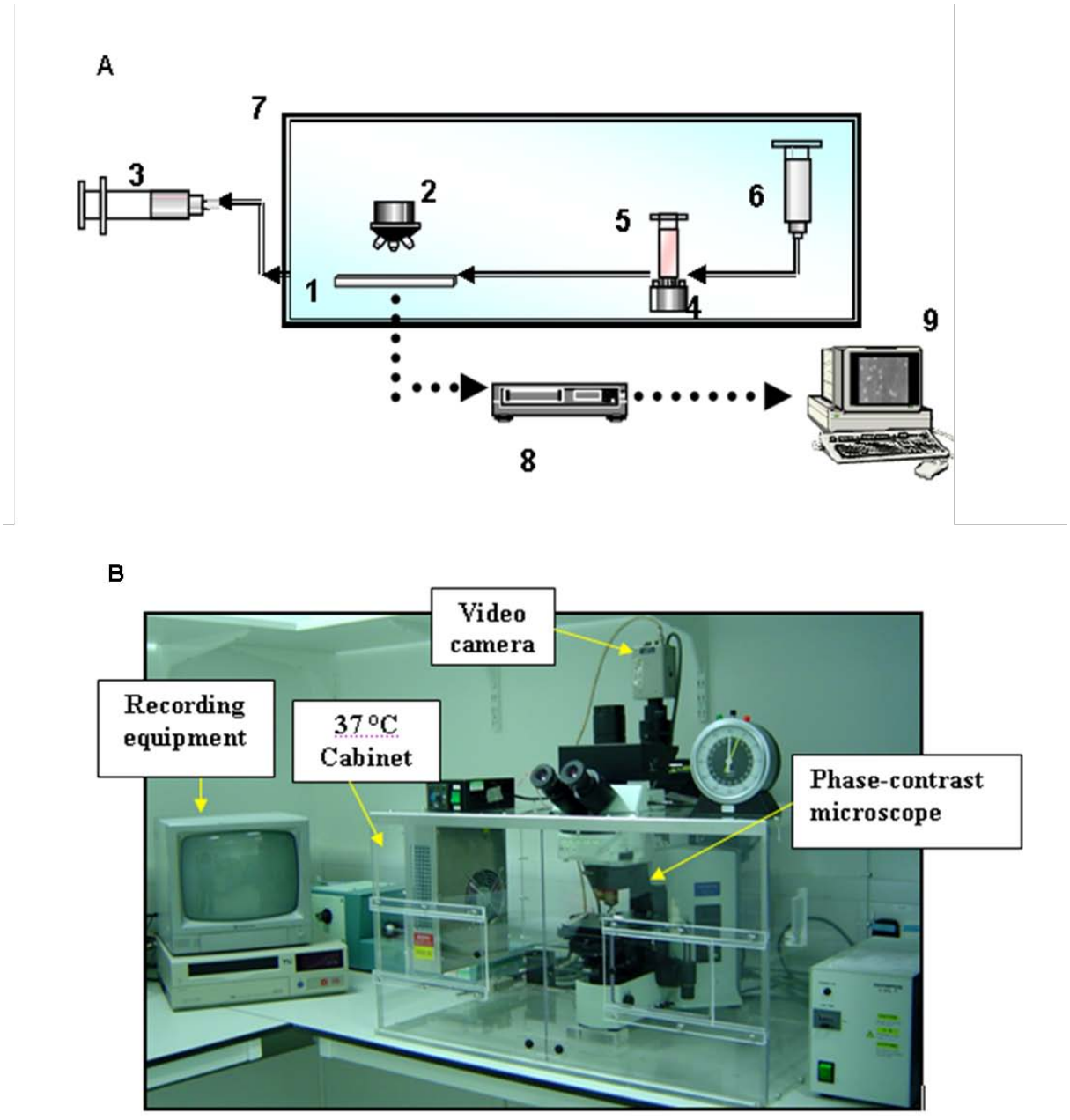


Figure 2.3 The flow based adhesion assay
(A) Microslides (1) containing EC were mounted on the stage of a video-microscope (2) and attached via silicone tubing to a 50ml glass syringe (3) and an electronic switching valve (4). Neutrophil suspension (5) or PBSA (6) were perfused through the microslides at a wall shear stress of 0.05 Pa. Experiments were conducted in a 37°C Perspex cabinet (7) and video recordings made (8,9). (**B**) The video-microscope and recording equipment in situ.

MCEC-1 were initially perfused with PBSA for washing and then with neutrophil suspension for 4 minutes, followed by a washout with PBSA. Video recordings were made after 1, 5 and 11 minutes of washout from a series of fields as selected at random along the centre line of each microslide.

In some experiments, the perfusion of wash buffer was prolonged up to 1 hour to allow neutrophil behaviour to be assessed over this longer period. For these experiments the shear stress was set at 0.05Pa, unless otherwise stated and the concentration of neutrophil suspension was $2x10^6$ cells/ml. MCEC-1 monolayers were initially perfused with PBSA and soon after with neutrophil suspension for 4 minutes, followed by a washout with PBSA. In some experiments flow was turned off after 20 minutes, simulating conditions from that time point onwards. Recordings of 8-10 random fields were made every 5 minutes in all microslides and the total number of adherent and transmigrated neutrophils was assessed as detailed below.

2.5.1.1.1 Video analysis of flow adhesion experiments

Video records were analysed off-line. Neutrophils adherent to the endothelial monolayer were categorized, depending on their behaviour, as rolling, arrested or transmigrated. The rolling adherent cells were phase-bright, spherical and rotated slowly over the surface. Arrested cells were considered the ones that were phase-bright, either stationary or transmigrating on top the endothelium, changing shape and spreading. On the other hand, transmigrated cells were phase-dark, migrating underneath the endothelium. These three categories of neutrophil behaviour were quantified and expressed as percentages of the total

number of adherent cells at every chosen time point after neutrophil perfusion. The recordings were analysed using the Image-Pro Plus software (Image-Pro Plus, USA).

2.5.1.2 Neutrophil adhesion assays on murine endothelial cells from lung (MLEC) and heart (MHEC)

Microslides with confluent MLEC or MHEC monolayers were removed from the flow culture system and were stimulated with TNF-α, and/or IL-1β at various concentrations for 4 hours at 37^{o}C and flow based adhesion assays up to 20 minute duration were performed, as previously described. The shear stress was set at 0.05Pa, and the concentration of murine neutrophil suspension was 2x10^{6} cells/ml.

2.5.2 Static adhesion assays

2.5.2.1 Co-culture of MCEC-1 and murine neutrophils in static cultures - Generating RPMNs from co-cultures of murine neutrophils and EC

MCEC-1 cultures derived from two 25 cm^{2} flasks were trypsinised and passaged into a previously gelatinised 6-well plate (from two 25 cm^{2} flask, 3 wells were coated). Monolayer was formed in each well after 24 hours. Upon reaching confluence, the monolayer in each well was treated with TNF- α (100U/ml) and IL-1β (0.5ng/ml) for 4 hours. After the 4-hour incubation, each well was washed with M199/BSA. Murine neutrophil preparations (~2x10^{6} cells/well) were then added. After 1 hour incubation, non-adherent neutrophils were washed out and MCEC-1 medium was added to each well. The plate was then mounted on a microscope stage and 10-20 random fields from each well were recorded. The 6-well plate was placed in the incubator at 37^{o}C for 1, 2, 4, and 24 hours and again random fields were recorded after this period of culture. All recordings were analysed off-line. The total number

of adherent cells and the number of transmigrated cells were determined and expressed as cells/mm^2.

2.5.2.2 Assaying the effects of constant flow on the rate of reverse migration

HUVEC were passaged into microslides, incorporated into a flow culture system for 24 hours to become confluent and then stimulated with cytokines (100U/ml TNF-α and 0.5ng/ml IL-1β) for 4 hours, as previously described. After the 4-hour incubation, all the microslides were rinsed with PBSA to remove the excess cytokines. At this time point 45μl of neutrophil suspension (7x10^6 cells/ml in HUVEC medium) was added in each microslide and incubated at 37^{o}C for 25 minutes, sufficient time for neutrophils to complete transmigration. This neutrophil concentration was chosen to yield a neutrophil/EC ratio of 6:1, in accordance with concentrations used in static cultures (Butler *et al*., 2005). The microslides were rinsed with PBSA to remove non-adherent neutrophils. As a control, one microslide was fixed with 2% formaldehyde straight after the washout. One microslide was placed in the incubator at 37^{o}C for 1 hour and another was attached to a glass dish in a continuous flow system (0.05 Pa) at 37^{o}C for 1 hour. The continuous flow system has been described by Sheikh *et al*. (2003). At the end of that time slides were washed again and fixed with 4% formaldehyde. The microslides were glued onto microscope slides, mounted onto a microscope stage and observed by phase contrast videomicroscopy. Ten random fields along the centre line of each microslide were recorded and analysed off-line. The total number of adherent cells and the number of transmigrated cells were determined and expressed as cells/mm^2. In some experiments the incubation time under flow conditions was extended to 2 or 4 hours. To identify the molecular mechanism(s) supporting reverse migration in other experiments, monoclonal blocking antibodies against adhesion molecules were introduced to the

microslides for 15 minutes after the first washout and prior to incorporation into a flow system.

2.6. Flow cytometry

2.6.1 Characterisation of MLEC and MHEC by flow cytometry

Murine primary endothelial cells (mECs) cultured on six-well plates were washed twice with Ca^{2+}/Mg^{2+} free HBSS and then were dissociated after a brief exposure to 0.5% trypsin in 0.02% EDTA. Following centrifugation at 400g for 5 minutes, the cells were re-suspended at $1x10^6$ cells/ml in ice-cold PBS2A (PBS/BSA 2%) and 100µl were transferred to each eppendorf tube. All antibodies were used at a 1 in 50 dilution, made up in ice-cold PBS2A, with the exception of the secondary antibody where the dilution was 1 in 10. After being washed twice in PBS2A the endothelial cells were incubated with 50µl of anti-ICAM-2, anti-CD31, anti-VE-Cadherin or rat anti-mouse IgG antibody (as negative control) for 30 minutes on ice. Following a double wash in cold PBS2A, all cells in the samples were incubated with 50µl of the secondary antibody for 30 minutes on ice. The samples were washed twice in cold PBS2A, re-suspended in 100µl of PBS2A and finally transferred in FACS tubes containing 300µl PBS2A. Samples were analysed using the Cyan flow cytometer (DAKO) and Summit version 4.3 software package (DAKO) and data were expressed as median fluorescence intensities (MFI).

2.6.2 Characterization of RPMNs-surface expression of adhesion molecules

In some experiments, after the 24-hour co-culture all the neutrophils on the apical surface were collected and centrifuged at 400g for 5 minutes. Then they were washed in ice

cold PBS2A and re-suspended at a final concentration of $1x10^6$ cells. Then 100µl were transferred into epperdorf tubes and centrifuged at 800g for 5 minutes. Each sample was incubated with 50µl of anti-CD16/32 (10µg/ml) for 20 minutes at 4^oC. After a wash step, the samples were incubated with anti-CXCR2 (1 in 10 dilution in PBS2A), anti-CD54 (1 in 500 dilution) or their IgG controls for 30 minutes on ice. The samples were washed twice, resuspended in 100µl of cold PBS2A and finally transferred into FACS tubes containing 300µl PBS2A. All samples were analysed using the Cyan flow cytometer (DAKO) and Summit version 4.3 software package (DAKO) and data were expressed as median fluorescence intensities (MFI).

2.7 Assessment of neutrophil viability and apoptosis

Neutrophil viability was estimated by Trypan blue exclusion, as this dye can only penetrate the membrane of dead cells and subsequently it stains them blue. 10µl of neutrophil suspension from each group were stained with an equal amount of Trypan blue dye for 5 minutes. Then, 10µl of the formed suspension were loaded on to a haemocytometer which was mounted on the microscope stage. The blue stained dead cells were counted and were expressed as a percentage of the total number of neutrophils.

One way to determine neutrophil apoptosis is by nuclear morphology observation. In order to determine the percentage of apoptotic neutrophils, 100µl of neutrophil suspension from each group was centrifuged onto glass slides at 10g for 3 minutes in a Shandon Cytospin II and stained with Diff Quick. They were then air-dried for at least 5 minutes before mounting a coverslip with Depex mounting fluid. Usually a condensed nuclei was an

indication of apoptosis. Those cells were counted and their numbers were expressed as a percentage of total number of neutrophils.

An alternative way to assess neutrophil apoptosis is by measuring the mitochondrial transmembrane potential. A reduction in the mitochondrial transmembrane potential is associated to apoptosis. The fluorochrome DiOC6 was used as its reduced uptake by mitochondria indicates apoptosis. In detail, 50μl of neutrophils ($1x10^6$ cells/ml) were incubated with 150μl of $DiOC_6$ (40nM) for 30 minutes at 37^oC. Ice-cold PBS was then added to stop the reaction. The cell suspension was centrifuged at 800g at 4^oC for 4 minutes, resuspended in 100μl of cold PBS and finally transferred to FACS tubes containing 200μl PBS2A. Each sample was analysed using the Cyan flow cytometer and Summit version 4.3 software package (DAKO). Data was presented as a percentage in reduction in Median Fluorescence Intensity (MFI) Data were presented as a percentage of neutrophils expressing low levels of $DiOC_6$.

For the assessement of neutrophil apoptosis, the mitochondrial dye JC-1 was also used. The JC-1 dye accumulates in the mitochondria of healthy cells as aggregates, which are fluorescent red in colour, but at the early stages of apoptosis, the mitochondrial potential is significantly reduced and the dye can not accumulate in the mitochondria. In this case JC-1 remains in the cytoplasm in a monomeric form which fluoresces green. These differences can be analysed with flow cytometry. Neutrophils from all groups were incubated with JC-1 in RPMI (0.01mg/ml) for 15 minutes at 37^oC. Each sample was transferred into a FACS tube, centrifuged at 400g for 5 minutes and washed once with ice cold PBS. Finally the samples were resuspended in 400μl of cold PBS and analysed using Cyan flow cytometry (DAKO), as

previously described. Data was presented as the percentage of cells whose red fluorescent aggregates decreased, compared to the fresh.

2.8 2-D DIGE electrophoresis

Sample preparation

Neutrophils from each group were collected (maximum $10x10^6$ cells) and washed twice with ice cold sucrose buffer. 200μl of lysis buffer was then added to each cell pellet. The lysis took place at 4^oC for 30 minutes with gentle agitation every 5 minutes. After a centrifugation at maximum speed at 4^oC for 20 minutes, the supernatant, which contained the protein fraction, was transferred to an eppendorf filled with 1.8ml 80% acetone and kept at -20^oC overnight. The following day after a wash with 80% acetone, protein pellets were gently dissolved in 50μl of labeling buffer and kept at -20^oC for at least 2 hours prior to any further use. The protein concentration was later determined by Bradford assay.

Labelling

All three vials of CyDye are transferred from -20^oC and allowed to warm in room temperature. Each vial contained 5nmol of Cy2, Cy3 or Cy5 dye. After CyDyes had been reconstituted in 5μl of anhydrous dimethylformamide (DMF) at a final concentration of 1mM, they were diluted 1:2 with DMF. Finally, 0.9μl of each dye was added to 30μg of protein lysate in 25μl of labelling buffer. Each sample was mixed, briefly centrifuged, and incubated for 30 minutes on ice in the dark. The reaction was finally terminated by the addition of 1 μl of lysine (10mM) and an equal amount of 2xIEF buffer was added, followed by a 10-minute

incubation on ice in the dark. Finally, each internal control and two samples were pooled at a final volume of 450μl in 1xIEF.

Isoelectric focusing (IEF)

Dry 24 cm polyacrylamide gel strips with an immobilized non-linear pH gradient 3-10 were used for first dimension separation. First, the protein samples were loaded into separate grooves of a reswelling tray and then the strips were carefully placed with the gel surface down to prevent any air being traped in the interface. At the end all strips were covered in paraffin oil (Drystrip cover fluid) and left to rehydrate for at least 10 hours. After that, they were transferred to a manifold in the IPGphor chamber (GE Healthcare, Uppsala, Sweden), with the gel side facing up and the acidic end towards the anode. Then damp electrode pads were applied at each end of the strips and the electrode assemblies were placed on them. Again, the strips were covered in paraffin oil before the IEF initiation of first dimension electrophoresis. The running conditions were 1) 100V for 2 hours, 2) gradient up to 1000V for 5 hours, 3) gradient up to 8000V for 3 hours and 4) 8000V for 2 hours. After IEF completion, the gel strips were transferred to a manifold for storage at -20^{o}C.

Gel formation and second dimension electrophoresis

Low fluorescent glass plates were assembled and placed in a vertical position inside the Ettan Dalt twelve separation unit (GE Healthcare, Uppsala, Sweden). Then 12% SDS solution was prepared and poured into the cassettes. Gels were left overnight, covered in wet tissue and cling film to keep them wet. The following day, strips were left at room temperature for 15 minutes. Firstly, 5ml of equilibration buffer containing 1% DTT was added to each strip for 15 minutes in gentle agitation, followed by a 15-minute incubation in

equilibration buffer with 2.5% iodoacetamide. Finally, strips were washed with 1x running buffer before being carefully placed to the gels.

The gel cassettes were placed inside the separation unit, previously filled with running buffer, were added and the lid was closed. The running conditions were 0.1W/gel for 1h, 0.2W/gel for 1h and 1W/gel overnight. The following day, after the completion of the electrophoresis, the gel cassettes were carefully rinsed with water and scanned with Typhoon 9400 Variable Mode Imager (GE Healthcare, Uppsala, Sweden). Each gel was scanned 3 times with different lasers. The acquired images were uploaded to DeCyder software (GE Healthcare, Uppsala, Sweden) for analysis.

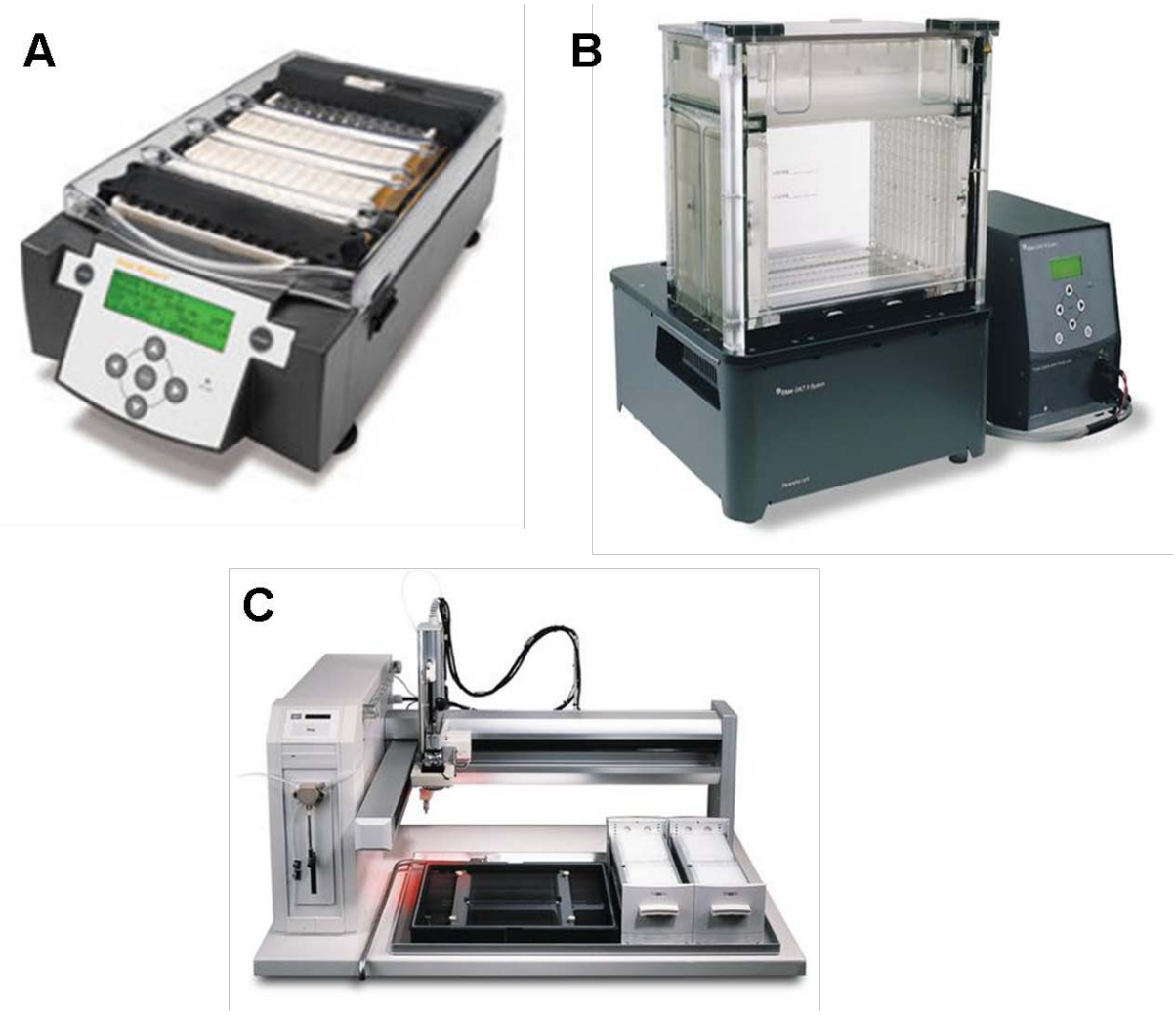


Figure 2.4 Equipment for 2D-DIGE experiments
The IPG phor chamber (A) was used to perform the isoelectric focusing, the Ettan Dalt twelve separation unit (B) for the second dimension electrophoresis and Ettan spot picker (C) for gel spot picking. All the equipment was from GE Healthcare, Uppsala, Sweden.

Spot picking

After the spots of interest were identified preparative gels were made. This time 200-250µg of total protein, without any previous staining, were dissolved in 450µl of 1xIEF buffer and loaded to gel strips exactly as before. The gel was immobilised on the surface of the small plate, using bindsilane. After the second dimension electrophoresis, gel cassettes were opened

and washed. The plate side with the gel was fixed with methanol/acetic acid for 1.5 hour with gentle agitation, followed by staining with Flamingo stain (1:20 dilution) for the same time. Finally, gels were scanned and the images were matched to the analysed DIGE images in the BVA analysis software. The gels were immersed in a tray filled with water and the spots of interest were picked using the automated Ettan spot picker (GE Healthcare, Uppsala, Sweden).The gel plugs were placed in 96-well plate in H_2O, until being analysed.

2.9 In-gel digestion and mass spectrometry analysis (I)

In-gel digestion and mass spectrometry analysis was outsourced to ServiceXS (Leiden, The Netherlands). The gel plugs were washed twice with water, twice with 50mM ammonium bicarbonate in 50% acetonitrile and dehydrated using 100% acetonitrile. The proteins were reduced with 10mM DTT and subsequently alkylated with 55mM iodoacetamide. The gel plugs were washed with 50mM ammonium bicarbonate, 50mM ammonium bicarbonate/50% acetonitrile and 100% acetonitrile and rehydrated with 10µl 50mM ammonium bicarbonate containing 50ng of trypsin. Gels plugs were incubated on ice for 30 minutes. When necessary, 50mM ammonium bicarbonate was added to completely cover them. Protein digestion by trypsin was allowed to proceed overnight at 37°C. Finally, the supernatant was acidified using TFA to a final concentration of 0.1%, desalted over a Zip-Tip (Millipore) and spotted directly on a MALDI target plate using 1ml α-cyano-4-hydroxycinnamic acid (HCCA, 0.3mg/ml in ethanol:acetone 2:1) as the Zip-Tip eluent. MS and MS/MS spectra were acquired on an Ultraflex™ II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany). The MS and MS/MS spectra were searched against the IPI_Human database using the MASCOT search algorithm (version 2.1) using mass

tolerances of 0.1 Da for MS and 0.5 Da for MS/MS. Carbamidomethylcysteine was taken as a fixed modification and oxidation of methionines as a variable modification. Furthermore, the search parameters allowed for 1 missed cleavage.

In- gel digestion and mass spectrometry analysis (II)

The gel plugs were washed twice with water, twice with 50mM ammonium bicarbonate in 50% acetonitrile for 45 minutes at 37^{o}C and finally dried. The proteins were reduced with 50mM DTT at 56^{o}C for 1 hour and subsequently alkylated with 100mM iodoacetamide at room temperature for 30 minutes. The gel plugs were washed with 40mM ammonium bicarbonate/10% acetonitrile for 15 minutes and then completely dehydrated. After that, these were incubated with 20µl of trypsin (12.5µg/ml) in 10% acetonitrile/40mM ammonium bicarbonate for 1hour at room temperature. 20µl of 10% Acetonitrile/40mM Ammonium Bicarbonate was added to each plug and these incubated at 37°C overnight. The following day, supernatant was collected and incubated with formic acid in water at 37°C for 1hour. Supernatant was collected and added to the previous one. 30µl of 3% formic acid in water was added again, followed by incubation at 37°C for 30 minutes. Supernatant was collected (final volume: 70-90µl) and stored at -20ºC or -80ºC.

The peptides were separated on a 75µm i.d. 15cm long C18 PepMap reverse phase column (3µm particle size) (Dionex) using a 3% - 35% solvent B gradient over 40 minutes (solvent A=2% acetonitrile/0.1% formic acid and solvent B= 99% acetonitrile/0.1% formic acid) at 350nl/minute using a Dionex U3000 nanobore HPLC system. The separated peptides were sprayed, using electrospray ionization, directly into a Bruker Amazon ion-trap mass spectrometer via a nanospray source equipped with a stainless steel Proxeon emmiter tip

(Bruker Daltonics, Bremen, Germany). A charge of 1.5kV was applied and the mass of the peptide was determined in the initial MS analysis. This data was used to select ions for MS/MS analysis where these were subjected to collision induced disassociation using He. The mass of the fragments determined and used to perform a database search. The most abundant 5 ions for each full scan were fragmented sequentially before another full scan was performed. This data dependent process selected ions for analysis twice, after which they were placed on an exclusion list for 1 minute to increase the number of low abundance fragmented ions. The spectra generated were analysed using the Mascot database search engine and the results processed by the ProteinScape software provided by Bruker (Bruker Daltonics, Bremen, Germany). Conditional carbamidomethylation of cysteine residues and oxidation of methionine residues were included and a minimum peptide score of 30 was employed.

2.10 Western blotting

Fresh and reverse migrated neutrophils were washed twice with ice cold PBS and lysed in sample buffer and boiled at 95^{o}C for 5 minutes. Protein samples were separated on 10-15% SDS-polyacrilamide gels, that were previously cast using mini gel apparatus (Mini-PROTEAN, Bio-Rad, UK). Gels were run for 1-2 hours at 150V. PVDF membranes were cut to gel dimensions, activated with 100% methanol and soaked in transfer buffer. After electrophoresis completion, the proteins on the gels were transferred to PVDF membrane, via wet transfer, according to manufacturer's protocol. After transfer, the membrane was washed in transfer buffer, and blocked for 2 hours at room temperature in blocking buffer. The membrane was then incubated with primary antibody diluted in blocking buffer overnight at 4°C, followed by 5 washes with wash buffer for 5 minutes. The blot was incubated in

secondary antibody (rabbit or mouse anti-human IgG-HRP, DAKO) for 1 hour in blocking buffer at room temperature. The membrane was washed 5 times for 5 minutes and then was incubated for 1 minute with ECL Western Blotting Substrate (Thermo Scientific Pierce, USA). After that, the membrane was exposed to Hyperfilm ECL (Amersham, UK). After the exposure, blots were stripped and re-probed with a primary antibody by using the Restore western blot stripping buffer (Thermo Scientific Pierce), followed by blocking for 1 hour with blocking buffer.

2.11 Statistical analysis

Data are presented graphically as the mean ± SEM for n independent experiments where $n \geq 3$. Variations between multiple treatments were evaluated using one-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison post-test when appropriate. Comparisons between specific treatment conditions were made by paired t test.

CHAPTER 3

NEUTROPHIL RECRUITMENT TO MURINE ENDOTHELIAL CELLS UNDER FLOW IN *IN VITRO* MODELS OF INFLAMMATION

3.1 Introduction

The process of leukocyte recruitment to inflamed endothelium has been extensively studied over previous years using well-established human *in vitro* models of inflammation (Cooke *et al*., 1993; Luu *et al*., 1999; Sheikh *et al*., 2005). However, the availability of human tissue is limited and/or expensive, especially since the introduction of the Human Tissue Act. These problems have been addressed to some extent by the use of animal models of inflammation, in which genetically homogenous strains of mice are commonly utilised. Moreover, the generation of transgenic and knockout mice provides a powerful tool for the investigation of mechanisms that underlie this process. Antibodies that block the function of adhesion molecules or chemokine receptors have been used extensively *in vitro* and *in vivo*. There is large literature on *in vivo* models of inflammation. These often use intravital microscopy techniques to enable the direct visualization of leukocyte recruitment at the site of inflammation (Baez, 1973). For example, post-capillary venules in the cremaster muscle can be stimulated with inflammatory cytokines such as TNF-α or IL-1β administered by intrascrotal injection. The muscle is subsequently exposed some hours post-stimulation with the circulation intact and leukocyte (typically neutrophil) adhesion is visualized under phase contrast or fluorescent microscopy (Dunne *et al*., 2002; Kunkel and Ley, 1996; Thompson *et al*., 2001).

Despite the valuable information that has arisen from the use of the *in vivo* models of inflammation there are some limitations. The *in vivo* system is too complex to ascribe effects to specific activating stimuli. For example, cytokines directly released in the circulation may change the phenotype of leukocytes over the period of stimulation, changing the nature of their interactions with vascular endothelial cells (Cotter *et al*., 2001; Itou *et al*., 2006). In

addition, stimulation of a tissue with a single cytokine such as TNF-α not only results in the activation of vascular endothelium by this agent, but also results in the activation of stromal cells, such as fibroblasts and macrophages, resulting in the release of a complex combination of inflammatory mediators that may act in addition or opposition to TNF-α. Another issue is the concordance between the regulation of inflammation in humans and mice. For example, *in vitro* studies have shown that L-selectin on murine neutrophils does not act as a ligand for E-selectin as it does on human neutrophils (Zollner *et al.*, 1997). In addition, not all human genes have homologues in the murine genome (Chinwalla *et al*., 2002; Emes *et al*., 2003). Thus, findings from *in vivo* animal models cannot necessarily be directly applied to leukocyte adhesion paradigms in human systems.

For all the above reasons, the establishment of murine *in vitro* models of inflammation is necessary. This allows the responses of endothelial cells from different species to be directly compared, because the same regimens of stimulation can be applied to them. Moreover, these are a more reliable tool to examine the role of individual molecules in the interactions between leukocytes and EC. However, there are only a very small number of studies using *in vitro* murine models of inflammation, and one major reason for this is the practical difficulties of isolating and culturing primary murine ECs (mECs) (Lidington *et al*., 2002). An additional problem lies in the small volume of blood and therefore low numbers of leukocytes that can be isolated from this source. Indeed, an isolation protocol which yields a very pure neutrophil suspension (>95%) from murine blood has been reported, but the total number of isolated cells is less than a $1x10^6$ per mouse at most (Cotter *et al.*, 2001), insufficient for the requirements of most *in vitro* adhesion assays, in particular those using flow methodology. It is possible to use neutrophils from bone marrow, where they are

abundant (Boxio *et al*., 2004; Chervenick *et al*., 1968). However, this raises issues about the maturity of the isolated cells. It has been demonstrated that morphologically mature, but functionally immature neutrophils are present in the human bone marrow (Berkow and Dodson, 1986). The obvious question is whether this also applies to murine neutrophils. Boxio *et al*. (2004) have shown that a large number of functionally competent neutrophils reside in the murine bone marrow. Performing functional studies they compared murine neutrophils from blood and bone marrow and found minor differences in degranulation and superoxide production between the two populations when stimulated with formylmethionyl-leucyl-phenylalanine (fMLF) (Boxio *et al.*, 2004). Moreover, mature bone marrow neutrophils are in a resting state straight after isolation and have the ability to be primed when treated with TNF-α (Itou *et al*., 2006).

Murine ECs have been isolated from various organs including heart (Lidington *et al*., 2002; Marelli-Berg *et al*., 2000), lung (Dong *et al*., 1997; Lim *et al*., 2003; Marelli-Berg *et al*., 2000), fat (Kajimoto *et al.*, 2010), lymph nodes (Toyama-Sorimachi, 1993) and brain (Song and Pachter, 2003). However, successful mEC isolation and propagation has proved a difficult task for a long time. The cost, time and effort in isolation procedure, the limited number of passages that mEC are capable of sustaining and poor levels of purity are some of the obstacles to overcome in order to use mECs in *in vitro* models of inflammation (Lidington *et al*., 2002; Lim and Luscinskas, 2006). An alternative strategy is the use of conditionally immortalised EC lines from murine tissues. For example, immortalised cardiac EC (MCEC-1) have been used by others, as well as ourselves. MCEC-1 cells are positive for typical EC markers (CD31, ICAM-2, E-selectin and endoglin) and sensitive to TNF-α and IL-1β treatment (Lidington *et al*., 2002).

Our purpose in this chapter was to establish murine *in vitro* models of inflammation using the MCEC-1 cell line or primary mECs and bone marrow neutrophils. The first step was to examine the sensitivity of murine EC monolayers to cytokine treatment in the presence of flow using flow-based adhesion assays. We have compared MCEC-1 with primary mECs from heart and lung, isolated following the protocol by Lim *et al*. (2006).

3.2 Methods

3.2.1 Flow-based adhesion assays with MCEC-1 cell line

In order to examine the recruitment of murine neutrophils to cytokine stimulated EC monolayers in murine *in vitro* models of inflammation under flow, experiments were performed using the flow-based adhesion assay methodology, as previously described in sections 2.4.1.1 and 2.5.1.1.

For the flow-based adhesion assays, microslides previously coated with gelatin were double seeded with MCEC-1 cells and cultured for 24 hours, at 37^{o}C, 5% CO_2 until they reached confluence. The monolayers were stimulated with TNF-α (100U/ml), IL-1β (0.5ng/ml) or a combination of these agents for 4 hours. Medium was changed every hour. Murine neutrophils were isolated from bone marrow using a 3-step Percoll density gradient. The lower cell fraction (70-80% mature neutrophils) was collected and resuspended at a final concentration of $2x10^6$ cells/ml. After 4-hour cytokine stimulation, the microslides were mounted on a phase-contrast microscope stage inside a heated box at 37^{o}C and connected to a pump and an electronic valve via silicon rubber tubing. The wall shear stress was set at 0.05 Pa by selecting a flow rate of 3.21 $\mu l/s^{-1}$.

Initially, a 4-minute neutrophil bolus was perfused over the MCEC-1 monolayers, followed by a washout with PBSA. Video recordings of 10 fields were taken at 5, 9 and 15 minutes from the beginning of the experiment. The adherent neutrophils were counted and classified as rolling (phase bright spherical cells, rotating over the surface), arrested (phase bright, firmly adherent cells) and transmigrated (phase dark, migrating cells underneath the monolayer). The total number of adherent neutrophils was expressed as the number of adherent neutrophils/$mm^2/10^6$ cells perfused and transmigration was expressed as the percentage of the adherent cells that transmigrated. Figure 3.1 shows the time line of the experimental procedure.

In some experiments, B-cell depletion of the neutrophil suspension was performed. The neutrophil suspension was incubated with anti-CD19 magnetic beads. $CD19^+$ cells, such as B-cells, were labelled with the beads and when the cell suspension was loaded onto a column placed in the magnetic field of a MACS separator; these were retained on the column, while the unlabeled cells ran through. As a result, this neutrophil fraction was depleted of B-cells.

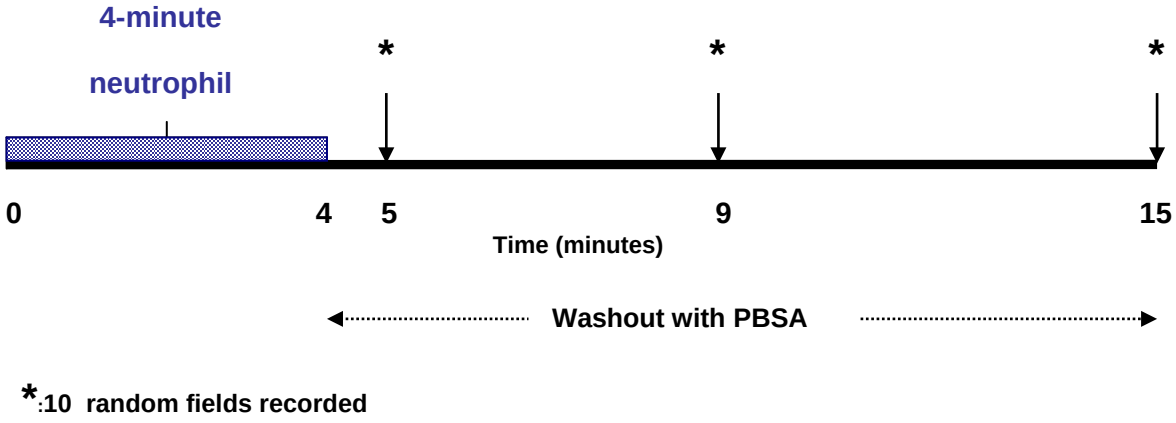


Figure 3.1 The time line of the flow based adhesion assay
After 4 minutes of neutrophil perfusion, PBSA washout was established and video recordings of 10 fields were taken at 5, 9 and 15 minutes after the beginning of neutrophil perfusion.

3.2.2 Characterization of MLEC and MHEC by flow cytometry

Following the protocol of Lim and Luscinskas (2006), we obtained EC that had a 'classical' EC cobblestone appearance. To verify that these cells constituted a pure population of endothelial cells, they were stained with antibodies against endothelial markers such as CD31, ICAM-2 and VE-cadherin. Table 3.1 shows the antibodies and dilutions used for mEC characterization.

Table 3.1 Antibodies and dilutions used for mEC characterization

ANTIBODY	ISOTYPE	DILUTION
APC anti-mouse CD31	Rat IgG	1:50
FITC anti-mouse ICAM-2	Rat IgG	1:50
Anti-mouse VE-cadherin	Rat IgG	1:50
FITC anti-rat IgG (as secondary against VE-cadherin)	Mouse IgG	1:10

3.2.3 Flow-based adhesion assays with mECs

After successfully establishing primary mEC cultures from heart and lung, we examined the ability of mECs to respond to cytokine treatment and recruit neutrophils from flow using titrations of activating stimuli. MLEC or MHEC were seeded on microslides precoated with 0.1% gelatin and 10μg/ml fibronectin and cultured for 24 or 48 hours, respectively. Confluent monolayers were stimulated with mTNF-α (at 2, 10, 100 and 500 U/ml), mIL-1β (at 0.01, 0.05, 0.5 and 2.5ng/ml) or a combination of agents (100U/ml and 0.5ng/ml, respectively). The flow-based adhesion assays were conducted, as previously

described, with the exception that an extra time point at 20 minutes from the beginning of neutrophil perfusion was introduced for collection of data by video microscopy. Video recordings were analysed off-line and the results were expressed as previously described.

3.3. Results

3.3.1 The effect of different cytokine treatments on neutrophil adhesion to MCEC-1 monolayers

Flow based adhesion assays were conducted on MCEC-1 cells after stimulation with cytokines. Neutrophil behaviour on cytokine stimulated EC could be categorised as rolling, arrested or transmigrated (Figure 3.2). Total adhesion levels and the percentage of transmigrated neutrophils, under different MCEC-1 cytokine treatment at the final time point of the experiment (15 minutes) are presented in Figure 3.3A. A surprising finding was the considerable levels of adhesion in the absence of inflammatory stimulus (198±42 cells/mm^2/10^6 perfused). Treatment with TNF-α, IL-1β or the combination of agents did not significantly increase levels of adhesion.

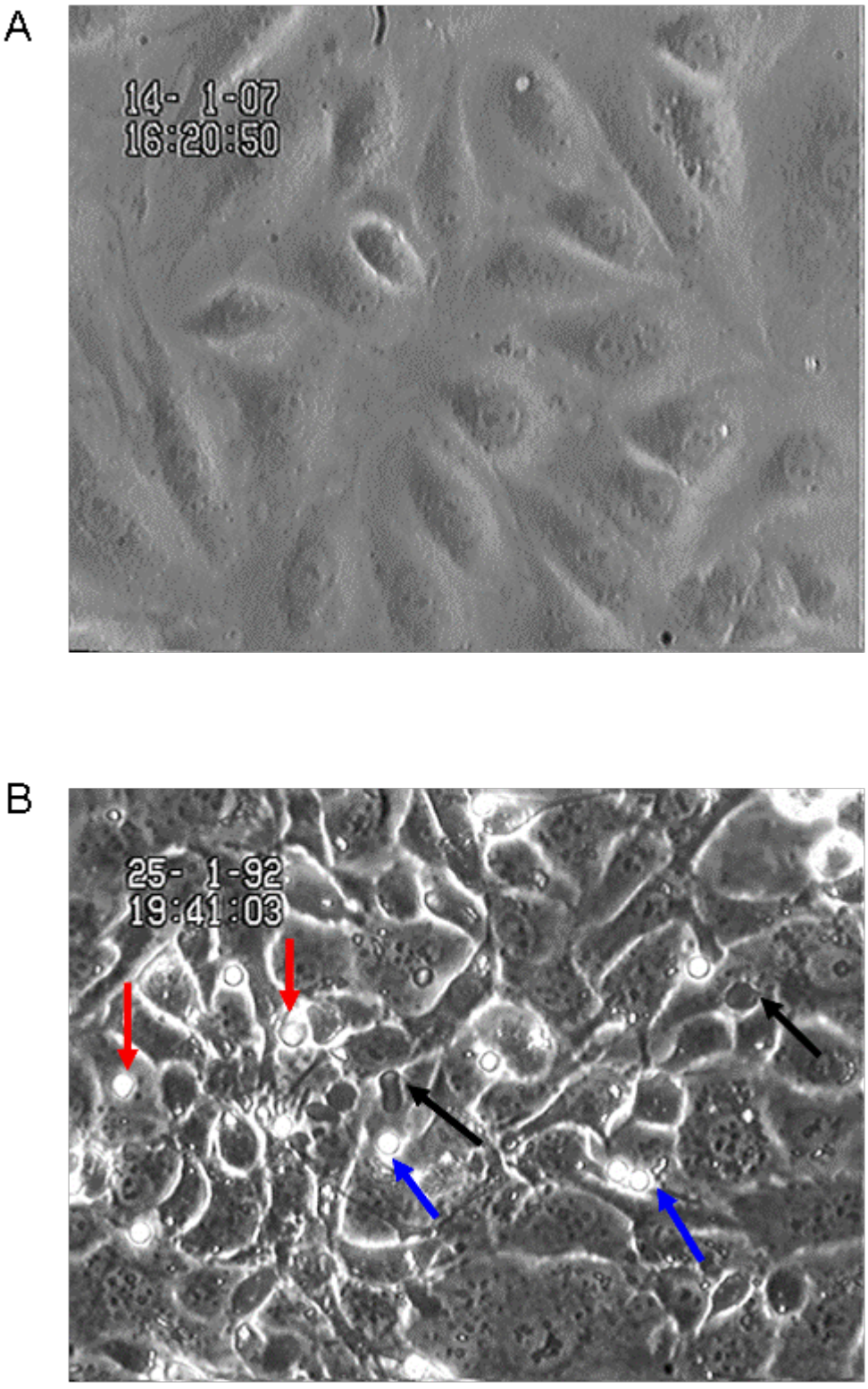


Figure 3.2 Images of MCEC-1 cultures (A) and cytokine stimulated (B) MCEC-1 monolayers after neutrophil perfusion
In (B), adherent neutrophils were categorized as rolling (blue arrows), arrested (red arrows) and transmigrated (black arrows).

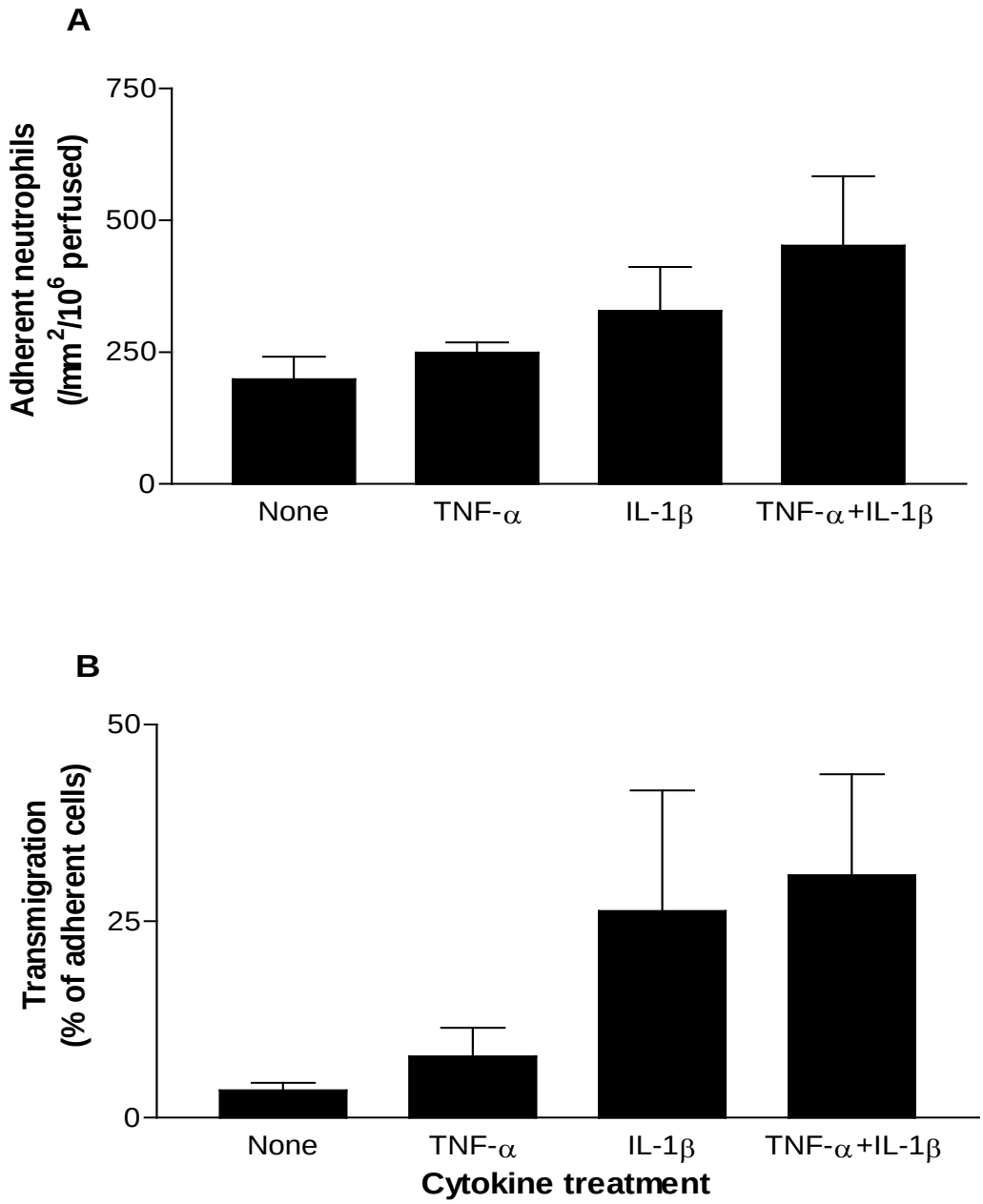


Figure 3.3 Effect of cytokine treatment on murine neutrophil adhesion (A) and transmigration (B) on MCEC-1 monolayers

MCEC- 1 monolayers in microslides were stimulated with TNF-α (100U/ml), IL-1β (0.5 ng/ml) or their combination for 4 hours and were incorporated in flow based-adhesion assays. After 4-minute neutrophil perfusion, non-adherent neutrophils were washed out and adherent (A) and transmigrated (B) neutrophils were counted at 15 minutes. Highest levels of adhesion and transmigration were obtained when the combination was used. In both cases, the differences were not statistically significant, as shown by one-way ANOVA. All data are the mean±SEM of three experiments.

Transmigration, expressed as a percentage of total adherent cells, followed the same trend. In the absence of inflammatory stimulus, 3.5±0.9% of the total number of adherent neutrophils had transmigrated at the final time point of the experiment. This percentage was 7.8±3.7%, with TNF-α treatment, and this was tripled with IL-1β. Transmigration was even higher when MCEC-1 monolayers were stimulated with TNF-α and IL-1β (31 ±13%, Figure 3.3B). However, ANOVA showed no significant differences between the responses to different cytokine stimulation regimens.

Figure 3.4 shows the effect of cytokine treatment of MCEC-1 monolayers on neutrophil behaviour. Adherent neutrophils were classified as rolling, arrested or transmigrated, based on their behaviour. The percentage of rolling neutrophils decreased over time in all treatments (Figure 3.4A). The majority of neutrophils at any time point were arrested. In the absence of treatment or stimulation with TNF-α, the percentage of arrested neutrophils remained stable over time. In other treatments, the number of arrested cells was reduced with time as the cells transmigrated (Figure 3.4B). The percentage of transmigration in unstimulated conditions and with TNF-α was low and stable, while treatment with IL-1β and the combination markedly increased transmigration over time, reaching a maximum at ~30% (Figure 3.4C). The different behaviour patterns show that an increasing number of rolling cells firmly arrested and the ones already stationary transmigrated over time.

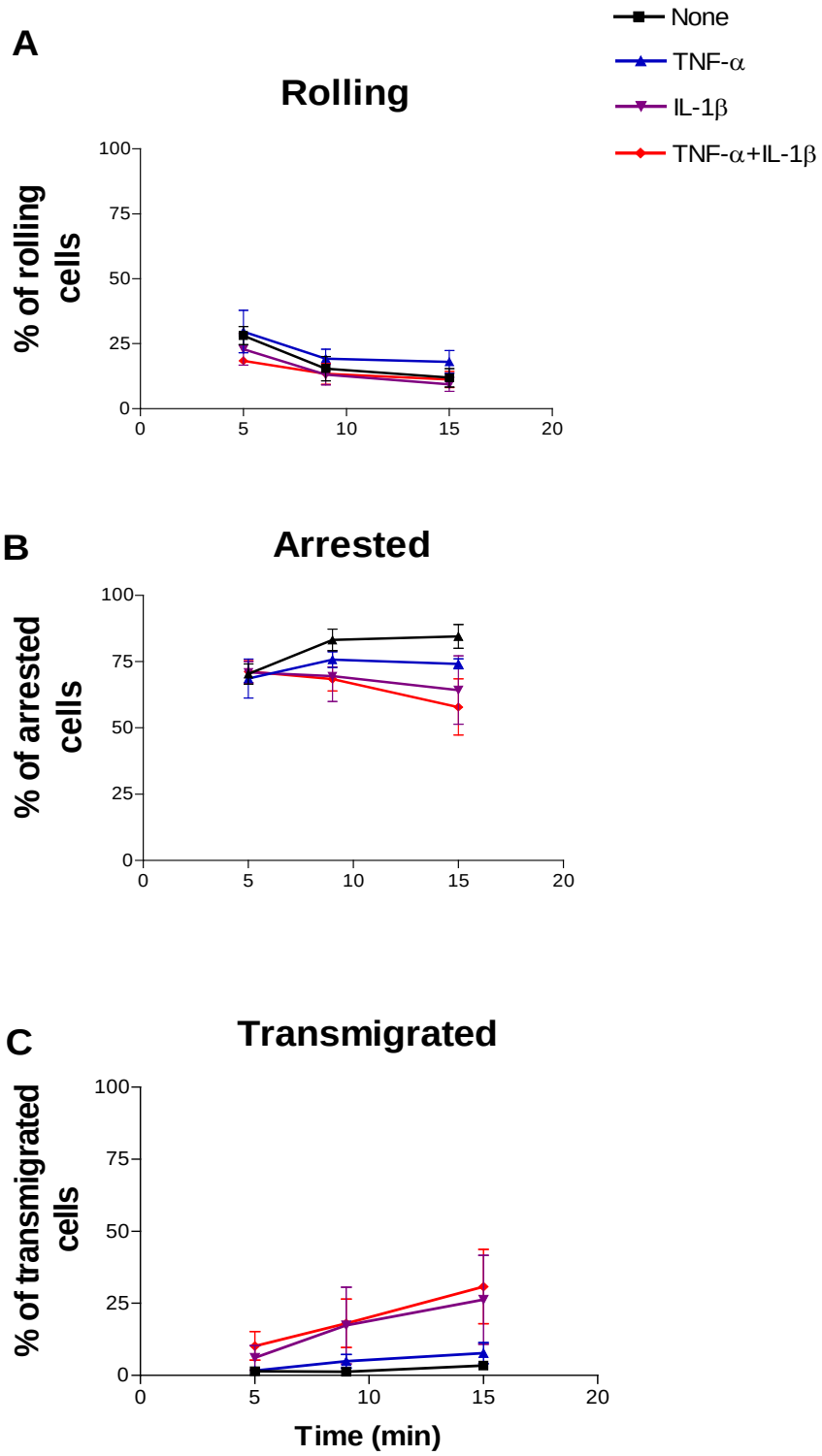


Figure 3.4 Effect of cytokine treatment of MCEC-1 monolayers on neutrophil behaviour Adherent neutrophils were categorized as rolling (A), arrested (B), and transmigrated (C). All data are the mean±SEM of three experiments.

3.3.2 The effect of B-cell depletion on total adhesion

Following the bone marrow isolation protocol for neutrophils we isolated the lower cell fraction from the 3-step Percoll gradient density. This fraction constitutes 70-80% mature neutrophils, 15-25% immature neutrophils and around 5% B-cells. We examined whether B-cell contamination is able to affect the numbers of adherent and transmigrated neutrophils. For this purpose, B-cell depletion was performed and the B-cell depleted neutrophils were used in flow-based adhesion assays.

As shown in Figure 3.5A adhesion levels were slightly higher when B-cell depleted neutrophils were used compared with those with the non-depleted. However, the difference between the two cell populations was not significant. Percentages of transmigration were almost the same over time reaching a maximum of 40% at 15 minutes (Figure 3.5B). These results from 4 experiments suggest that B-cell contamination does not affect the overall adhesion and transmigration.

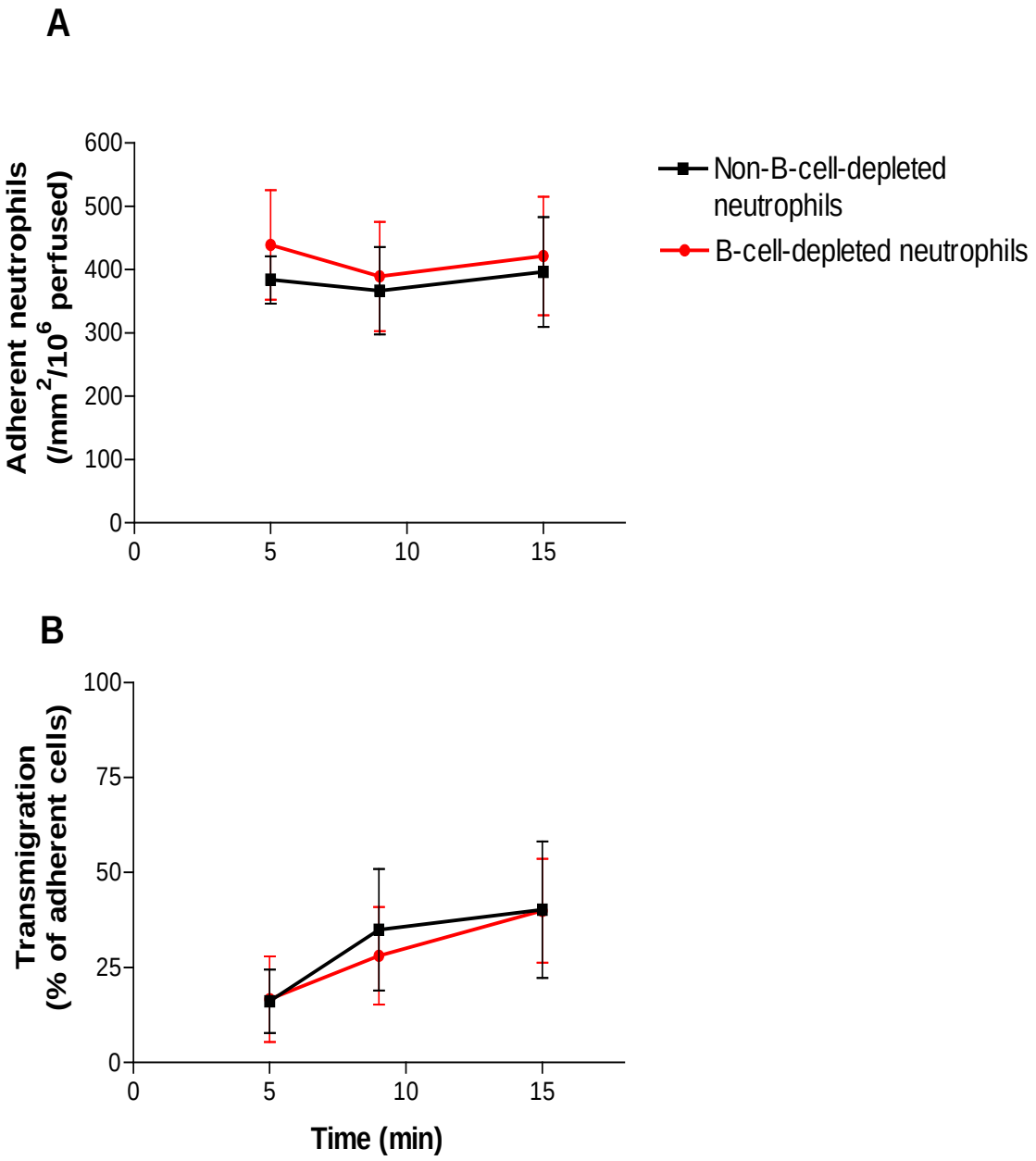


Figure 3.5 Effect of B-cell depletion in adhesion and transmigration
B-cell depleted and non-B-cell depleted neutrophils were perfused over TNF-α and IL-1β stimulated monolayers. B-cell depletion did not have an effect on adhesion (A) and transmigration (B). All data are the mean±SEM of four experiments.

3.3.3 Surface expression of ICAM-2, CD31 and VE-cadherin on MLECs and MHECs

Murine primary ECs from heart and lung showed the classical cobblestone appearance (Figure 3.6A and B). In order to characterize the cells, surface expression of ICAM-2, CD31 and VE-cadherin was measured (Figure 3.6C-H). Table 3.2 presents data from two experiments with MHECs and five from MLECs.

Table 3.2 Surface expression of ICAM-2, CD31 and VE-cadherin on MLECs and MHECs expressed as Median Fluorescence Intensity (MFI) and % of positive cells

	MLEC (n=2)		**MHEC (n=5)**	
	MFI	**% Histogram**	**MFI**	**% Histogram**
ICAM-2	17.5 ±7.4	77.1±9.5	34±9.6	89.8 ±5.8
CD31	84±23.2	94.5±2	16.5±11.2	32±24
VE-cadherin	38±27.2	87.6±11.6	12.5±1.6	34.5±7

Approximately 77, 94.5 and 87.6% of the MLEC population expressed ICAM-2, CD31 and VE-cadherin, respectively. However, mEC marker surface expression was different in MHEC from 2 experiments. Here, 89% of the cells expressed ICAM-2, but only 31.9% and 34% of cell population were positive for CD31 and VE-cadherin, respectively (Figure 3.6C, D and E).

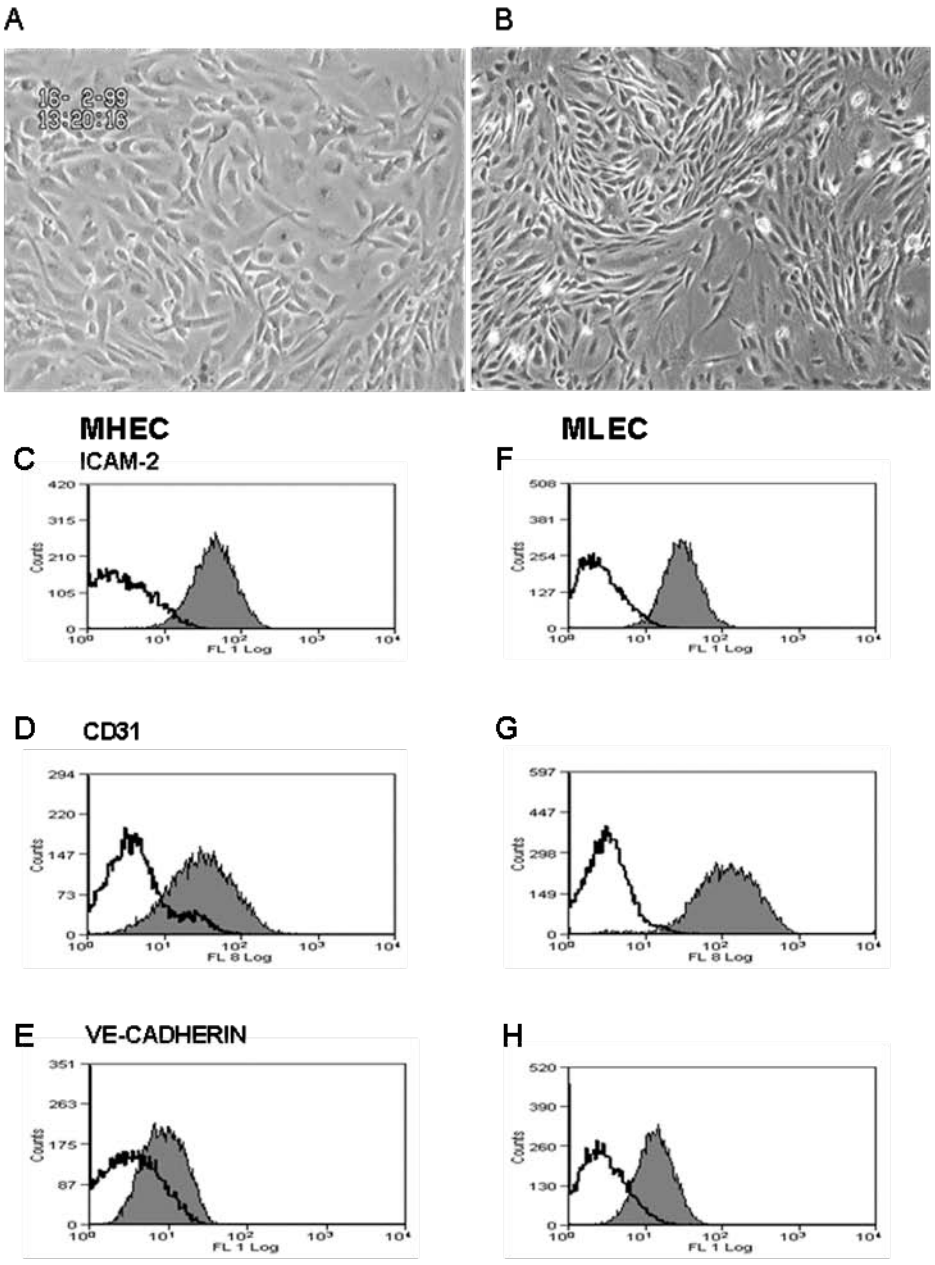


Figure 3.6 Morphological appearance and surface expression of ICAM-2, CD31 and VE-cadherin on MHECs and MLECs

MHECs (A) and MLECs (B) show the classical EC cobblestone appearance. MHECs and MLECs were positive for ICAM-2 (C and F), CD31 (D and G), and VE-cadherin (E and H), respectively. Data are representative from two (MHEC) and five experiments (MLEC).

3.3.4 The effect of different cytokine treatment of MHEC monolayers on neutrophil recruitment and behaviour under flow.

In order to investigate the effect of different concentrations of various cytokines on the ability of MHEC monolayers to recruit neutrophils, 20-minute flow-based adhesion assays were conducted. MHEC monolayers in microslides were stimulated with different concentrations of IL-1β (0.01, 0.05, 0.5 and 2.5ng/ml), TNF-α (2, 5, 10 and 100U/ml) or a combination of agents (0.5ng/ml and 100U/ml respectively), for 4 hours. Adhesion and transmigration was analysed and neutrophils were categorised as rolling, arrested and transmigrated, based on their behaviour.

MHEC responded to IL-1β treatment in a dose dependent manner. The two lower doses resulted in similar adhesion levels, but as IL-1β concentration increased, so did the neutrophil adhesion. There was a significant dose dependent effect on adhesion ($p<0.001$, by ANOVA). Adhesion levels in each treatment remained relatively stable after 10 minutes (Figure 3.7A). The dose dependent effect of IL-1β treatment on neutrophil adhesion on MHEC monolayers can be seen more clearly at the final time point of the experiment ($p<0.01$, by ANOVA). The highest level of adhesion (661±72 adherent cells/mm^2/10^6 perfused) was reached in the highest IL-1β concentration (2.5ng/ml) (Figure 3.7B). IL-1β treatment also had an effect on transmigration in a concentration dependent manner ($p<0.001$, by ANOVA). However, treatment with a dose higher than 0.5ng/ml did not result in an increase in transmigration efficiency, which reached a peak at ≈50% (Figure 3.7C).

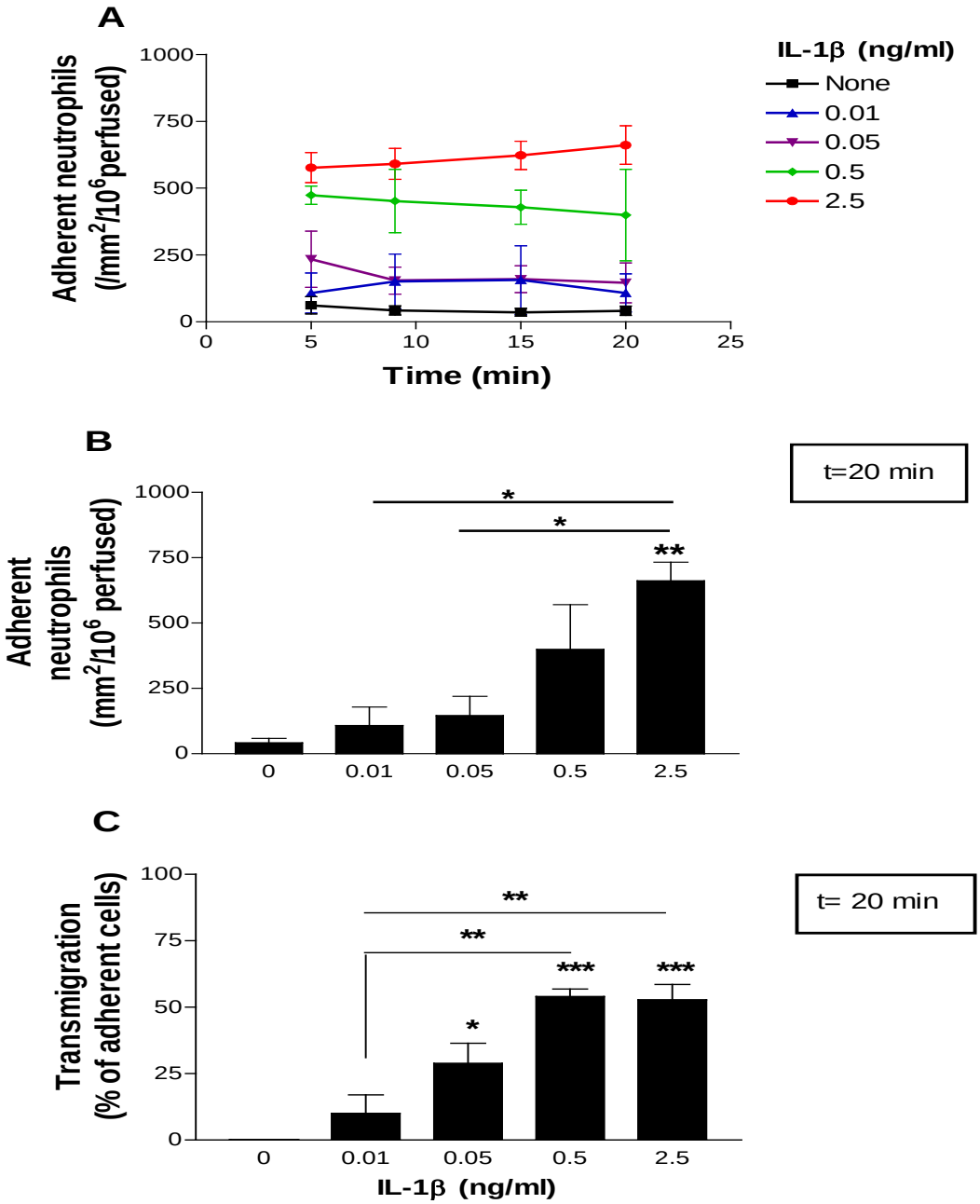


Figure 3.7 Effect of MHEC treatment with different concentrations of IL-1β on neutrophil adhesion (A and B) and transmigration (C)

MHEC monolayers in microslides were stimulated with different concentrations of IL-1β (0.01, 0.05, 0.5 and 2.5ng/ml) for 4 hours. (A) ANOVA showed a significant effect (P<0.001) of IL-1β dose treatment on adhesion at all time points, P<0.001. (B) Dose of IL-1β had a significant effect on adhesion levels at 20 minutes, P<0.01, by ANOVA, *, $P<0.05$, **, P<0.01 by Bonferroni's Multiple Comparison Test. (C) ANOVA showed a significant effect (P<0.001) of IL-1β dose on % of transmigration at 20 minutes. *, P<0.05, **, P<0.01 and ***, P<0.001, by Bonferroni's Multiple Comparison Test.

The behaviour of adherent neutrophils in all IL-1β concentrations over time is illustrated in Figure 3.8. The percentage of rolling cells decreased over time at all concentrations (Figure 3.8A). At 5 minutes, around 50% of adherent neutrophils were firmly arrested and this percentage decreased over time in all concentrations apart from the lowest (Figure 3.8B). The opposite pattern was observed in the percentages of transmigrated neutrophils as these behaviours are reciprocal, i.e. arrested cells go on to migrate over time.

Findings were different when TNF-α was used to activate MHEC. Again, MHEC responded to TNF-α treatment in a dose dependent manner ($p<0.001$, by ANOVA). This time though, adhesion levels slightly decreased over time. This finding suggests that adherent cells detached from EC monolayer (Figure 3.9A). Overall adhesion at 20 minutes increased with higher TNF-α concentrations, reaching a maximum at 500U/ml (Figure 3.9B). But by far the most striking result was the very low transmigration observed even at high concentrations of TNF-α. MHEC treatment with TNF-α concentration higher than 10U/ml could induce transmigration that only reached ≈5% (Figure 3.9C). The majority of adherent cells (50-75%) were rolling at 5 minutes and this percentage remained stable over time with minor changes between the different concentrations (Figure, 3.10A). The same pattern was true for the percentage of firmly arrested cells (25-50% of adherent neutrophils) (Figure, 3.10B).

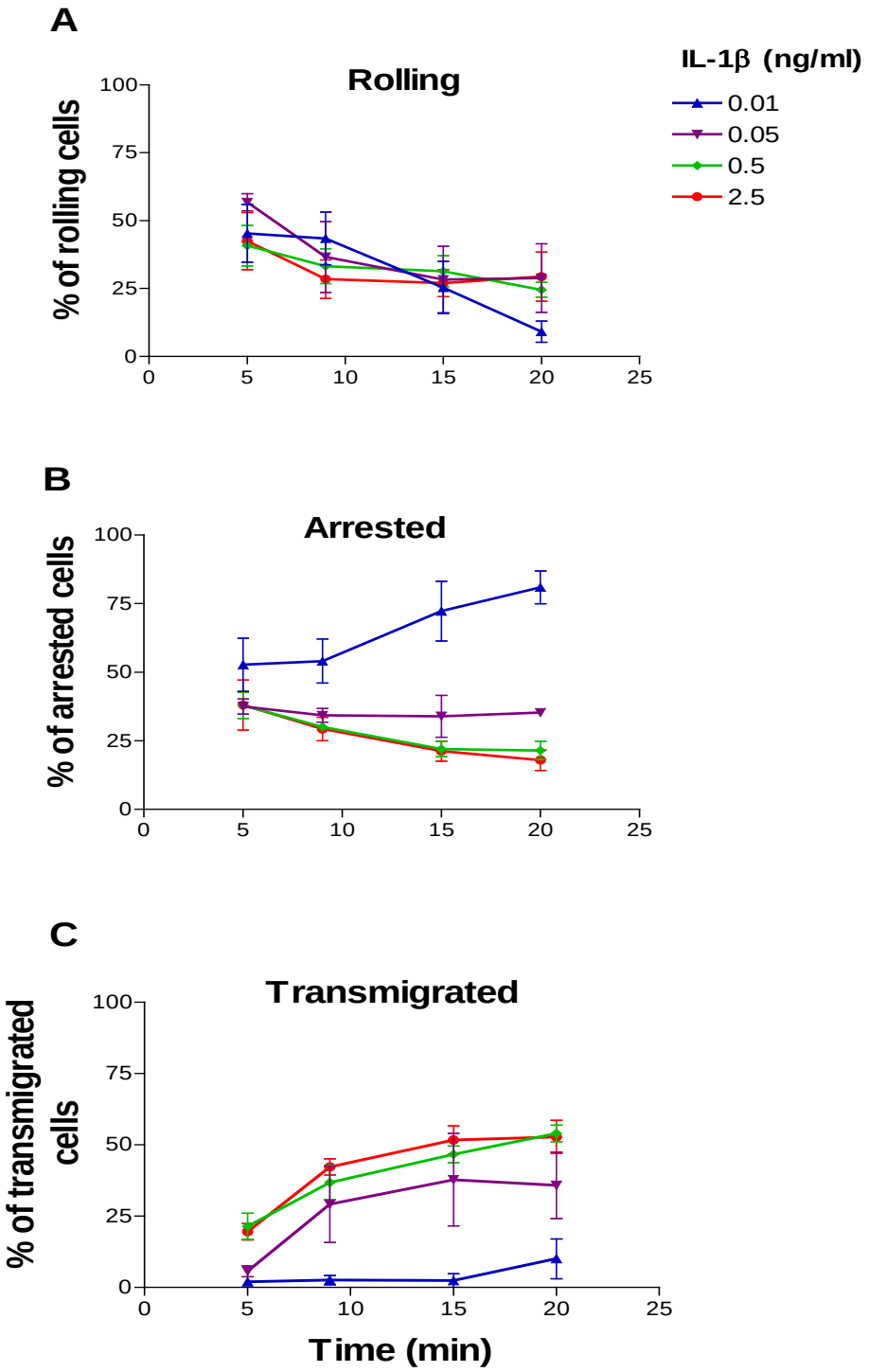


Figure 3.8 Effect of different doses of IL-1β stimulated MHECs on neutrophil behaviour
Adherent neutrophils were categorized as rolling (A), arrested (B) and transmigrated (C). All data are the mean±SEM of three experiments.

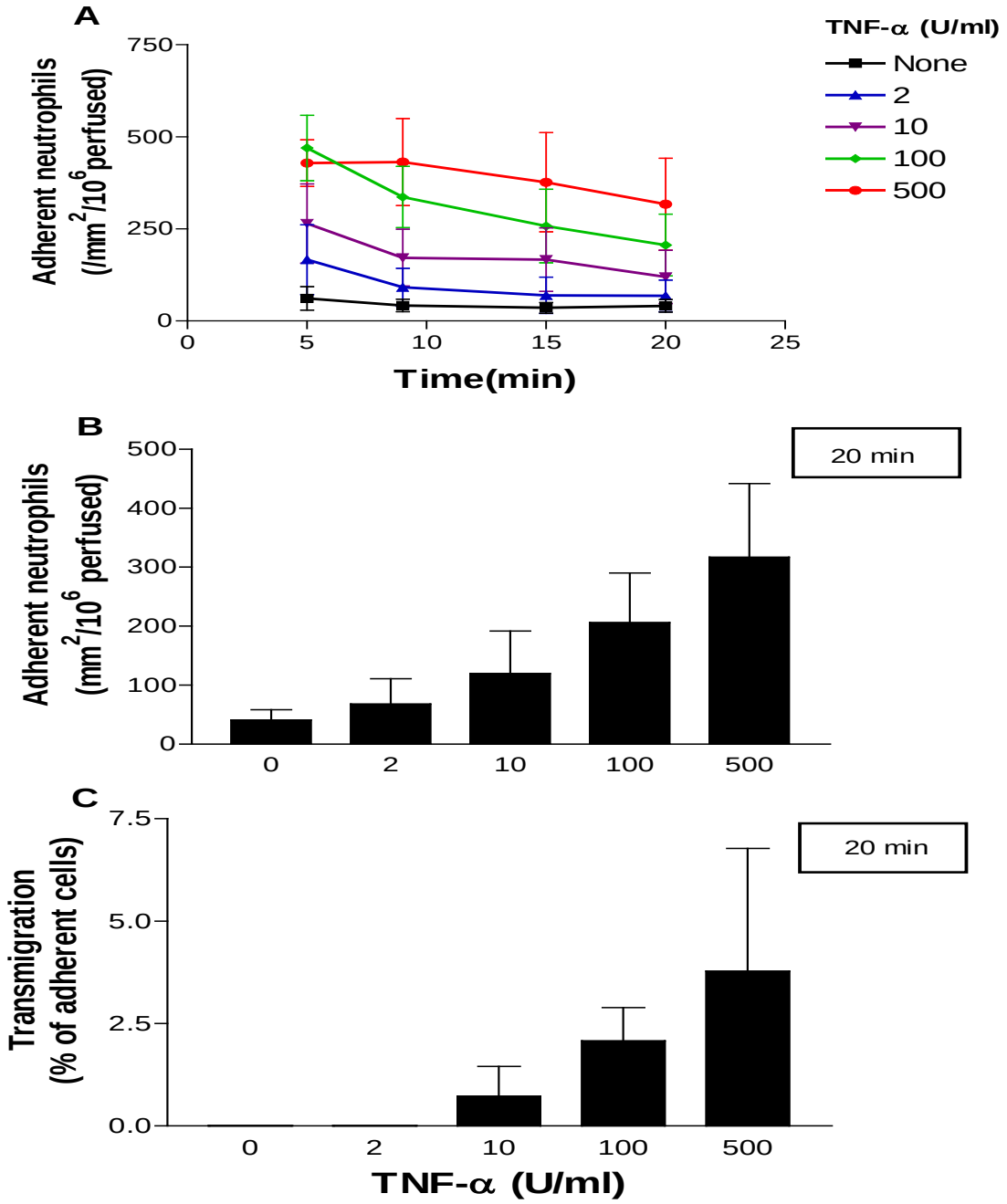


Figure 3.9 Effect of MHEC treatment with different concentrations of TNF-α on neutrophil adhesion (A and B) and transmigration (C)

MHEC monolayers in microslides were stimulated with different concentrations of TNF-α (2, 5, 10 and 100U/ml) for 4 hours. (A) ANOVA showed a statistically significant effect of TNF-α dose treatment on adhesion at all time points,$P<0.001$. (B) Despite the gradual increase of adhesion with higher concentrations, the TNF-α. dose treatment did not have a statistically significant effect on adhesion at 20 minutes. (C) Very low transmigration (5%) was observed in the highest TNF-α concentration at the final time point. All data are the mean±SEM of three experiments.

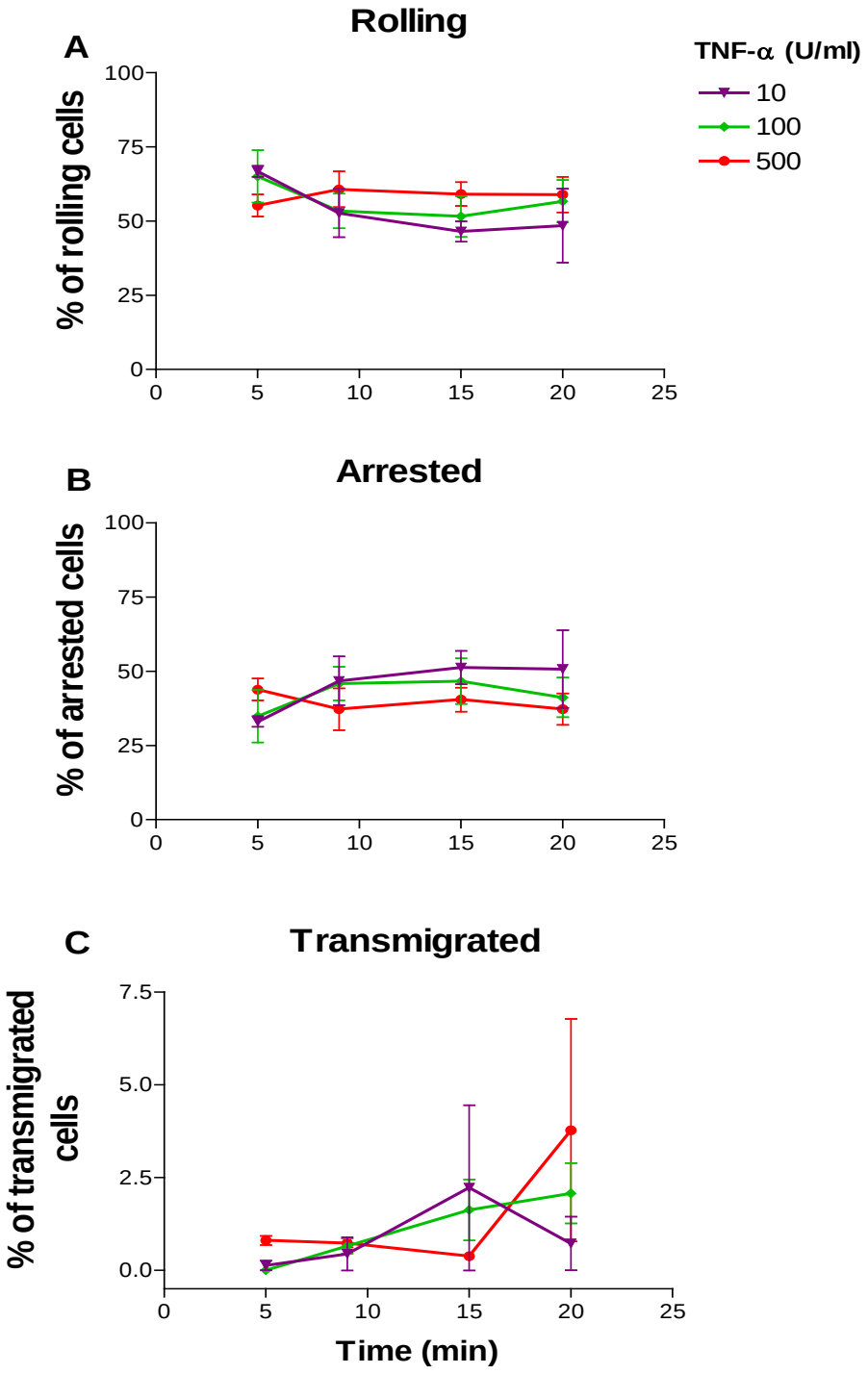


Figure 3.10 Effect of different doses of TNF-α stimulated MHECs on neutrophil behaviour

Adherent neutrophils were categorized as rolling (A) arrested (B) and transmigrated (C). All data are the mean±SEM of three experiments.

We also compared the highest dose of TNF-α and IL-1β stimulation with treatment using a combination of cytokines. Treatment with cytokines always induced increased levels of adhesion compared to unstimulated EC. However, there was no significant difference between the levels of adhesion using single or combined cytokines for EC stimulation (Figure 3.11A). There was still, a significant effect of cytokine treatment on transmigration ($p<0.01$, by ANOVA). Very few neutrophils transmigrated when TNF-α alone was used as inflammatory stimulus. More than 50% of cells transmigrated, when MHEC were stimulated with IL-1β or the combination of agents and the difference in levels of migration between the two activation regimens was not significant (Figure 3.11B). Data are the mean of 3 experiments.

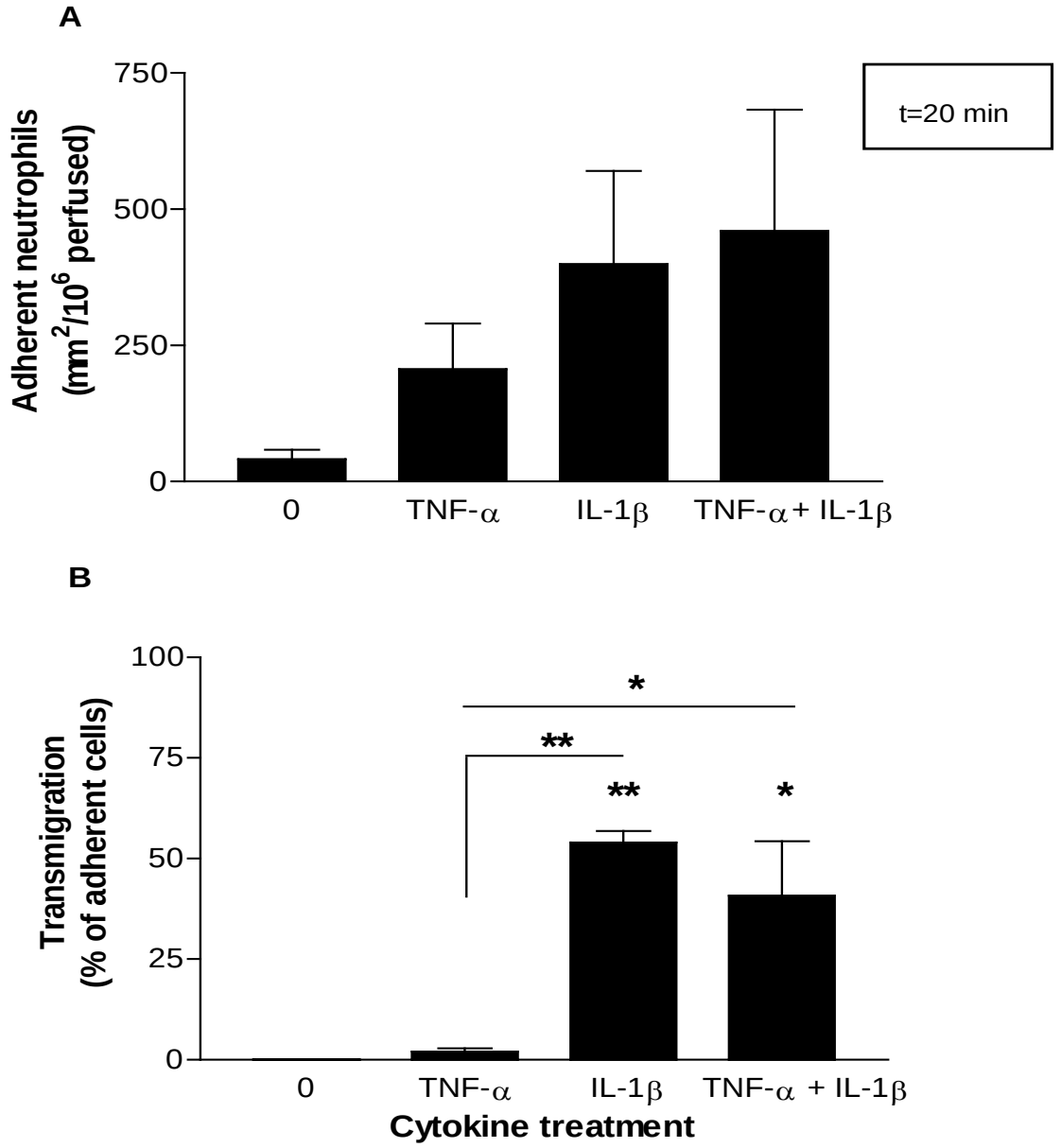


Figure 3.11 Effect of cytokine treatment on neutrophil adhesion (A) and transmigration (B) on MHEC monolayers

MHEC monolayers in microslides were stimulated with TNF-α (100 µg), IL-1β (0.5ng/ml) or their combination for 4 hours and were incorporated in flow based-adhesion assays. After 4-minute neutrophil perfusion, non-adherent neutrophils were washed out and adherent (A) and transmigrated (B) neutrophils were counted at 20 minutes. (A) ANOVA showed no statistically significant effect of different cytokine treatment on adhesion at 20 minutes. (B) There was a significant effect of cytokine treatment on transmigration ($P<0.01$, by ANOVA). *, $P<0.05$, **, $P<0.01$, by Bonferroni's Multiple Comparison Test. All data are the mean±SEM of three experiments.

3.3.5 The effect of cytokine treatment of MLEC on neutrophil recruitment and behaviour under flow

Three cytokine titration experiments were conducted on MLECs. MLECs responded to IL-1β treatment in a dose dependent manner. As IL-1β concentration increased, so did the levels of neutrophil adhesion, although the two higher doses resulted in similar adhesion levels. Overall, there was a significant dose dependent effect on adhesion ($p<0.001$, by ANOVA). Adhesion levels in the unstimulated condition and the lowest dose slightly decreased over time indicating unstable binding of neutrophils to the monolayer. Higher doses were able to stabilise adhesion (Figure 3.12A). After 20 minutes, the highest levels of adhesion (720±171 cells/mm^2/10^6 perfused) were observed at the maximum IL-1β concentration (Figure 3.12B). There was significant effect of IL-1β treatment in the percentage of transmigrated neutrophils at 20 minutes ($p<0.001$, by ANOVA). Treatment with the higher concentration resulted in maximum transmigration; around 75% of adherent neutrophils had transmigrated at 20 minutes (Figure 3.12C).

The behaviour of adherent neutrophils in all IL-1β concentrations over time is illustrated in Figure 3.13. In contrast with MHEC, the dose and the percentage of rolling cells were inversely proportional at 5 minutes. The percentage of firmly arrested cells decreased over time in all concentrations, apart from the concentration of 0.01ng/ml (Figure 3.13B). The opposite pattern was observed in transmigration, which increased in all doses over time. There was a very significant effect of dose treatment on transmigration ($p<0.001$, by ANOVA). The two highest concentrations of IL-1β led to 75% transmigration at 20 minutes (Figure 3.13C).

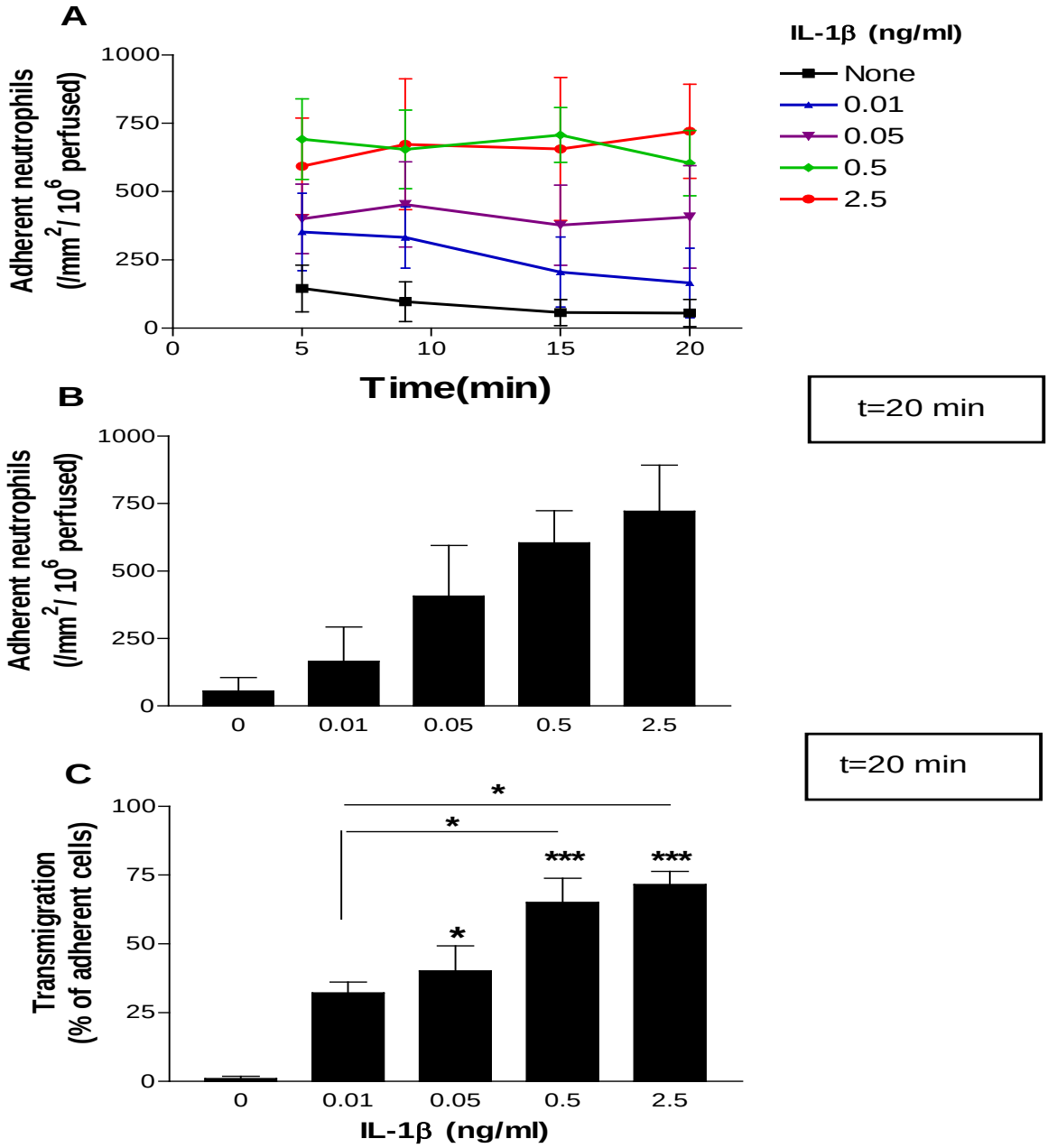


Figure 3.12 Effect of MLEC treatment with different concentrations of IL-1β on neutrophil adhesion (A and B) and transmigration (C)

MLEC monolayers in microslides were stimulated with different concentrations of IL-1β (0.01, 0.05, 0.5 and 2.5ng/ml) for 4 hours. (A) ANOVA showed an extremely significant effect (P<0.001) of IL-1β dose treatment on adhesion at all time points, P<0.001. (B) IL-1β dose treatment had a significant effect on adhesion levels at 20 minutes, P<0.05, by ANOVA. (C) ANOVA showed an extremely significant effect (P<0.001) of IL-1β dose treatment on % transmigration at 20 minutes. *, P<0.05 and ***, P<0.001, by Bonferroni's Multiple Comparison Test. All data are the mean±SEM of three experiments.

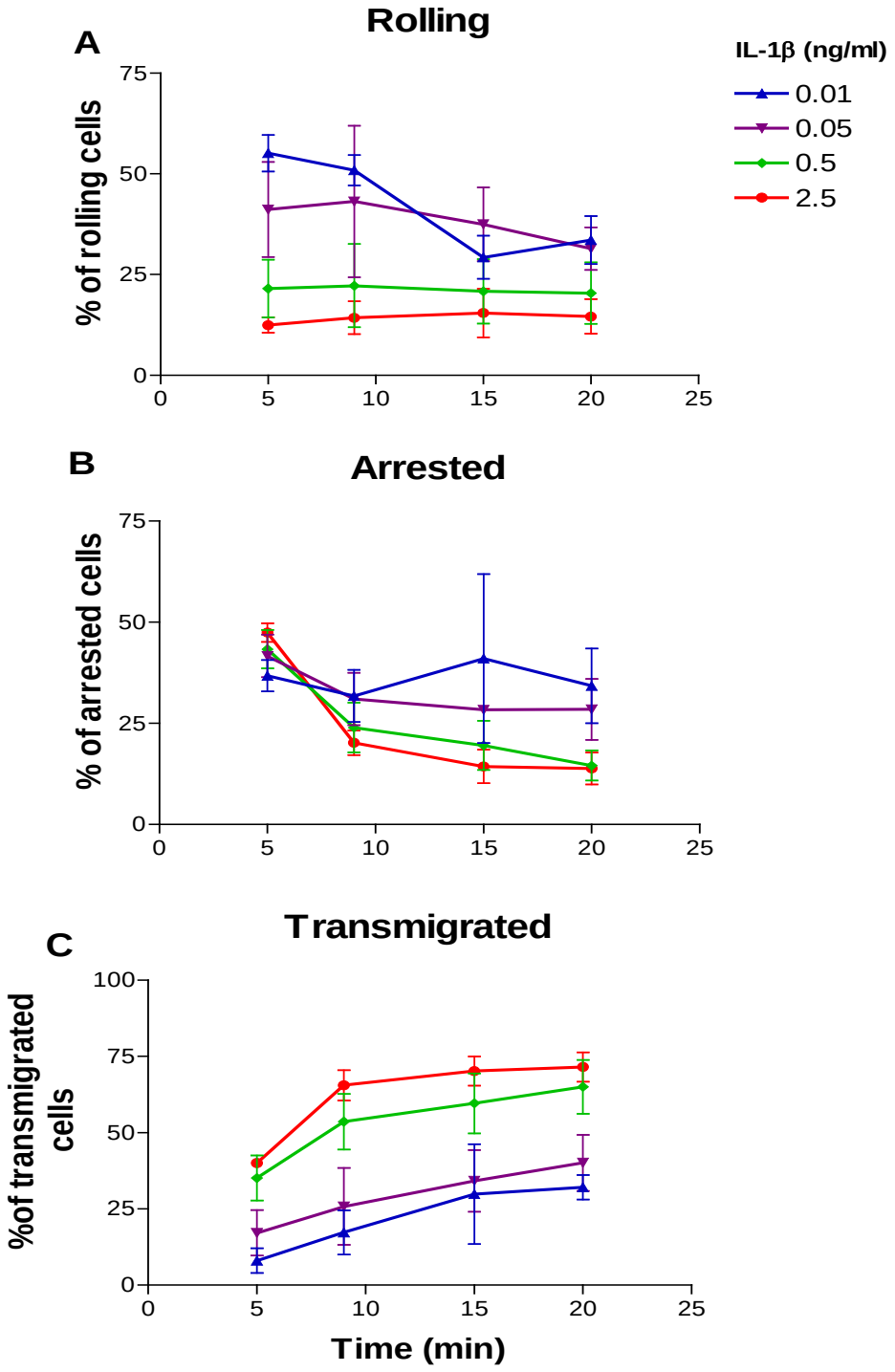


Figure 3.13 Effect of different doses of IL-1β stimulated MLECs on neutrophil behaviour

Adherent neutrophils were categorized as rolling (A) arrested (B) and transmigrated (C). All data are the mean±SEM of three experiments.

MLEC responded to TNF-α treatment in a concentration dependent manner ($P<0.001$, by ANOVA). Adhesion levels in all concentrations slightly decreased over time indicating that adhesion of neutrophils to the monolayer was not stable. The adhesion response saturated at 100U/ml TNF-α (Figure 3.14A and B). There was a significant effect of dose in adhesion ($p<0.05$, by ANOVA). Stimulation with TNF-α supported very low levels of transmigration (≈5%) even at the highest concentration (Figure 3.14C). The majority of adherent neutrophils (around 75%) were rolling unless 500U/ml of TNF-α was used, when the majority were firmly arrested (Figure 3.15A and B).

We also compared the treatment with each cytokine at the highest concentration alone or in combination. EC stimulated with TNF-α or the combination of cytokines supported the same levels of adhesion, but the highest levels of adhesion were observed with IL-1β alone (Figure 3.16A). There was a significant effect of cytokine treatment to transmigration ($p<0.01$, by ANOVA). Very low levels of transmigration were observed with TNF-α stimulation. More than 50% of cells transmigrated when MLEC were stimulated with IL-1β and this was not significantly altered by use of the combination of cytokines (Figure 3.16B). Data are the mean of 3 experiments.

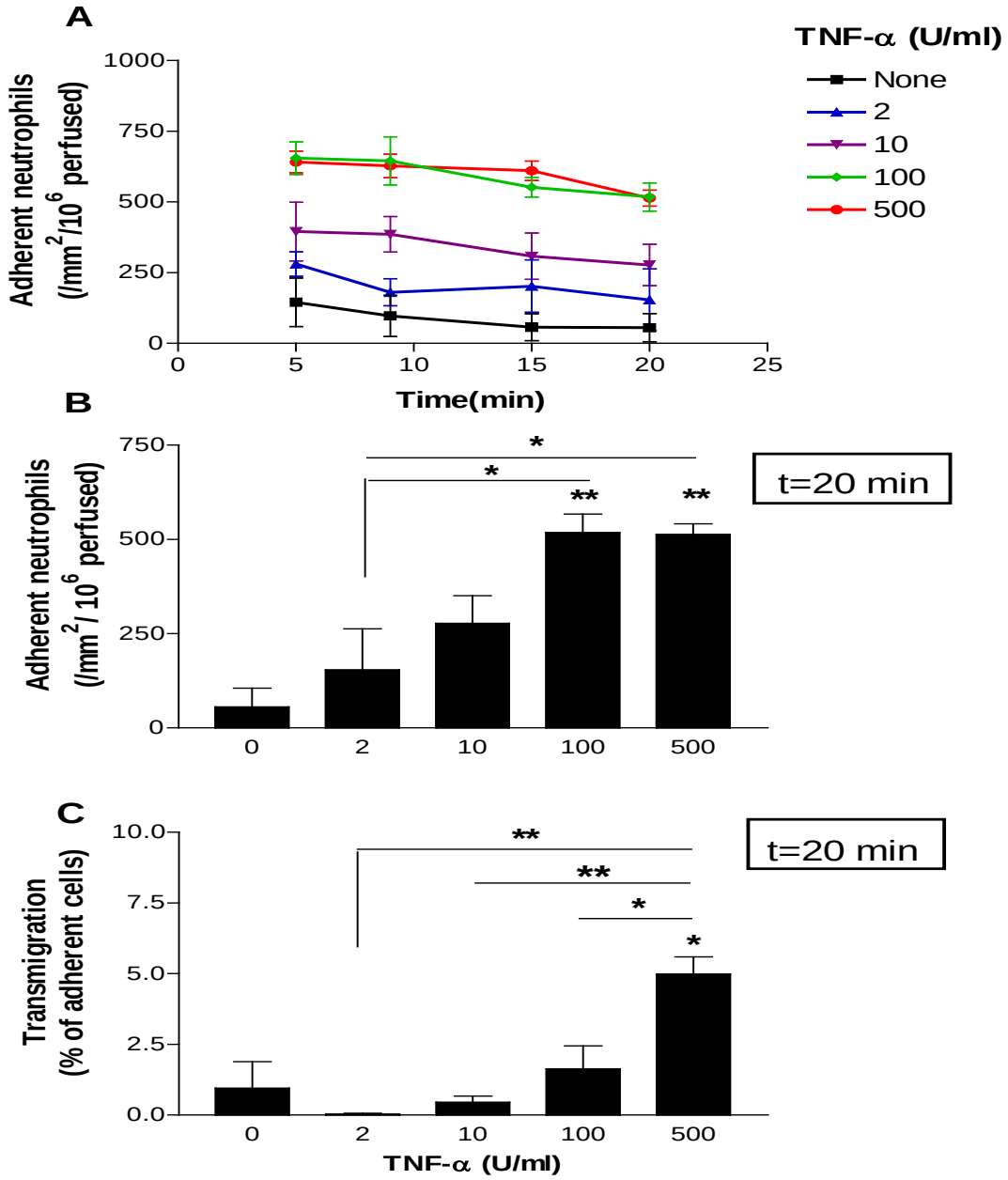


Figure 3.14 Effect of MLEC treatment with different concentrations of TNF-α on neutrophil adhesion (A) and B) and transmigration (C)

MLEC monolayers in microslides were stimulated with different concentrations of TNF-α (2, 5, 10 and 100U/ml) for 4 hours. (A) ANOVA showed an extremely significant effect (P<0.001) of TNF-α dose treatment on adhesion at all time points. (B) TNF-α dose treatment had a significant effect on adhesion levels at 20 minutes, P<0.01, by ANOVA, (C) ANOVA showed a significant effect (P<0.01) of TNF-α dose treatment on % transmigration at 20 minutes. *, P<0.05, **, P<0.01, by Bonferroni's Multiple Comparison Test. All data are the mean±SEM of three experiments.

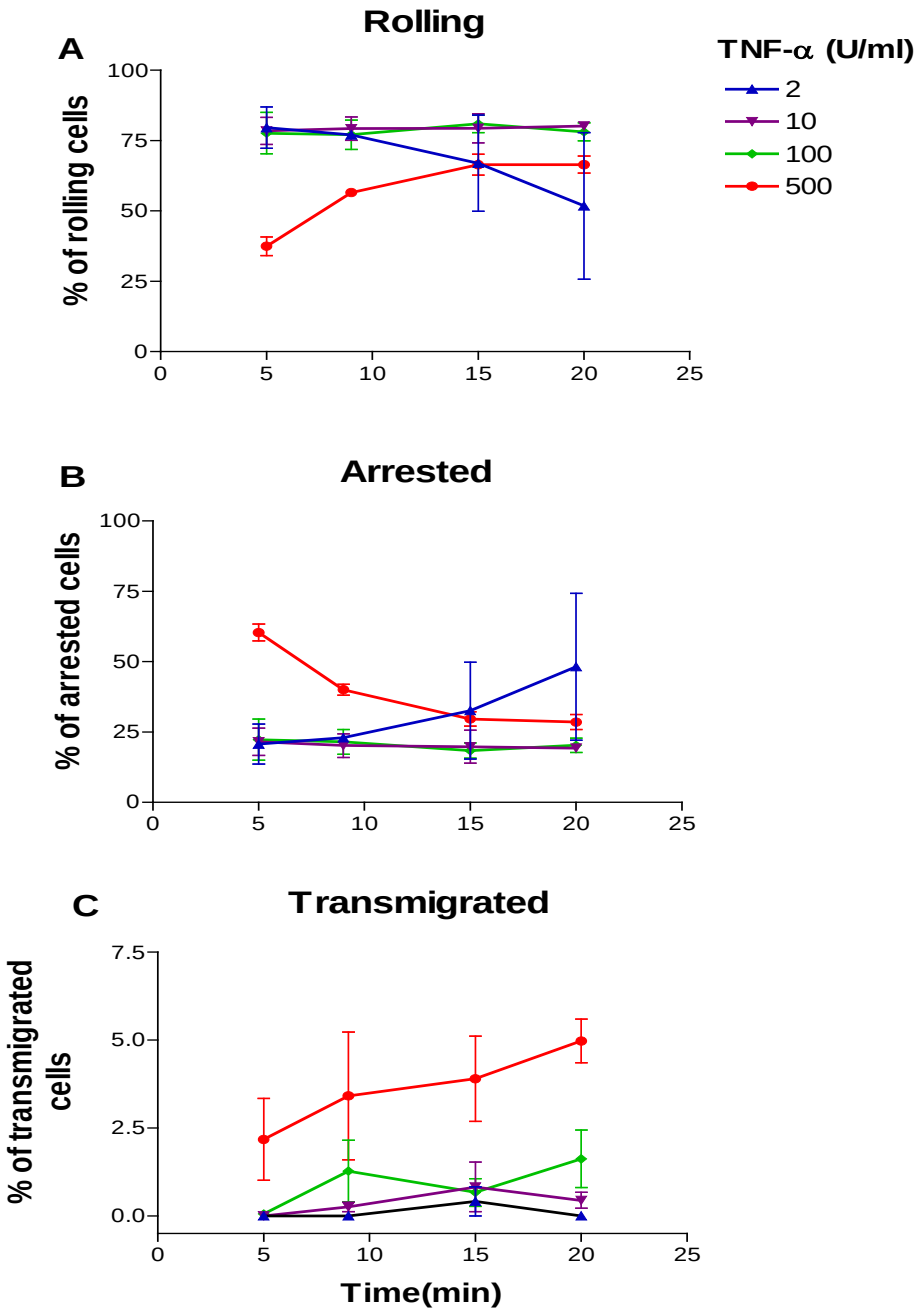


Figure 3.15 Effect of different doses of TNF-α stimulated MLECs on neutrophil behaviour

Adherent neutrophils were categorized as rolling (A), arrested (B) and transmigrated (C). All data are the mean±SEM of three experiments.

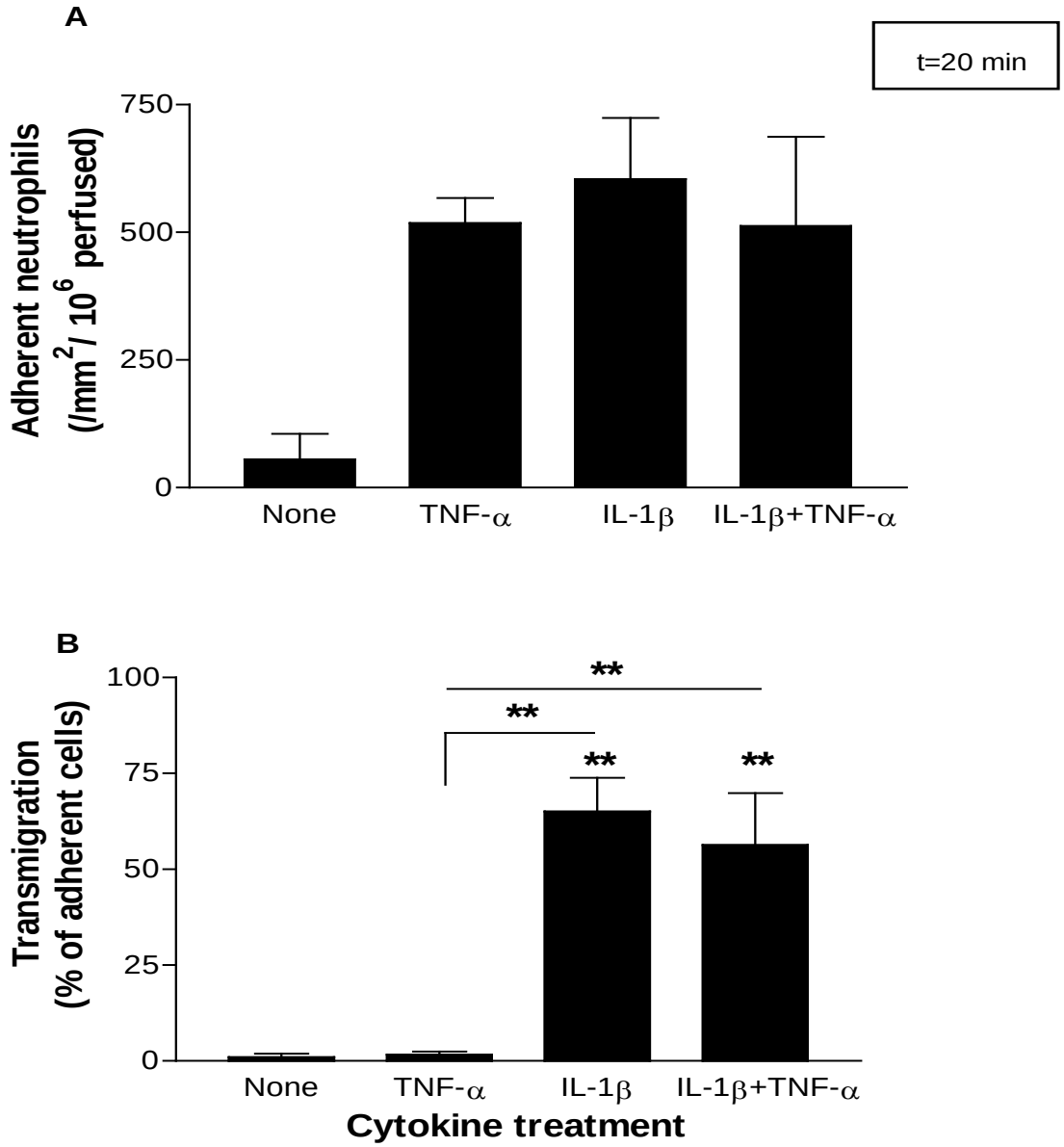


Figure 3.16 Effect of cytokine treatment on neutrophil adhesion (A) and transmigration (B) on MLEC monolayers

MLEC monolayers in microslides were stimulated with TNF-α (100U/ml), IL-1β (0.5ng/ml) or their combination for 4 hours and were incorporated in flow based-adhesion assays. After 4-minute neutrophil perfusion, non-adherent neutrophils were washed out and adherent (A) and transmigrated (B) neutrophils were counted at 20 minutes. (A) ANOVA showed an effect of different cytokine treatment on adhesion at 20 minutes, $P<0.05$. (B) There was an extremely significant effect of cytokine treatment on transmigration ($P<0.001$, by ANOVA). **, $P<0.01$, by Bonferroni's Multiple Comparison Test. All data are the mean±SEM of three experiments.

3.3.6 Direct comparison of neutrophil adhesion to MHEC and MLEC stimulated with different regimen of cytokines

By presenting the data detailed above in a different format we here present a direct comparison of the levels of adhesion and transmigration to the different ECs after stimulation with TNF-α, IL-1β or the combination of agents. This analysis showed that the responses of the two endothelial cells were remarkably consistent to all stimulation regimens and there was no significant differences in adhesion or transmigration when comparing MHEC and MLEC for any given cytokine regimen (Figure 3.17).

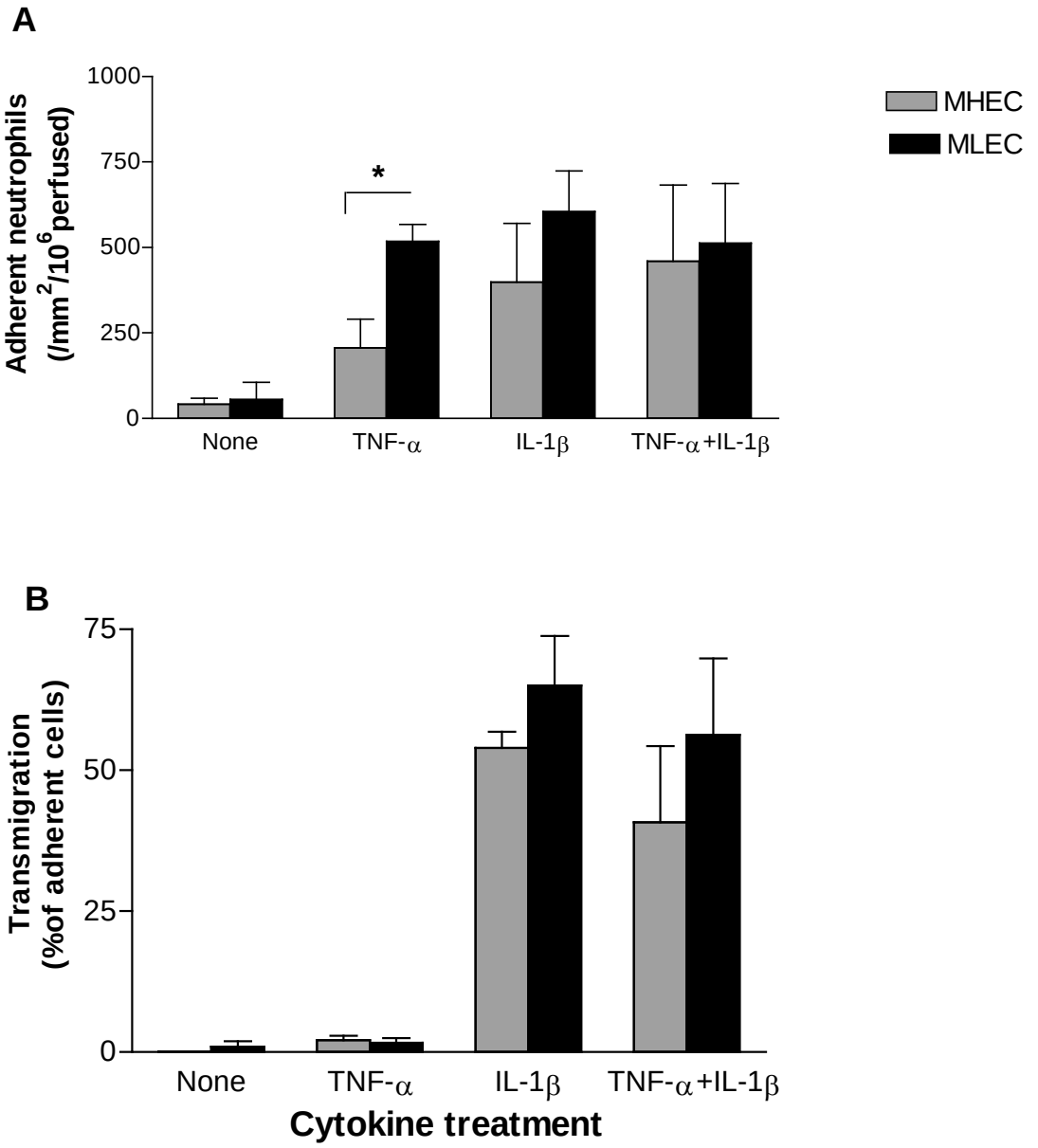


Figure 3.17 Comparison on adhesion and transmigration levels between different cytokine treatment on MHECs and MLECs

Adhesion levels (A) and % transmigration (B) were higher in MLECs (black bars) than in MHECs (grey bars) when they were stimulated with IL-1β or the combination,*, $P<0.05$, by Bonferroni's Multiple Comparison Test. Data are from 3 experiments in MHECs and MLECs respectively.

3.4 Discussion

In this chapter we described the development of three murine *in vitro* models of inflammation which can be used to investigate the recruitment of murine neutrophils to cytokine stimulated murine EC monolayers.

Initially we used the MCEC-1 cell line in our flow-based adhesion assays and we verified its responsiveness to cytokine treatment at a functional level *in vitro*. Treatment with IL-1β induced higher levels of adhesion and transmigration than treatment with TNF-α, and both readouts were further increased by the combination of cytokines. These findings are in accordance with those from the group who generated the MCEC-1 cell line (Lidington *et al*., 2002). It has been demonstrated that MCEC-1 cells express endothelial markers such as CD31, ICAM-2 and endoglin and respond to cytokine treatment. Their surface expression of E-and P-selectin, VCAM-1 and ICAM-1 was significantly induced after 6 hours when a combination of IL-1β and TNF-α was used as inflammatory stimulus (Lidington *et al*., 2002). Another interesting finding was the fact that unstimulated MCEC-1 monolayers were able to support considerable levels of neutrophil adhesion. This could be associated with the high constitutive expression of VCAM-1 in MCEC-1 cells as murine neutrophils have been described to express α4β1, the counter ligand for this receptor (Bowden *et al*., 2002; Lidington *et al*., 2002).

Bone marrow neutrophil isolation is accompanied with <5% contaminating B cells. The depletion of these cells does not modulate the behaviour of neutrophils in our assays. In fact, the smaller size of lymphocytes compared with neutrophils enables their discrimination with relative ease under phase contrast microscopy, and an experienced user is able to exclude

the very small number of lymphocytes from the analysis. Moreover, there was significant cell loss through the magnetic immuno-separating columns and an increased risk of inducing activation on the neutrophils finally isolated. For all the above reasons, we believed that B-cell depletion was unnecessary and we did not perform it routinely.

Here we have successfully isolated primary murine EC from heart and lung tissue. Both MLEC and MHEC cells were found positive for CD31, ICAM-2 and VE-cadherin. However, CD31 and VE-cadherin expression was higher in MLEC than MHEC cells. Relatively low CD31 expression on cardiac ECs was found in previous studies (Lidington *et al*., 2002; Marelli-Berg *et al*., 2000). This may be because MHEC cultures generally require more time to reach confluence than MLEC. After characterisation of mECs we tested the ability of these cells to recruit neutrophils from flow after cytokine stimulation. Both MHEC and MLEC responded to these cytokines in a dose dependent manner in most cases and showed the same patterns between cytokine treatment with TNF-α or IL-1β. However, our previous observations and the direct data comparison between them have raised some differences. Overall, the adhesion levels and the percentage of transmigrated neutrophils were higher in MLECs than MHECs under the same cytokine treatment. An explanation could lie in the differential induction of adhesion molecules upon TNF-α stimulation. Performing fluorescent immunoassay, Lim and Luscinskas (2006) have shown that CD31, ICAM-1 and ICAM-2 induction upon stimulation was higher in MLECs, while VCAM-1 and P-and E-selectin expression was higher in MHEC (Lim and Luscinskas, 2006).

The differences between adhesion and transmigration levels within the two cell cultures could be attributed to the intrinsic properties of the cells derived from different

vascular beds. It has been demonstrated with cDNA arrays that different biomechanical and humoral conditions acting upon the vascular endothelium from various organs has an effect on functional phenotype (Garcia-Cardena *et al.*, 2001). Furthermore, dual radiolabel methodology has demonstrated marked differences in the constitutive expression of VCAM-1 and ICAM-1 within brain, heart and intestine (Henninger *et al*., 1997). Interestingly, using immunohistochemistry techniques, VCAM -1 was found expressed in individual ECs of small and medium size vessels in the lung in the absence of inflammation (Fries, 1993).

Further evidence indicating different phenotypic characteristics between lung and heart EC comes from a study on T cell recruitment by these cells when stimulated with TNF-α. T-helper cells where perfused over TNF-α stimulated MLECs and MHEC in an *in vitro* flow assays. More Th1 cells arrested to MHECs than MLEC (87% than 27%, respectively) and transmigrated through them after 15 minutes (35.7% than 23.5%). The Th1 adherent cells exhibited a rolling phenotype on MLECs (Lim *et al.*, 2003). The same group found that MHECs but not MLECs produced RANTES after TNF-α stimulation. Finally, blocking studies revealed that the differences in T helper recruitment between MHECs and MLECs are attributed to the differential expression of VCAM-1 and RANTES in these cells (Lim *et al.*, 2003).

By far the most striking finding was the lack of response of MLEC and MHEC to TNF-α stimulation. Adhesion reached similar levels in both cytokine treatments but these decreased over time with TNF-α treatment, suggesting that a number of neutrophils initially captured from flow were able to detach from ECs over time. While more than half of adherent neutrophils had transmigrated at 20 minutes when the highest dose of IL-1β was used, hardly

any cells migrated in response to TNF-α. This was rather a surprising result, because the majority of adherent neutrophils (around 75%) were arrested on MCEC-1 monolayers treated with a lower dose of TNF-α (100U/ml) and TNF-α is an excellent stimulus for driving the adhesion and migration of human neutrophils to human EC (Luu *et al*., 1999). One possible explanation could be that a higher TNF-α dose is required to induce transmigration on stimulated mECs. A dose more than the double the highest used here has been reported to induce ≈30% of murine neutrophils to transmigrate on primary cardiac endothelial cells in *in vitro* parallel plate flow assays (Allport *et al.*, 2002). A reason for the low sensitivity of these cells to TNF-α stimulation could be explained by low density of TNF-α receptors. For example, a dramatic reduction in neutrophil adhesion and transmigration occurs in TNF-α receptor II (TNFR-II) deficient mice (Chandrasekharan *et al.*, 2007). Whether isolation and culture of mEC leads to loss of TNF-α receptors still needs to be ascertained.

Our findings could also be interpreted to indicate that TNF-α and IL-1β induce very different responses in primary murine endothelial cells. For example, injection of the cytokines into rabbit skin elicits responses with distinct kinetics. Administration of TNF-α results in rapid neutrophil recruitment (30 minutes), a process that does not require protein synthesis. IL-1β however induces a slower response (3-4 hours) and protein synthesis is required for neutrophil recruitment (Rampart. *et al*., 1989). Additional evidence for disparity of response is available for intravital studies in the cremaster muscle of the mouse. Neutrophil recruitment is inhibitable using anti-CD31 when TNF-α is used to stimulate the vasculature, but this is not the case when IL-1β is used (Thompson *et al.*, 2001). Similar observations were made after blocking $\alpha_6\beta_1$ integrin (Dangerfield *et al.*, 2005), ICAM-2 and JAM-A (Woodfin *et al.*, 2009). Finally, it has been demonstrated using well-characterized pharmacological

interventions (e.g. use of antagonists) that IL-1β (but not TNF-α) induced adhesion and transmigration of neutrophils is LTB_4 and PAF dependent (Young, 2002).

In this chapter, we have examined the ability of a cell line and primary ECs to recruit neutrophils, under different stimuli, in order to establish a murine *in vitro* model of inflammation that would enable us to investigate this process and reverse migration. The MCEC-1 cell line was characterized by constitutive levels of neutrophil recruitment and low levels of transmigration. The use of primary cells is preferred because these require stimulation with cytokines for neutrophil recruitment. However, stimulation with TNF-α did not give satisfactory results, as it did not induce stable adhesion and there was very little transmigration. In contrast, stimulation with IL-1β was more promising, as it could induce high levels of adhesion with consistently high proportion of transmigrating cells. Therefore, we chose to use mECs, with IL-1β routinely used as an inflammatory stimulus of choice.

CHAPTER 4

GENERATION OF MURINE REVERSE MIGRATED NEUTROPHILS *IN VITRO* AND INVESTIGATION OF THEIR FATE *IN VIVO*

4.1 Introduction

Human neutrophils are released from bone marrow and enter the blood circulation where they have a half life of ≈6.5 hours (Mauer *et al*., 1960). Under physiological conditions, neutrophils die by apoptosis and are cleared by the macrophages of liver and spleen (Doerschuk *et al*., 1987; Ohgami *et al*., 1989; Saverymuttu *et al*., 1985; Savill, 1997; Skretting *et al*., 1988). Clearance of neutrophils is performed by the Kupffer cells in the liver and presumably, although they have not been identified experimentally, by the red pulp macrophages in the spleen (Rankin, 2010; Weiss, 1974). Interestingly, P-selectin, is up-regulated on Kupffer cells in the liver under inflammatory conditions and might play a role in neutrophil clearance by this tissue during inflammation (Shi *et al*., 1998).

A number of studies have demonstrated that bone marrow might also play a role in neutrophil clearance under physiological conditions (Lovas *et al*., 1996). When mice were injected with ^{111}In senescent neutrophils, these migrated to various organs, depending on their activation and maturation state. Infused blood neutrophils migrated in equal numbers between bone marrow and liver, peritoneal exudate neutrophils localised primarily to the liver, while bone marrow derived neutrophils migrated preferentially to their tissue of origin (the bone marrow) 4 hours post-injection (Suratt *et al*., 2001). Another study has suggested an equal contribution of liver, spleen and bone marrow to neutrophil clearance (Furze and Rankin, 2008).

Neutrophil survival *in vitro* has been extensively investigated. Human neutrophils undergo spontaneous apoptosis after 18-36 hours *in vitro* (Scheel-Toellner *et al*., 2004) , but there is strong evidence that these cells exhibit prolonged survival under inflammatory

conditions, probably to maintain their immune functions. Peripheral neutrophils from patients with systemic inflammation became apoptotic relatively slowly *in vitro* (Ertel *et al*., 1998; Jimenez *et al*., 1997; Ottonello *et al*., 2002). Similarly, neutrophils isolated from mice, previously injected with pro-inflammatory agents showed a delay in apoptosis *ex vivo* (Coxon *et al*., 1996). Moreover, neutrophils isolated from an inflammatory milieu appear to also have a prolonged survival. Human neutrophils isolated from inflammatory exudates were protected against the pro-apoptotic effect of the combination of TNF-α and cycloheximide (Hotta *et al*., 2001). In addition, neutrophils isolated from the bronchoalveloar lavage fluid, peritoneal cavity and cerebrospinal fluid of experimental animals, exhibited a delay in apoptosis *ex vivo* compared to peripheral blood derived neutrophils (Coxon *et al*., 1996; Coxon *et al*., 1999; Watson *et al*., 1997). Interestingly, there is evidence that soluble factors produced at the site of inflammation are able to delay apoptosis. TNF-α is capable of delaying apoptosis (Colotta *et al*., 1992; Hotta *et al*., 2001). GM-CSF, produced by endothelial cells, is able to prolong neutrophil survival (Colotta *et al*., 1992). In addition, an anti-apoptotic role for IL-8, LTB4, C5a and LPS has also been reported (Kettritz *et al*., 1998; Klein *et al*., 2001; Murray *et al*., 2003; Perianayagam *et al*., 2002).

Subsequently, the process of neutrophil adhesion and transmigration was examined for a potential role in modulation of neutrophil survival. It was shown that apoptosis of resting or fMLP activated neutrophils in the presence of soluble fibrinogen was reduced through the binding of neutrophil β_2-integrin (Rubel *et al*., 2003). Similarly, neutrophils adherent to IL-1β-stimulated endothelial monolayers exhibited prolonged survival through neutrophil β_2-integrin binding to ICAM-1 (Ginis and Faller, 1997). Additionally, the interaction of α_M- or β_2-integrin chains with ICAM-1 resulted in a delay in apoptosis (Whitlock *et al*., 2000).

Transmigration also plays a role in neutrophil survival. It has been demonstrated that neutrophils are rescued from apoptosis when transmigrated through TNF-α+IL-1β treated-endothelial monolayer on filters and collected onto a non-adhesive surface. Neutrophils survived from six to eight hours in the presence of cytokines (Coxon *et al*., 1999). Recently, it was found that neutrophil transmigration across EC stimulated with increasing concentrations of TNF-α and the levels of apoptosis were inversely correlated, i.e. the greater the inflammatory stimulus the less prone to apoptosis migrated cells became (McGettrick *et al*., 2006).

Other evidence that interactions with EC delay apoptosis comes from a study on once transmigrated neutrophils that were able to reverse transmigrate back across an EC monolayer *in vitro* in 24 hours (Buckley *et al*., 2006). These cells were found to be non-apoptotic and functionally distinct compared to naïve circulating neutrophils. Reverse migration of neutrophils not only challenges the very well established perception of neutrophils being short-lived cells which die in the tissue after recruitment, but also provides evidence for the cells ability to transmigrate twice across an EC in a bi-directional manner. This phenomenon has also been demonstrated *in vivo* in transgenic Zebrafish models of inflammation. Thus, by performing wounding assays, neutrophils were observed to bi-directionally migrate between the site of inflammation and the vasculature (Mathias *et al*., 2006). It is suggested that this process might be an additional mechanism for clearance of neutrophils from tissues that are constantly recruiting these cells during immune surveillance (gut, lung, and peritoneum). There is also evidence suggesting this reverse migration increases in frequency in chronic inflammatory diseases (Buckley *et al*., 2006). Reverse migration has also been demonstrated for monocytes *in vivo* (Bradfield *et al*., 2007) and lymphocytes *in vitro* (McGettrick *et al*.,

2009).

Reverse migrated neutrophils (RPMNs) exhibit an altered phenotype compared to naïve circulating neutrophils. These cells could adhere to TNF-α stimulated HUVEC from flow with reduced efficiency compared to peripheral blood neutrophils, but only 1% of recruited cells could transmigrate, suggesting that *in vivo* they were unlikely to re-enter a site of inflammation. Buckley *et al*. (2006) have shown that RPMNs were rheologically more rigid than the naïve cells and might have a tendency for mechanical entrapment in small vessels. RPMNs were also able to produce greater levels of ROS after fMLP stimulation, indicating that they were functionally primed. RPMNs also had a distinct surface phenotype (CXCR1 low, ICAM-1 high) compared to peripheral blood cells before (CXCR1 high, ICAM-1 low) or after activation (CXCR1 low, ICAM-1 low). Interestingly, RPMNs were also readily distinguished from tissue resident neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis or cells that had migrated once across EC (CXCR1 low, ICAM-1 low). A very interesting finding in this study was the presence of cells carrying this phenotype in the blood of healthy individuals (~0.25%). More importantly, the frequency of these cells was increased to 1-2% in the blood of patients with inflammatory disease, such as rheumatoid arthritis and atherosclerosis (Buckley *et al*., 2006).

In this chapter, we aim to characterize murine RPMNs. For this reason, these were generated in 24-hour static co-cultures with mECs. Their viability and apoptosis levels were quantified. CXCR2 (as an antibody against mCXCR1 was not available) and ICAM-1 surface expression was measured. Previous studies have not been able to demonstrate the fate of these cells *in vivo*. Here, we attempt to investigate the fate of murine RPMNs using adoptive

transfer methodology. RPMNs from EYFP (Enhanced Yellow Fluorescent Protein) mice were injected into WT mice that were later sacrificed. Blood and single cell suspensions from bone marrow, spleen, liver and lung were analysed for the presence of any EYFP cells, by flow cytometry.

4.2 Methods

4.2.1 Generation of mRPMNs using MCEC-1 and primary mEC cultures.

MCEC-1 cells from two 25 cm^2 flasks were seeded in a 6-well plate pre-coated with gelatin (0.1%). The cultures were confluent after 24 hours. The monolayer in each well was stimulated with 100U/ml TNF-α and 0.5ng/ml IL-1β for 4 hours. Cultures were washed twice and $2x10^6$ murine neutrophils in DMEM/BSA were added to each well. After 1 hour incubation at 37^oC, 5%CO_2, non-adherent neutrophils were removed by a wash with DMEM/BSA and 2ml of MCEC-1 medium supplemented with TNF-α and IL-1β (100U/ml and 0.5ng/ml, respectively) was added to each well. The plate was then mounted on a microscope stage and 10 random fields from each well were video-recorded. The 6-well plate was placed in the incubator at 37^oC, 5%CO_2 for 24 hours. Random fields from each well were captured on video after 2, 4 and 24 hours. All recordings were analysed off-line and reverse transmigration was expressed as a percentage of the total number of neutrophils that had transmigrated at 1 hour.

Static adhesion assays with MHECs and MLECs were performed, as previously described with minor alterations. This time, IL-1β (2.5ng/ml) was used to stimulate the MHEC and MLEC monolayers in 25 cm^2 flasks. In addition, mEC complete medium without

any cytokines was introduced to the flasks after the removal of non-adherent neutrophils. As control, $3x10^5$ neutrophils were added in a well and cultured at 37^oC, $5\%CO_2$ for 24 hours. Neutrophils were cultured in MCEC-1 medium with TNF-α and IL-1β in experiments with MCEC-1 cells and mEC complete medium without any cytokines in those with primary mECs.

4.2.2 Culture of neutrophils in different media

Freshly isolated bone marrow neutrophils ($3x10^5$ cells) were added in a well of a six-well plate and cultured in PBSA or MCEC-1 medium supplemented with TNF-α, IL-1β or their combination for 24 hours. The following day, each culture was observed under phase contrast microscopy and intact phase bright cells were counted. Results were presented as a percentage of reduction in neutrophil numbers. Additionally, the cells from each culture condition were collected, centrifuged at 400g for 5 minutes and stained with Trypan Blue.

4.2.3 Measurement of neutrophil viability

Neutrophil viability was assessed by Trypan blue exclusion. Trypan blue is a dye that only penetrates the membrane of dead cells and stains them blue. Equal amounts (10μl) of each neutrophil sample and dye were mixed for 5 minutes. Then, 10μl of the formed suspension was loaded on a haemocytometer. Intact alive and blue stained dead cells were counted under phase contrast microscopy and viability was expressed as a percentage of live cells from the total number of neutrophils.

4.2.4 Measurement of neutrophil apoptosis

Neutrophil apoptosis was assessed in three ways. First, it was measured by

observation of nuclear morphology. Cytospin samples were stained with Diff Quick and observed under a light microscope. The presence of a condensed nucleus in a cell is a typical sign of apoptosis. Apoptotic neutrophils were counted and their numbers were expressed as a percentage of total number of neutrophils.

An additional way to assess neutrophil apoptosis is the measurement of the cells' mitochondrial transmembrane potential. The mitochondrial transmembrane potential is reduced in apoptotic cells. The reduction of DiOC6 fluorochrome uptake by mitochondria in cells that are undergoing apoptosis can be measured by flow cytometry. In detail, 50μl of neutrophils (1x 10^6 cells/ml) were incubated with 150μl of DiOC6 (40nM) for 30 minutes at 37^{o}C. The reaction was stopped by adding ice-cold PBS and the cell suspension was centrifuged at 800g at 4^{o}C for 4 minutes. Each sample was analysed using the Cyan flow cytometer and Summit version 4.3 software package (DAKO). Data was presented as a percentage in reduction in Median Fluorescence Intensity (MFI).

The mitochondrial dye JC-1 was also used for neutrophil apoptosis assessment. JC-1 accumulates in aggregates in the mitochondria of non-apoptotic cells. These aggregates are fluorescent red in colour. At the early stages of apoptosis, the mitochondrial potential is significantly reduced and the dye remains in the cytoplasm in a monomeric form which fluoresces green. The difference in red/green JC-1 fluorescence ratio can be analysed with flow cytometry. Each sample was incubated with JC-1 in RPMI (0.01mg/ml) for 15 minutes at 37^{o}C, centrifuged at 400g for 5 minutes, washed once with ice cold PBS, resuspended in 400μl of cold PBS and analysed by Cyan flow cytometry, as previously described. Data was presented as the percentage of cells whose red fluorescent aggregates decreased, compared to

the fresh.

4.2.5 Characterization of mRPMNs - Surface expression of CXCR2 and ICAM-1

Fresh neutrophils, mRPMNs and 24-hour control neutrophils were collected and centrifuged at 400g for 5 minutes. Around 10^5 cells per sample were resuspended in ice cold PBS2A and centrifuged at 800g for 5 minutes. Each sample was incubated with 50μl of anti-mouse CD16/CD32 antibody for 20 minutes at 4^oC, in order to block any Fc-mediated reactions. After a wash step, the samples were incubated with antibodies against CXCR2 and ICAM-1 or their IgG controls for 30 minutes on ice. Table 4.1 shows the antibodies and dilutions used for FACS staining. Each sample was washed twice and transferred into a FACS tube in cold PBS2A (400μl final volume). All samples were analysed using the Cyan flow cytometer (DAKO) and Summit version 4.3 software package (DAKO). Results were expressed as median fluorescence intensities (MFI) and percentage of cells positive for the specific surface marker.

Table 4.1 Antibodies and dilutions used for mRPMN characterization

Antibody	ISOTYPE	DILUTION
Anti-mouse CD16/CD32	Rat IgG2a, λ	1:50
APC anti-mouse CXCR2	Rat IgG2a	1:10
PE anti-mouse ICAM-1	Rat IgG2b,κ	1:500

4.2.6 Adoptive transfer experiments

Adoptive transfer experiments were performed in order to investigate the fate of mRPMNs. Isolated bone marrow neutrophils from EYFP mice (C57/BL6 strain) were used in static assays for the generation of a fluorescent mRPMN population. Reverse migrated

neutrophils were collected, centrifuged at 400g for 5 minutes, and finally resuspended in 200µl HBSS at different concentrations. Wild type (C57/BL6) mice were injected intravenously (IV) with different numbers of EYFP mRPMNs (up to 1 x 10^6 cells) and sacrificed by cardiac puncture, after 4 or 24 hours. The blood from these mice was collected (300-800µl) and centrifuged at 800g for 10 minutes. The cell pellet was resuspended in 1ml of PBS and then 3ml of red cell lysis buffer was added. After centrifugation at 400g, for 5 minutes, the samples were washed and resuspended in PBS2A on ice.

Prior to dissection, mice were perfused with PBS through right atrium in order to remove any remaining blood cells from the organs. The tissues of interest (bone marrow, spleen, lungs and liver) were dissected and processed in order to obtain single cell suspensions. Bone marrow cell suspension was prepared following the same protocol for neutrophil isolation, excluding percoll centrifugation step. Spleen was mechanically disrupted, filtered through a 70µm nylon mesh cell strainer and rinsed with 10ml RPMI/EDTA (15mM). The cell suspension was centrifuged at 400g for 5 minutes and resuspended in 1ml of PBS. Next, red cell lysis buffer was added and the cells were centrifuged at 400g for 5 minutes. After a wash with RPMI/EDTA solution, cells were resuspended in PBS2A.

Lungs were minced with a scalpel and tissues were incubated in 15ml type 1 collagenase solution at 37^{o}C for 45 minutes. At the end of the incubation, 15ml of RPMI/EDTA was added and the preparation was filtered twice through a cell strainer. After centrifugation at 400g for 5 minutes, red cell lysis buffer was added, and after two washes, cells were resuspended in PBS2A.

Liver was immersed in a petri dish filled with collagenase. Approximately 10ml of collagenase solution was injected into different parts of the liver, using 1ml syringe and a 25G needle. Then, liver was cut into small pieces and these were incubated with collagenase at 37^{o}C for 30 minutes. After filtration through a cell strainer, red cells were lysed and the remaining cells were diluted in PBS2A, as previously described. All cell suspensions were at a final concentration of 1x10^{6} cells/ml. In some experiments, fresh EYFP neutrophils (1 or 3x10^{6}) were injected into WT mice that were sacrificed by cardiac puncture after 4 hours. In these experiments, single cell suspensions were fixed in 4% PFA and analysed on the following day.

At least 5x10^{3} cells were analysed for EYFP expression in the gate for reverse migrated neutrophils (CXCR2 low/ ICAM-1 high) in fluorescence isothiocyanate (FL1) channel by flow cytometry. The samples were analysed, as previously described. As EYFP cells are highly fluorescent (their MFI is between 100 and 700), we can estimate the number of EYFP cells present in the total cell population from each organ.

4.3 Results

4.3.1 Generation of mRPMNs in cytokine stimulated MCEC-1 cultures under static conditions

In order to generate mRPMNs, MCEC-1 monolayers were stimulated with TNF-α (100U/ml) and IL-1β (0.5ng/ml), for 4 hours. Around 2x10^{6} neutrophils were added to each well. The co-cultures were placed inside an incubator for 1 hour, at 37^{o}C, 5% CO_2. Then, non

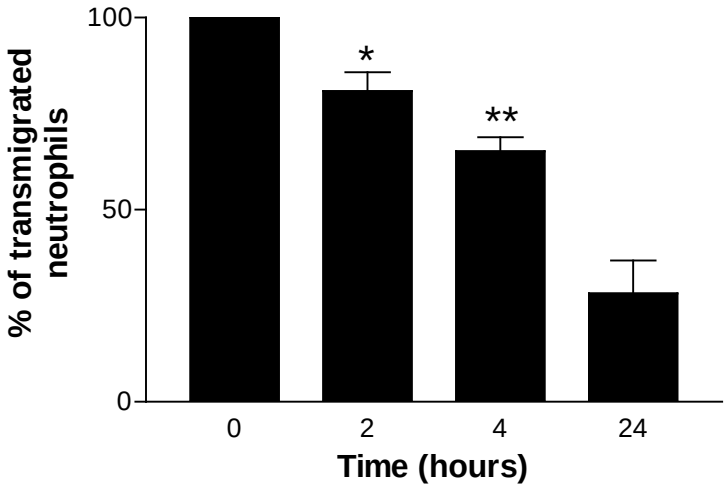


Figure 4.1 Decrease in transmigrated neutrophils in static cultures over a 24–hour period

$2x10^6$ neutrophils were added to MCEC-1 monolayers stimulated with TNF-α (100U/ml) and IL-1β (0.5ng/ml) for 4 hours. After 1 hour co-culture, non-adherent neutrophils were removed and the number of transmigrated neutrophils at 0, 2, 4 and 24 hours was determined under phase contrast microscopy. Transmigration at 2, 4 and 24 hours was expressed as a percentage of the number of transmigrated neutrophils at 0 hours. The number of transmigrated neutrophils decreased over time. ANOVA showed a significant correlation between transmigration and time ($P<0.001$). *, $P<0.05$, **, $P<0.01$, by Bonferonni's Multiple comparison test. All data are the mean±SEM of 3-4 experiments.

4.3.2 Assessment of murine neutrophil survival in 24-hour culture in different media

Following the static assay protocol, we added MCEC-1 medium with TNF-α and IL-1β into the co-cultures after the removal of non-adherent neutrophils. However, the low survival of neutrophils in 24-hour control group, led us to examine the survival of neutrophils in different media. Twenty four-hour cultures of neutrophils in various media were observed under phase contrast microscopy and intact phase bright cells were counted. After 24 hours, 50% of neutrophils cultured in PBSA were alive. Culture with TNF-α alone resulted in the survival of just 30% of the cells, while this was doubled with IL-1β alone. The combination led to a slight increase in cell viability, compared with PBSA (Figure 4.2).

All the cells from each co-culture were collected, centrifuged at 400g for 5 minutes and stained with Trypan Blue. Around 90% of RPMNs were alive, compared to 54% of PBSA. In the cultures with MCEC-1 medium, 55% of cells were alive with the cytokine combination, 72 % with IL-1β and only 31% with TNF-α (data not shown).

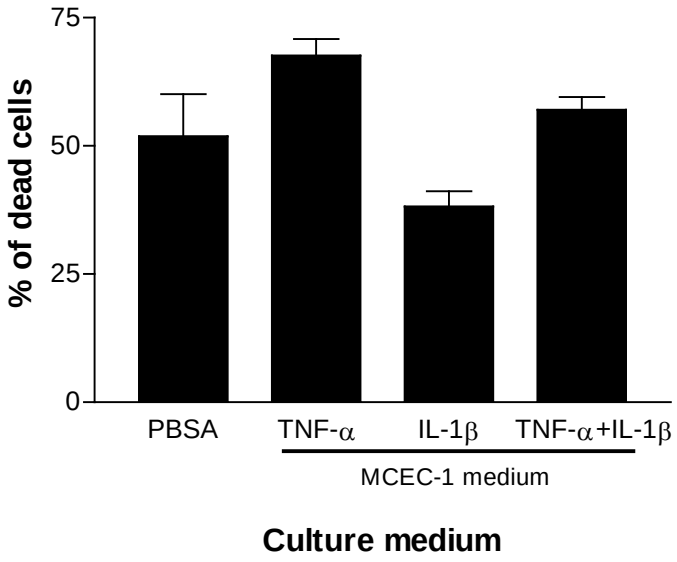


Figure 4.2 The effect of culture with different media on neutrophil viability
Bone marrow neutrophils were cultured in PBSA or MCEC-1 medium supplemented with TNF-α and IL-1β or their combination for 24 hours. The following day, each culture was observed under phase contrast microscopy and intact phase bright cells were counted. Results were presented as a percentage of neutrophil number reduction. All data are the mean+SEM of three experiments.

4.3.3 Generation of mRPMNs in IL-1β stimulated MHEC and MLEC cultures under static conditions

Comparing transmigration on IL-1β stimulated MHECs and MLECs, 35±10% and 42±2% of the neutrophils added to MHECs and MLECs respectively, had transmigrated after 1 hour of co-culture (Figure 4.3A). The numbers of transmigrated neutrophils followed the same pattern as with MCEC-1 static co-cultures over time. Transmigration levels were very similar between MHEC and MLEC at each time point. In MHEC co-cultures, 63.1±2.4% of neutrophils remained transmigrated at 2 hours, then 42.1±3.2% at 4 hours and 30.9±7.5% at 24 hours (Figure 4.3B). In MLEC co-cultures, 49.8±3.7% of neutrophils remained transmigrated at 2 hours, then 36.7±3.4% at 4 hours and 25.4±6.2% at 24 hours (Figure 4.3C). In both cases, ANOVA showed a significant effect of time on the number of transmigrated neutrophils ($P<0.001$). At 24 hours, all mRPMNs were collected, centrifuged and checked for their viability. With Trypan Blue exclusion, around 90% of mRPMNs were alive from both cultures. The same percentage of 24-hour control cells was viable.

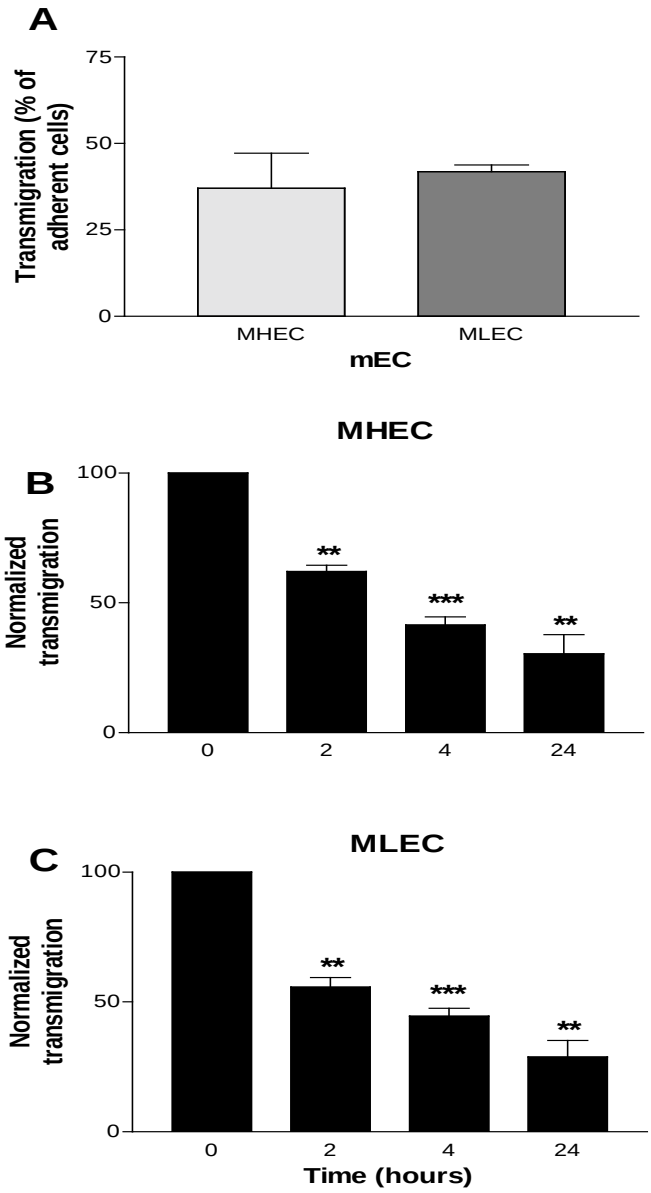


Figure 4.3 Transmigration on IL-1β stimulated MHECs and MLECs
$2x10^6$ neutrophils were added to MHEC or MLEC monolayers previously stimulated with 2.5 ng/ml IL-1β for 4 hours. After 1 hour co-culture, the number of transmigrated neutrophils at 0, 2, 4 and 24 hours was determined under phase contrast microscopy. Transmigration at 2, 4 and 24 hours was expressed as a percentage of the number of transmigrated neutrophils at 0 hours. (A) Almost equal levels of transmigration across MHECs and MLECs were observed. The number of transmigrated neutrophils decreased over time in MHEC and MLEC monolayers (B and C respectively). ANOVA showed a very significant correlation between transmigration and time in both cases (P<0.001). **, P<0.01, ***, P<0.001, by Bonferonni's Multiple comparison test. All data are the mean+SEM of four experiments.

4.3.4 Measurement of apoptosis in RPMNs and 24-hour control neutrophils

After defining the percentage of dead cells, we examined whether the intact cells from each group were undergoing apoptosis. Apoptosis was assessed by nuclear morphology observation and staining with DiOC6 and JC-1.

Cytospin samples from each group, stained with Diff Quick, were observed under light microscopy and cells were categorised in non-apoptotic and apoptotic, based on the presence of a condensed nucleus (Figure 4.4A and B). Less than 1% of neutrophils were found apoptotic in fresh samples. This percentage was 3.5±0.6% in RPMNs and almost doubled in the 24-hour control population (Figure 4.4C). ANOVA showed an effect of different conditions ($P<0.05$) on the percentage of apoptosis between the three groups. Results are shown from 5 experiments. Interestingly, mRPMNs had a typical 'human' polymorphonuclear appearance rather than the murine ring shaped nucleus.

Cells from each group were incubated with DiOC6 and the reduction in their mitochondrial potential was measured by flow cytometry. DiOC6 uptake expressed as median fluorescence intensity was reduced by 10.9±18.7% in RPMNs and 38.4±18.7% in the 24-hour control population, compared with fresh neutrophils (Figure 4.5A, B and C). Again, ANOVA showed an effect of different conditions ($P<0.05$) on DiOC6 uptake between the three groups.

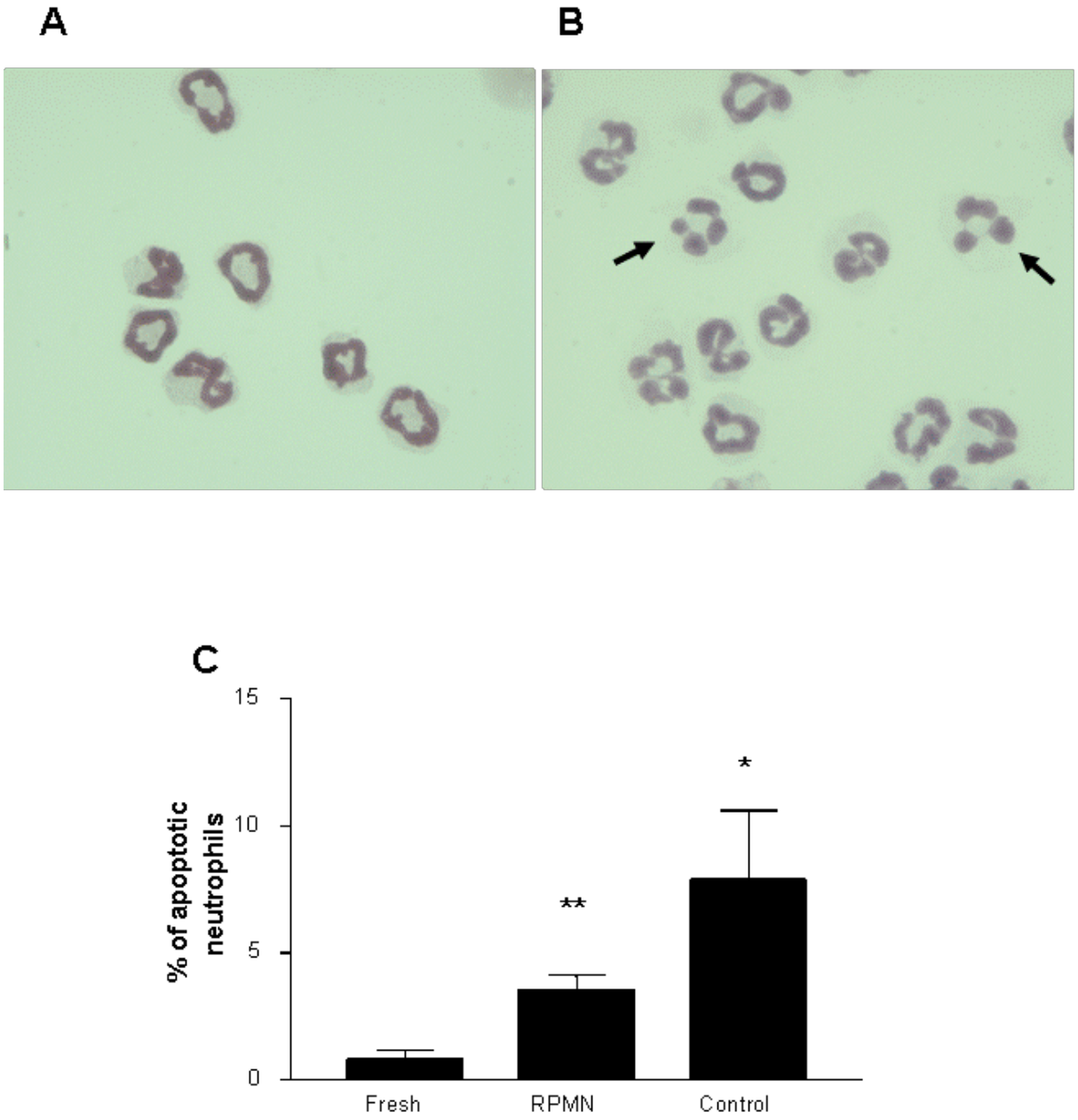


Figure 4.4 Assessment of apoptosis by observation of nuclear morphology
Cytospin samples from fresh (A) and RPMNs (B), were observed under light microscopy and cells were determined as non-apoptotic and apoptotic (with black arrows), based in the presence of a condensed nucleus. (C) Percentage of apoptotic neutrophils in fresh, RPMNs and 24h control neutrophils. RPMNs exhibited a slight increase in apoptotic cells numbers, compared to the 24-hour control population. ANOVA showed an effect of different conditions on neutrophil apoptosis ($P<0.05$). *, $P<0.05$, **, $P<0.01$, at control and RPMNs compared to fresh, respectively, by students't-test. All data are the mean+SEM of five experiments.

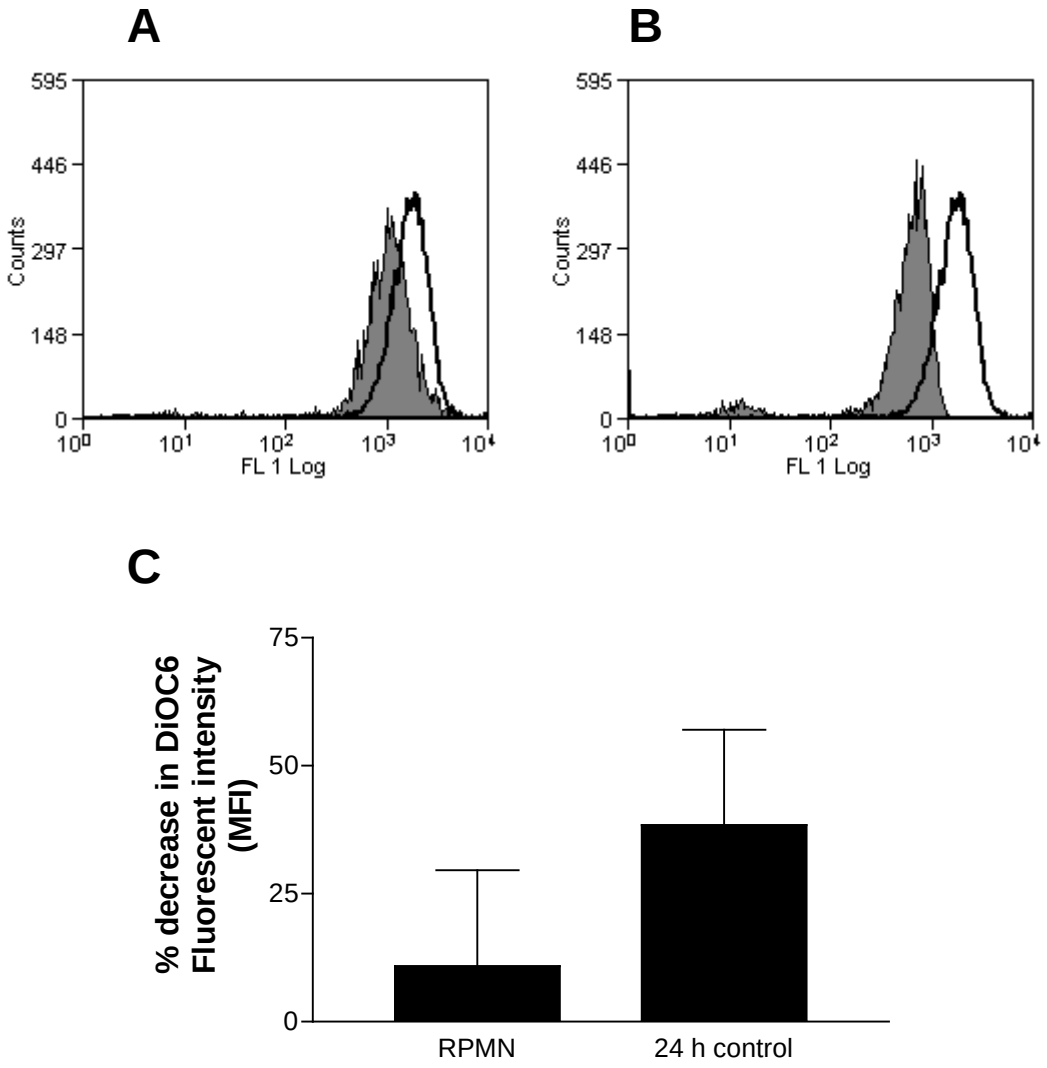


Figure 4.5 Apoptosis assessment by measurement of reduction in DiOC6 uptake
Cells from each group were incubated with DiOC6 (40nM) for 30 minutes at 37^{o}C and the reduction in their mitochondrial potential was measured by flow cytometry. (A and B) Each histogram represents the DiOC6 uptake reduction in RPMNs (A) and 24 h control neutrophils (B), compared with the fresh. RPMNs and control cells uptake in grey compared with that of fresh in white. (C) MFI decrease was higher in control cells than in RPMNs, compared to fresh neutrophils. ANOVA showed an effect of different conditions on DiOC6 uptake (P<0.05). All data are the mean±SEM of three experiments.

Finally, the cells were stained with JC-1 for 15 minutes at 37^{o}C, 5% CO_2. Fluorescent red aggregates started to decrease in numbers in RPMNs and control cells, compared to fresh (Figure 4.6A, B and C). Low fluorescent red aggregates were around 2.5% in fresh, 4.5% in RPMNs and 13% in the control population (Figure 4.7). Despite the trend, the results were not statistically significant.

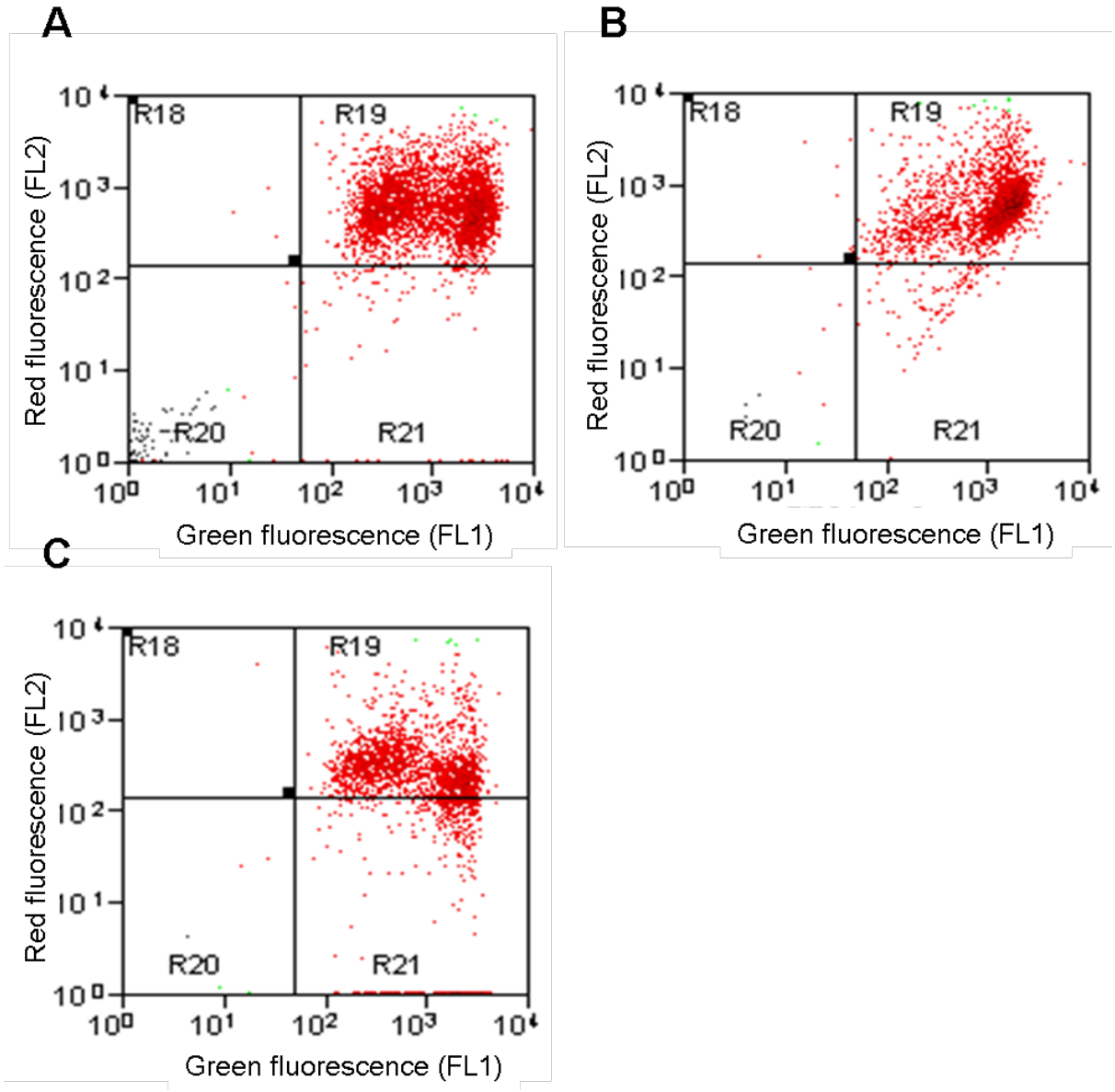


Figure 4.6 Measurement of the reduction of red fluorescent JC-1 aggregates in neutrophils

Red fluorescence/green fluorescence plots of fresh (A), RPMNs (B) and 24-hour control (C) neutrophils stained with JC-1 for 15 minutes at 37^{o}C, 5% CO_2. The decrease in FL2/FL1 ratio is indicative of early apoptosis. Results shown are representative of three experiments.

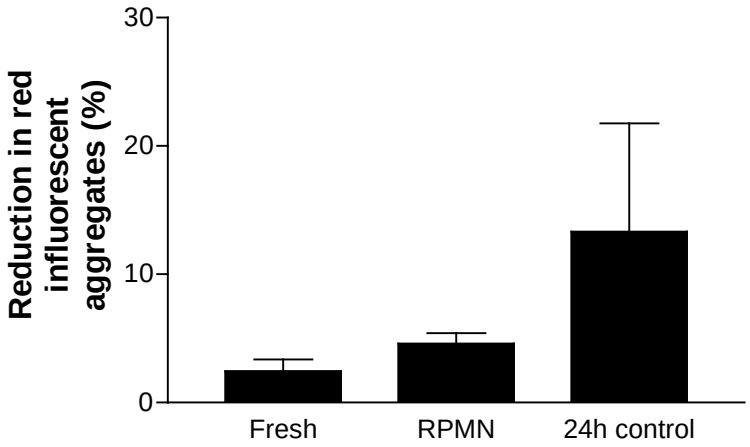


Figure 4.7 Assessment of neutrophil apoptosis by measurement of the reduction of red fluorescent JC-1 aggregates

Fresh, RPMNs and 24-hour control neutrophils were stained with JC-1 for 15 minutes at 37^{o}C, 5% CO_2. Low fluorescent red aggregates were around 2.5% in fresh, 4.5% in RPMNs and 13% in control population. No statistical significance was found between the different condition and red fluorescent JC-1 aggregates reduction. All data are the mean+SEM of three experiments.

4.3.5 Characterization of mRPMNs by flow cytometry

As with human RPMNs we hypothesised that mRPMNs would have a different phenotype from fresh neutrophils or the 24-hour control population. As a first step, we assessed the surface expression of CXCR2 and ICAM-1 on mRPMNs and compared these to fresh and 24h plastic cultured control neutrophils. As shown in Table 4.2 and Figure 4.4, CXCR2 expression almost doubled in mRPMNs (13.9±3.7, compared to 7.7 ± 2.3 in fresh), while it slightly increased in the control population (7.9±1.5). Interestingly, ICAM-1 expression significantly increased in mRPMNs (more than 20 fold), compared to fresh and control cells. In addition, the percentage of cells expressing ICAM-1 markedly increased, from 50 ± 0.8% in fresh to 83.6±11.3% in reverse migrated neutrophils. ANOVA showed an effect of different conditions to ICAM-1 expression, both in MFI and percentage of positive cells ($P<0.05$).

Table 4.2 Surface expression of CXCR2 and ICAM-1 in fresh, reverse migrated and 24-hour control neutrophils

	FRESH		**REVERSE MIGRATED**		**24 H CONTROL**	
	MFI	**%Histogram**	**MFI**	**%Histogram**	**MFI**	**%Histogram**
CXCR2	7.7 ± 2.3	53.1 ±4.1	13.9±3.7	59.4±8.3	7.9±1.5	52.4±4.1
ICAM-1	3.5±0.003	50 ± 0.8	83.3±28.8	83.6±11.3	4.1±1.3	48.9±7.3

$P<0.05$, by ANOVA in ICAM-1 expression. All data are the mean+SEM of three experiments.

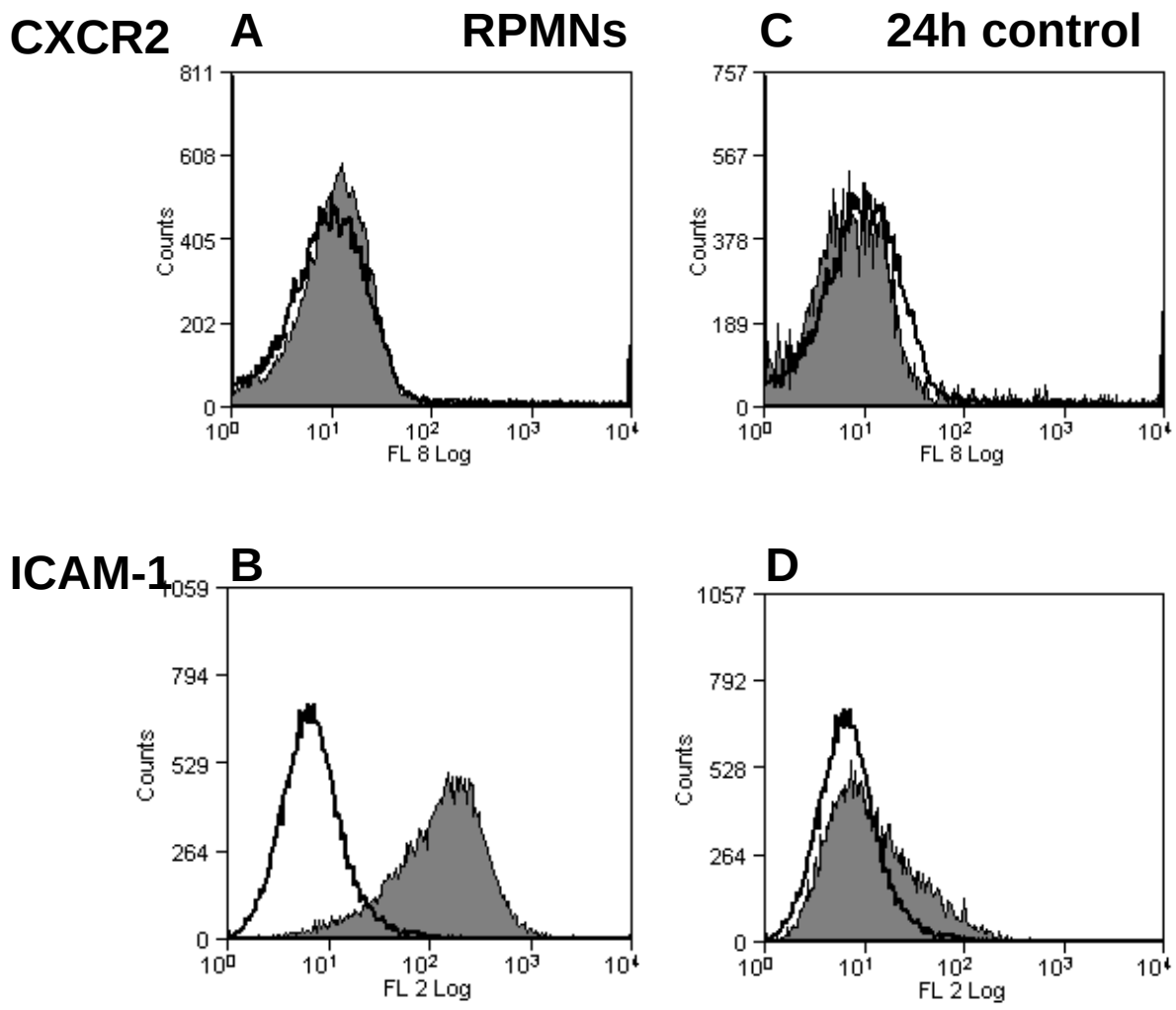


Figure 4.8 Surface expression of CXCR2 (A, C) and ICAM-1 on RPMNs and 24-hour control neutrophils compared to fresh

The expression of CXCR2 of RPMNs (A) and control (C) was quantified by flow cytometry. Similarly for ICAM-1 in RPMNs (B) and control (D). Each histogram represents the expression of the specific marker (in grey) compared to fresh (in white). ICAM-1 expression was significantly increased in RPMNs. Results shown are representative of three experiments.

4.3.6 Adoptive transfer experiments

In an attempt to examine the fate of reverse migrated neutrophils *in vivo*, EYFP RPMNs were injected into WT mice. These were sacrificed after 4 or 24 hours. Blood and single cell suspensions from bone marrow, spleen, liver and lungs were analysed for the presence of any EYFP cells, by flow cytometry. Table 4.3 shows the MFI and % positive cells in injected mice, compared to control WT mice. In most cases, less than 2% had an increased MFI compared to the control. But the MFI of these cells did not correspond to the EYFP fluorescent intensity (MFI: 100-700). However, a very small number of cells in the EYFP fluorescent intensity range borderline was detected in the bone marrow 4 hours post-injection (Figure 4.9).

Table 4.3 Median fluorescence intensities and % of histogram in WT and injected mice 4 and 24 hours post injection with EYFP RPMNs

4 HOURS				**24 HOURS**			
WILD TYPE		**1x10^6 INJECTED**		**WILD TYPE**		**1x10^6 INJECTED**	
MFI	**%**	**MFI**	**%**	**MFI**	**%**	**MFI**	**%**
BLOOD							
2.90±0.52	1.07±1.03	2.60±0.82	0.70±0.67	3.81	2.2	2.85	0.08
BONE MARROW							
2.96	0.81±0.01	3.18±0.12	0.95±0.05	4.09	1.38	3.95	1.77
SPLEEN							
3.15±0.40	0.56±0.30	3.02±0.27	1.40±0.80	4.24	0.88	4.24	0.51
LIVER							
5.77±2.40	1.57±0.21	5.13±3.00	1.17±0.62	7.84	4.83	6.54	1.51
LUNG							
2.96±0.58	0.51±0.08	2.99±0.43	0.81±0.58	3.29	0.18	4.09	0.27

Results are the means±SEM from two experiments in the 4hour post-injection group. One 24-hour post-injection experiment was performed.

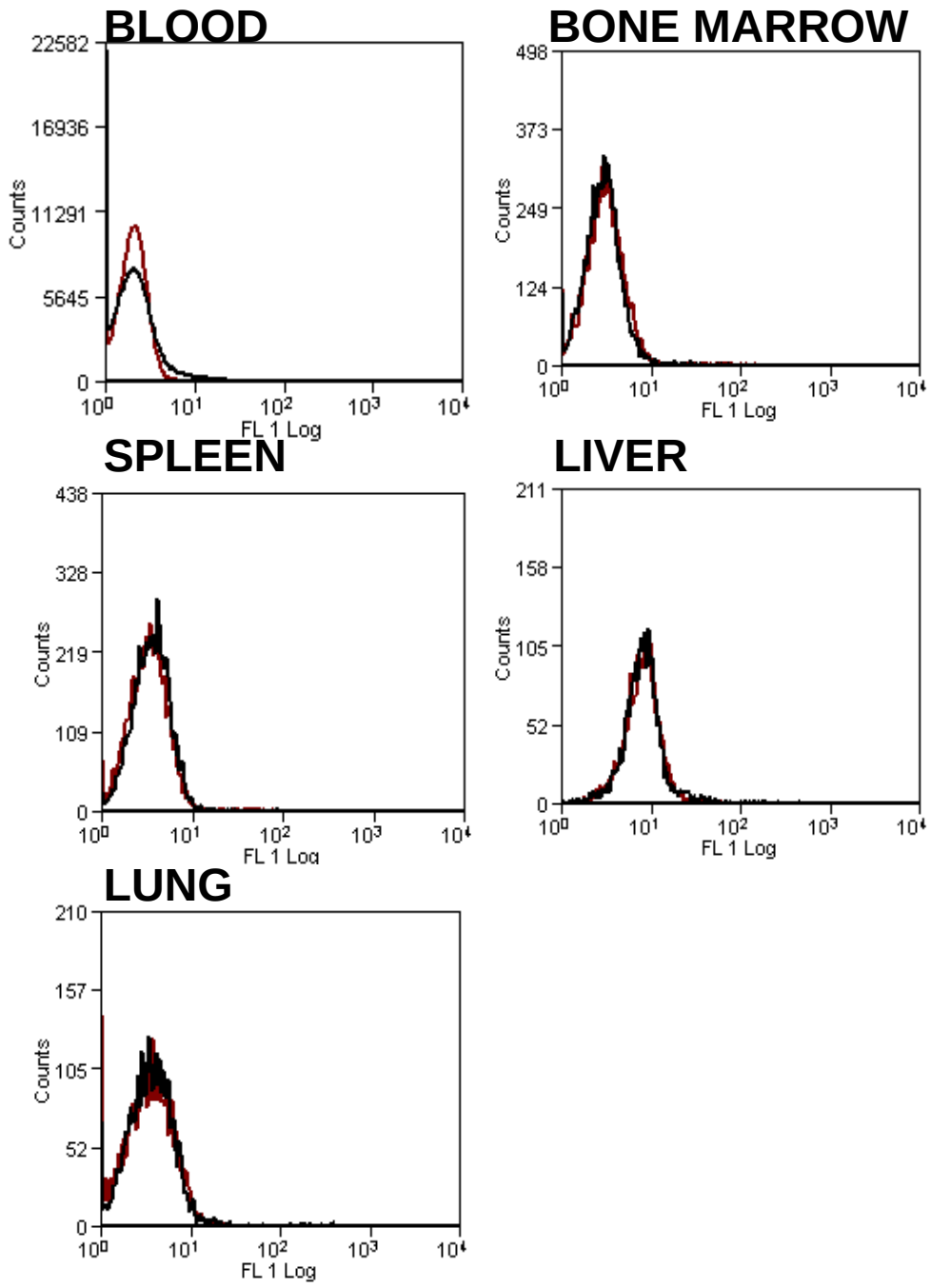


Figure 4.9 Detection of EYFP RPMNs in blood, bone marrow, spleen, liver and lungs, 4 hours post injection

1x 10^6 EYFP RPMNs were injected into WT mice Histograms represent the presence of any EYFP RPMNs in blood, bone marrow, spleen, liver and lungs, 4 hours post injection. Contours in black and brown represent the single cell suspensions from WT control and injected mice organs respectively. Results shown are representative of two experiments.

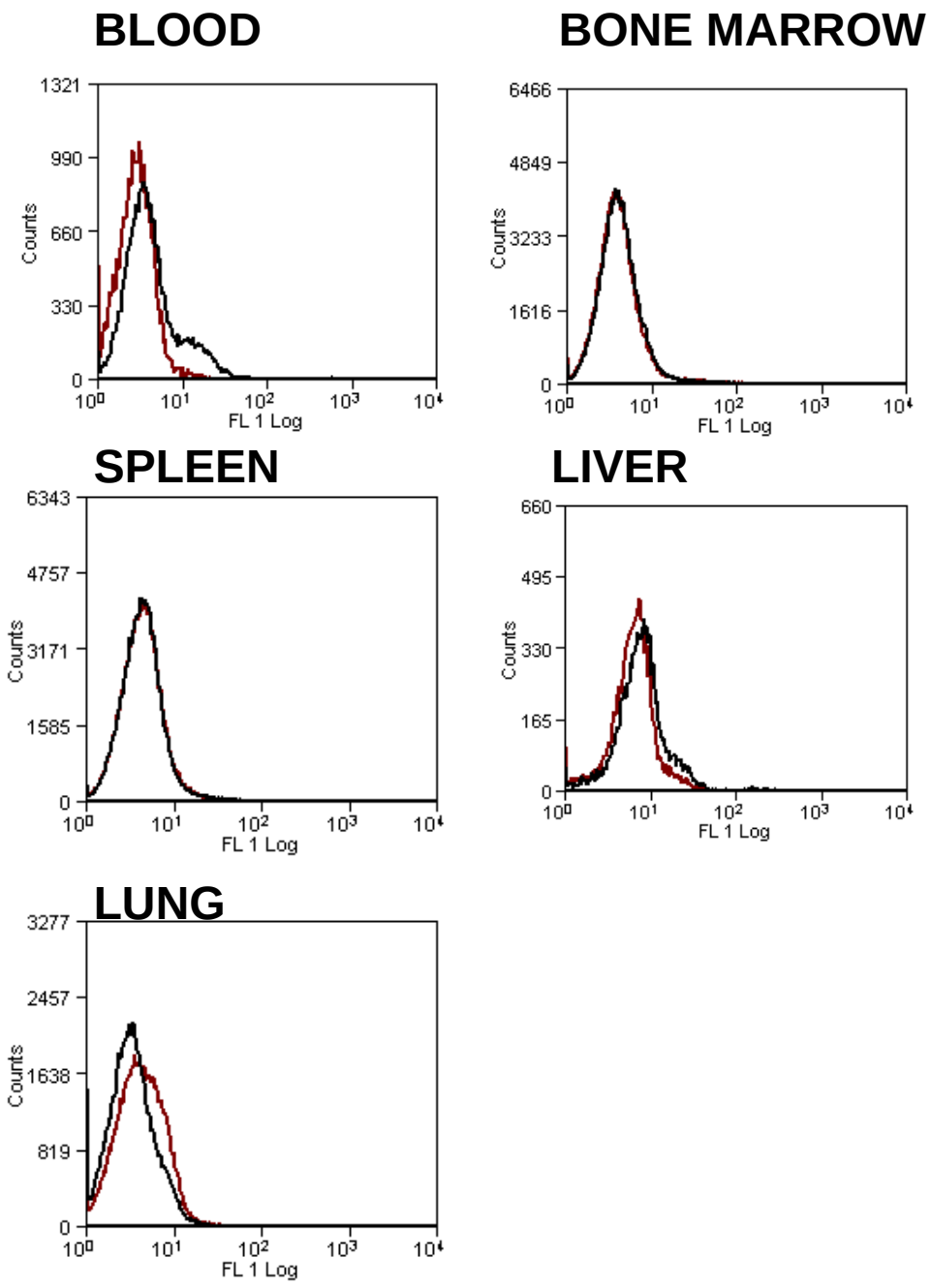


Figure 4.10 Detection of EYFP RPMNs in blood, bone marrow, spleen, liver and lungs, 24 hours post injection

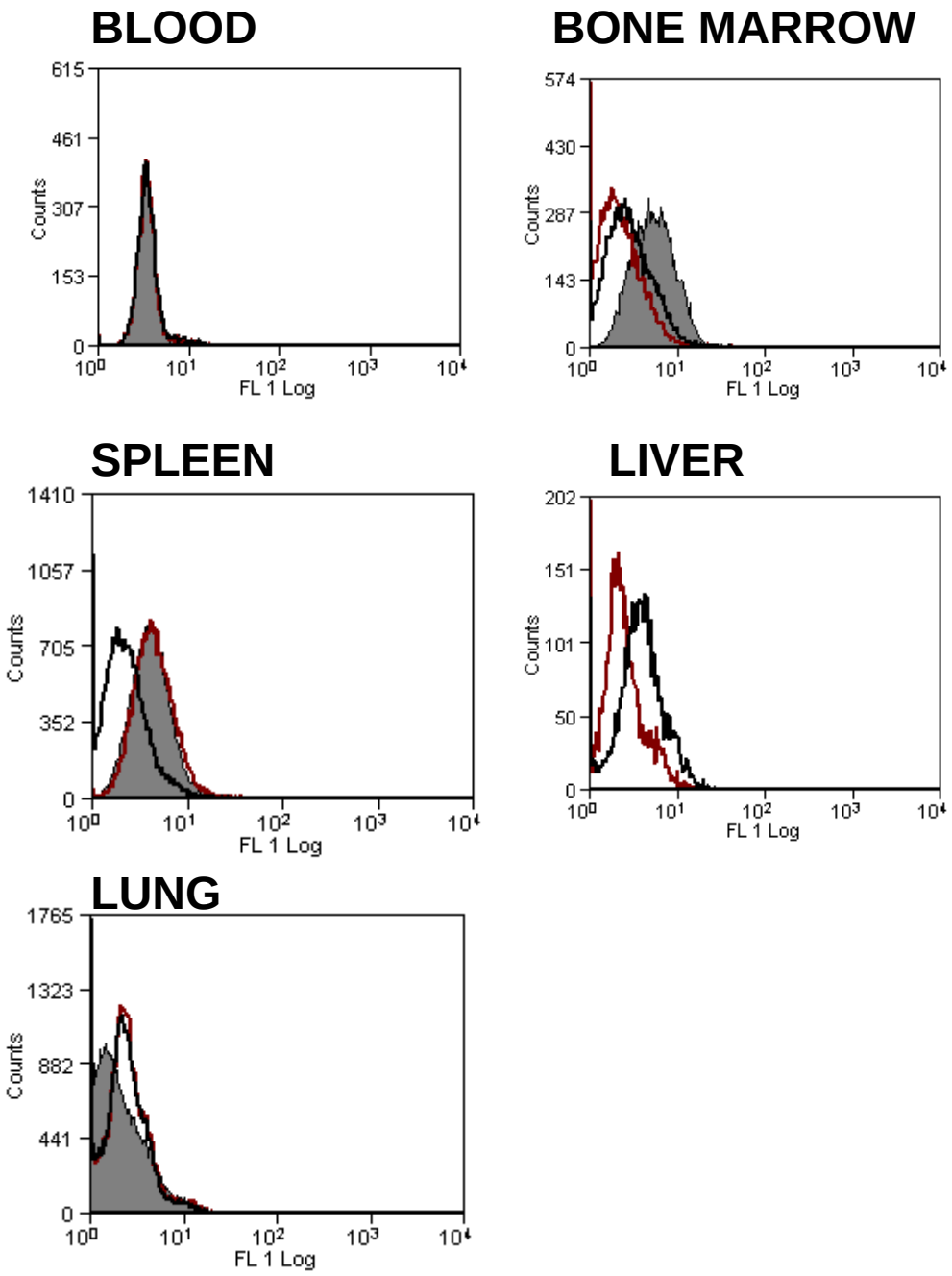
Histograms represent the presence of any EYFP RPMNs in blood, bone marrow, spleen, liver and lungs 24 hours post injection. Contours in black and brown represent the single cell suspensions from WT control and injected mice organs respectively. Results shown are from one experiment.

In order to examine whether those findings were due to the technical limitations of the methodology we used, WT mice were injected with freshly isolated EYFP neutrophils (at 1 and $3x10^6$ cells), sacrificed after 4 hours and analysed as previously described. Table 4.4 shows the MFI and % cells with higher MFI than the WT control, from 1 experiment. In bone marrow and spleen there was an increase in positively staining cells (22.49 and 8.22% respectively). It is difficult to draw firm conclusions in regard of the identity of these cells as we found that fixation dramatically altered the fluorescent profile of EYFP cells. However, the distribution of the histograms is highly suggestive that at 4h post transfer, RPMN are localised in the liver, spleen and lung.

Table 4.4 Median fluorescence intensities and % of histogram in WT and injected mice with 1 and 3x 10^6 fresh EYFP neutrophils

WILD TYPE		**$1x10^6$ INJECTED**		**$3x10^6$ INJECTED**	
MFI	**%**	**MFI**	**%**	**MFI**	**%**
BLOOD					
3.41	3.33	3.41	3.07	3.41	3.34
BONE MARROW					
2.56	5.84	1.99	2.53	5.08	22.49
SPLEEN					
2.14	2.46	4.4	11.44	4.09	8.22
LIVER					
3.81	11.24	2.21	2.32		
LUNG					
2.14	2.51	2.21	3.17	1.72	3.04

Results are from one experiment.



4.11 Detection of fresh EYFP neutrophils in blood, bone marrow, spleen, liver and lungs, 24 hours post injection

Histograms represent the presence of any EYFP fresh neutrophils in blood, bone marrow, spleen, liver and lungs, 4 hours post injection. Contours in grey represent the single cell suspensions from WT control mouse, while contour in black and brown line represent the single cell suspensions from mice injected with $1x10^6$ and $3x10^6$ EYFP neutrophils respectively. Results shown are from one experiment.

4.4. Discussion

In this chapter, we studied the reverse migration of murine bone marrow neutrophils *in vitro*. Murine RPMNs were generated in static co-cultures with murine ECs and characterized *in vitro*, based on CXCR2 and ICAM-1 surface expression. Their viability and apoptosis levels were also assessed. At a later stage, we performed a small number of preliminary adoptive transfer experiments, in an attempt to investigate their fate *in vivo*.

First, static assays on cytokine stimulated MCEC-1 or primary mECs were conducted. Around 33% and 40% of cells added in MCEC-1 or primary mEC monolayers respectively had transmigrated in the absence of any wall shear stress. Compared with our flow-based studies, these data indicate that flow is not essential for transmigration of neutrophils in this system, but it does increase the efficiency of transmigration. Thus, as described in chapter 3 (section 3.3.4 and 3.3.5), most neutrophils had transmigrated by 15-20 minutes, where as here less than 50% had migrated after 1h. This slower rate of neutrophil migration under static regimens has been previously reported in assays performed on TNF-α stimulated human ECs (Butler *et al*., 2005; Kitayama *et al*., 2000).

In all cases, the number of transmigrated neutrophils decreased over time in the 24-hour co-cultures. Simultaneously, the number of phase bright neutrophils followed exactly the opposite trend. In other words, these had reverse migrated across a mEC monolayer in 24 hours *in vitro*, a process very similar to that described for human neutrophils by Buckley *et al*.,(2006). We wanted to verify that these cells were rescued from apoptosis after being in contact with endothelial cells as other studies have suggested (Buckley *et al*., 2006; McGettrick *et al*., 2006). Therefore, viability and apoptosis levels were measured in

mRPMNs, compared with fresh and 24-hour culture control neutrophils. 90% of mRPMNs were alive, as determined by Trypan Blue exclusion. Assessment of nuclear morphology revealed that 3.5% of these cells were apoptotic. Incubation with DiOC6 showed that 11% of mRPMNs exhibited a reduction in their mitochondrial membrane potential. Interestingly, incubation with JC-1 demonstrated that 4.5% of the cells were apoptotic. The levels of apoptosis vary between assays as they report on different stages of the process. For example, nuclear morphology reports on the late stages of apoptosis, while changes in the mitochondrial membrane potential report on early changes in the process of apoptosis. However, differences in the apoptosis levels were found between DiOC6 and JC-1 staining. DiOC6 uptake from the cells depends on the state of mitochondria. If mitochondria are depolarized, DiOC6 could be distributed to other intracellular membranes, such as endoplasmic reticulum, introducing small changes in the total fluorescence intensity of the cells. That could make DiOC6 a less reliable probe than JC-1 for $\Delta\Psi$ changes assessment in intact cells (Salvioli *et al*., 1997). Overall, the above findings demonstrate that mRPMNs show a delay in apoptosis when compared to cells aged on plastic, as previously shown with human RPMNs (Buckley *et al*., 2006).

Another interesting finding was the fact that neutrophil survival is affected by culture medium and more importantly by the presence of various cytokines in it. When murine neutrophils were cultured in MCEC-1 medium in the presence of TNF-α and IL-1β, slightly more than 50% were alive after 24 hours. In the presence of TNF-α alone 30% were alive. Interestingly, neutrophil viability was higher with IL-1β than the combination. These findings suggest that TNF-α induces apoptosis in murine neutrophils. The role of TNF-α in neutrophil apoptosis remains unclear. There are a number of studies indicating that TNF-α delays

apoptosis of human neutrophils (Kilpatrick *et al*., 2006; Lindemans *et al*., 2006; van den Berg *et al*., 2001;Walmsley *et al*., 2004). However, other studies have shown an opposite effect (Maianski *et al*., 2003; Murray *et al*., 1997; Salamone *et al*., 2001). It has been proposed that the effect of TNF-α on apoptosis depends on the culture conditions and cytokine concentration (Salamone *et al*., 2001; van den Berg *et al*., 2001). IL-1β is thought to delay apoptosis (Colotta *et al*., 1992). However, in later experiments, where neutrophils were cultured in complete mEC medium without any cytokines we found around 90% of neutrophils were viable after 24 hours. This strongly implies that in our assays, TNF-α is pro-apoptotic to murine neutrophils, while IL-1β did not have the same effect.

Another important aspect of this work was the characterization of mRPMNs, as human neutrophils that have twice transmigrated across an EC monolayer in opposite directions are known to exhibit an altered phenotype compared to fresh or plastic cultured neutrophils. Here we examined the surface expression of cytokine receptor CXCR2 and the adhesion molecule ICAM-1, based on the markers used in previous studies on human RPMNs (Buckley *et al*., 2006). We could not verify the down-regulation of CXCR2 in mRPMNs which would have mirrored the process in human RPMNs, however, levels of CXCR2 were at the limit of detection in our system and it is thus difficult to draw firm conclusions in regard of this marker. In contrast, ICAM-1 expression was increased 20 fold in mRPMNs compared to fresh. ICAM-1 is a surface glycoprotein that is a member of the immunoglobulin superfamily (Springer, 1990). During the process of reverse migration, ICAM-1 production is induced, leading to this marked increase in their surface expression. The change in ICAM-1 expression in murine RPMNs is dramatically greater than that seen in human equivalents. ICAM-1 up-regulation in these cells has been suggested to facilitate their engagement with

the macrophages of the liver and spleen, and therefore permit their removal from the circulation (Buckley *et al*., 2006). ICAM-1 is involved in the firm adhesion of neutrophils on EC. Ligands for ICAM-1 are the β_2 integrins (LFA-1, Mac-1) (Kishimoto *et al*., 1989; Springer, 1990). It is possible that ICAM-1 up-regulation on the surface of RPMNs may result in their removal from the circulation, especially from sites at various organs that express β_2 integrins.

Finally, adoptive transfer experiments were performed in order to examine the fate of RPMNs *in vivo*. Our preliminary data did not give a clear indication about where RPMNs have migrated to. Cells in the lower MFI borderline of EYFP cells were detected in the bone marrow 4 hours post injection. This could be attributed to the high auto-fluorescence of the cells in that particular tissue. The signals were so low it was hard to draw firm conclusions. However, if we were measuring cells in the bone marrow it would agree with the theory that bone marrow is a site of neutrophil clearance (Rankin, 2010; Suratt *et al*., 2001). However, we suspected that the assay used here was not sensitive enough when using $1x10^6$ adoptively transferred cells. When $3x10^6$ cells were injected (in a single experiment), cell suspensions from spleen, liver and lung exhibited distinct peaks, indicating the presence of RPMNs. However, it would not be technically feasible to use concentrations of RPMNs above $1x10^6$ per mouse, as this would represent an unfeasible effort in the production of cells for transfer.

Still, our findings in a single experiment using higher numbers of transferred cells indicate that RPMNs are rapidly recognised and cleared from the circulation. Investigation of the fate of mRPMNs needs to be performed using more sensitive *in vivo* imaging techniques such as IVIS whole animal imaging facility, now available in Birmingham. This assay is not

invasive and allows the observation of neutrophil trafficking into all the organs of the mouse at different time points. Moreover, it is probable that the increased sensitivity of this system would allow lower numbers of mRPMNs to be used. For the above reasons, this adoptive transfer methodology will be pursued in the future using this alternative technology.

CHAPTER 5

THE EFFECT OF SHEAR STRESS AND NITRIC OXIDE ON NEUTROPHIL RECRUITMENT AND REVERSE MIGRATION

5.1 Introduction

There is a large variation in the phenotype and properties of endothelial cells throughout the vascular tree and in different organs. These differences are seen in EC morphology, permeability, secretory potential and gene expression patterns (Aird, 2003; Aird, 2007; Chi *et al*., 2003). Variation can also be seen in the ability of ECs to recruit leukocytes. For example, under physiological conditions, ECs lining the high endothelial venules (HEV) of secondary lymphoid tissue have the ability to constantly recruit circulating lymphocytes. In contrast, ECs in the post-capillary venules near a site of inflammation only recruit leukocytes to sites of inflammation after being activated, by inflammatory mediators such as cytokines (Nash *et al*., 2004).

There is increasing evidence that EC phenotype is conditioned by the haemodynamic environment, such that shear forces acting upon EC can convey important regulatory signals important for determining phenotype. The (bio)chemical and cellular environment also appears to play a critical role in this process (Nash *et al*., 2004). Thus, the physicochemical environment can affect the sensitivity of ECs to inflammatory cytokines and therefore their ability to recruit leukocytes (Nash *et al*., 2004). For example, it has been demonstrated that leukocyte recruitment by HUVEC in response to TNF-α, is inversely related to wall shear stress at which they had been conditioned for 24h (Sheikh *et al*., 2003; Sheikh *et al*., 2005) . Leukocyte recruitment occurs at sites with low wall shear stress (Atherton and Born, 1972). Leukocytes are recruited to ECs lining the post-capillary venules, but not to those in their adjacent arterioles, where the wall shear stress is higher. In addition, leukocyte recruitment may take place in sites through the vascular tree, where the flow has been disturbed (Caro *et al*., 1971). It appears that disturbance in flow may facilitate leukocyte recruitment or increase

ECs sensitivity to cytokines (Nash *et al*., 2004). Thus, ECs covering atheromatous plaques, which occur at areas of low average shear stress, express P-selectin and VCAM-1 in contrast to adjacent vessel segments exposed to high shear laminar flow (Davies *et al*., 1993; Johnson-Tidey *et al*., 1994). In addition, signalling responses and activation induced by TNF-α are modified in ECs previously cultured under flow for short or prolonged periods (Surapisitchat *et al*., 2001). In our own group Sheikh *et al* showed a graded loss of response of EC to TNF-α when the cells were activated after conditioning for 24 hours at increasing wall shear stress (Sheikh *et al*., 2004; Sheikh *et al*., 2003). In contrast, under static conditions there was abundant adhesion and migration of flowing neutrophils. At intermediate shear stress (0.3Pa), levels of adhesion were unaffected but migration was significantly less efficient. At high shear stress (2.0 Pa) neutrophils were not even recruited from flow. These authors showed that loss of IL-8 and E-selectin gene expression was responsible for the reduction in neutrophil recruitment.

It is also worthy to note that ECs also respond rapidly to changes in wall shear stress by regulating a series of genes (Chen *et al*., 2001; McCormick *et al*., 2001; Nagel *et al*., 1994; Passerini *et al*., 2003; Sampath *et al*., 1995; Walpola *et al*., 1995). It has been demonstrated that shear stress activates a variety of regulatory molecules, such as the membrane K^+ channel (Olesen *et al*., 1988), G proteins (Gudi *et al*., 1996), intracellular Ca^{2+} (Ando *et al*., 1988), cGMP (Ohno *et al*., 1993), mitogen-activated protein kinases (MAPKs, such as ERKs and JNKs) (Li *et al*., 1996; Tseng *et al*., 1995), small GTPases (Ras) (Li *et al*., 1996) and focal adhesion proteins (FAKs) (Li *et al*., 1997; Li *et al*., 1996). After the trigger of MAPK and FAK signaling cascades, a number of transcription factors such as AP-1, NF-κB（Davis *et al*., 2004; Lan *et al*., 1994）, Sp-1, and Egr-1 (Khachigian *et al*., 1997) are activated. These factors

induce transcription genes involved in the growth, adhesion, vasoactivation and monocyte attraction in endothelial cells (Chien *et al*., 1998). Shear stress is also believed to be one of the most potent inducers of nitric oxide (NO) production (Li *et al*., 2004).

NO is a biologically active compound with many functions, such as vasodilation through vascular smooth muscle relaxation, inhibition of platelet aggregation, neurotransmission and immune regulation (Ignarro *et al*., 1987). NO activates guanylate cyclase generating cyclic guanosine monophosphate (cGMP) that acts as an intracellular second messenger (Murad, 1999). Endogenous NO is produced during the generation of L-citruline from L-arginine. This reaction is catalysed by nitric oxide synthases (NOS). There are three mammalian NOS isoforms: the neuronal (nNOS or NOS-I), inducible (iNOS or NOS-II) and the endothelial (eNOS or NOS-III). nNOS and eNOS are constitutively expressed in healthy tissue, Ca^{2+} dependent and produce relatively small amounts of NO. In contrast, iNOS is inducible in a variety of cell types (NK cells, DCs, mast cells, monocytes, macrophages, eosinophils and neutrophils) upon inflammation and Ca^{2+} independent (Alderton *et al*., 2001). Inducible NOS in activated macrophages produces relatively large amounts of NO that acts as a cytotoxic agent against microorganisms and tumor cells (Bogdan *et al*., 2000; Nathan and Shiloh, 2000; Pervin *et al*., 2001; Stuehr and Nathan, 1989; Xie *et al*., 1996). There is also a direct link between NOS impairment and endothelial dysfunction in cardiovascular diseases such as atherosclerosis. Under oxidative stress, eNOS enzymology changes by producing more peroxynitrite (ONOO-) and finally O_2^- contributing further to endothelial dysfunction (Beckman *et al*., 1990; Ischiropoulos and Al-Mehdi, 1995; Wever *et al*., 1998).

One of NO major functions is the regulation of interactions of circulating leukocytes and platelets with the vessel wall. There is evidence that NO may effect the leukocyte ability to adhere to microvascular endothelium (Kubes *et al*., 1991; Tsao *et al*., 1996). Endothelial NOS inhibitors, such as L-NAME, were able to increase neutrophil adhesion 15 times (Kubes *et al*., 1991). In mesenteric post-capillary venules of mice lacking eNOS or nNOS, the levels of rolling and overall adherent leukocytes significantly increased, even in the absence of any inflammatory stimulus. Interestingly, in an acetic acid induced model of acute colitis, iNOS deficient mice exhibited increased neutrophil infiltration and impaired ability to resolve inflammation (McCafferty *et al*., 1997). Similar observations were made after LPS administration in cremasteric postcapillary and liver post sinusoidal venules, as well in the lungs, of iNOS deficient mice. These data indicate that, in the mouse at least, iNOS–produced NO may play a homeostatic regulatory role in inflammation (Hickey *et al*., 2001). NO has been shown to inhibit platelet aggregation and secretion *in vitro* (Stamler *et al.*, 1989). When platelets were treated with L-NAME, platelet aggregation increased (Freedman *et al*., 1997) . It has also been shown that NO could inhibit platelet adhesion to endothelial monolayers (Radomski et al., 1987). In some experiments with L-NAME treated ECs, there was a marked increase in platelet adhesion on them. In contrast, when exogenous NO was added to the cultures under flow this was inhibited (de Graaf *et al*., 1992). This NO-induced inhibition of platelet activation is a result of the subsequent increase of cyclic nucleotides cAMP levels (Radomski *et al*., 1987; Wang *et al*., 1998).

The above effects of NO on leukocyte recruitment can be associated with shear stress, as the latter induces the synthesis of the former. The role of shear stress in NO production has been extensively investigated. *In vitro* studies, using flow channels and stretch apparatus have

demonstrated that ECs under shear stress or mechanical strain respond by increasing their eNOS mRNA levels and NO production (Kuchan *et al*., 1994; Ranjan *et al*., 1995; Uematsu *et al*., 1995). In another study it was found that laminar flow could induce NO synthesis by HUVECs, which was dependent on shear stress levels, yet turbulent flow did not have the same effect (Noris *et al*., 1995). It has been demonstrated that, at a molecular level, laminar shear stress increases NF-κB subunits p50/p65 binding to shear stress response element (SSRE) on eNOS promoter, and subsequently the transcription of the enzyme (Davis *et al*., 2004). In the same study, it was shown that mutation of the SSRE dramatically impaired eNOS promoter activity, otherwise induced by shear stress.

The molecular mechanisms supporting the reverse migration of neutrophils are unknown. In this chapter, we examined the effect of shear stress on the ability of EC monolayers to retain transmigrated neutrophils. As shear stress induces the production of NO production (Kuchan *et al*., 1994; Ranjan *et al*., 1995; Uematsu *et al*., 1995) and NO has been shown to regulate neutrophil recruitment (Hickey *et al*., 2001; Kubes *et al*., 1991; Lefer *et al*., 1999), we addressed the question whether this shear stress effect on reverse migration was mediated via NO production. We used a NO donor, diethylamine NONOate (DEANO) and a NOS inhibitor (L-NAME) to study the effect of NO in neutrophil recruitment and reverse migration under the absence of shear stress.

5.2 Methods

5.2.1 Flow based adhesion assays

Prolonged duration flow assays (up to 45 minutes) were performed. Three microslides were seeded with HUVEC. Upon confluence, monolayers in two of them were stimulated with TNF-α (100U/ml) and IL-1β (0.5ng/ml) for 4 hours, while the other was untreated to serve as control. In the meantime, human neutrophils were isolated from whole blood, as previously described in chapter 2 and resuspended at a final concentration of $2x10^6$ cells/ml. After 4 hours, the microslides were incorporated in a flow assay system. The shear stress was set at 0.05 Pa. HUVEC monolayers were initially perfused with PBSA and next with neutrophil suspension for 4 minutes, followed by a washout with PBSA. In one of the two microslides with cytokine stimulated HUVEC, the flow was turned off after 8 minutes, simulating static conditions from that time point onwards. Recordings were made every 10 minutes in all microslides and the total numbers of adherent and transmigrated neutrophils were determined.

5.2.2 Assessment of the effect of continuous shear stress on reverse transmigration rate

HUVEC monolayers in microslides were stimulated with 100U/ml TNF-α and 0.5ng/ml IL-1β for 4 hours. After the 4-hour incubation, all the microslides were rinsed with PBSA to remove any excess cytokines. At this time point approximately $2x10^5$ neutrophils in M199/BSA were added to each microslide. This neutrophil concentration was chosen to yield a neutrophil/EC ratio of 6:1, in accordance with concentrations used in other static assays (Butler et al., 2005). The microslides were then placed in an incubator at 37^oC for 25 minutes, sufficient time to complete transmigration. The microslides were rinsed with PBSA to remove non-adherent neutrophils. As control, one microslide was fixed with 2% paraformaldehyde

(PFA) straight after the washout. One microslide was placed in the incubator for at 37^{o}C for 1 hour, while another was attached to a glass dish in a continuous flow system (0.05 Pa) at 37^{o}C for the same time period. At the end of that time, these were washed and fixed with 2% PFA (Figure 5.1). The microslides were glued onto microscope slides, mounted onto a microscope stage and observed by phase contrast videomicroscopy. Ten random fields along the centre line of each microslide were recorded and analysed off-line. The total number of adherent cells was determined and expressed as cells/mm^2.

In some experiments the incubation time in static and under flow conditions was 2 or 4 hours. In some other experiments, monoclonal blocking antibodies against CD31, CD99, JAM-C and VE-cadherin (10μg/ml) were introduced to the co-cultures in microslides for 15 minutes prior their incorporation to a flow system (Figure 5.2).

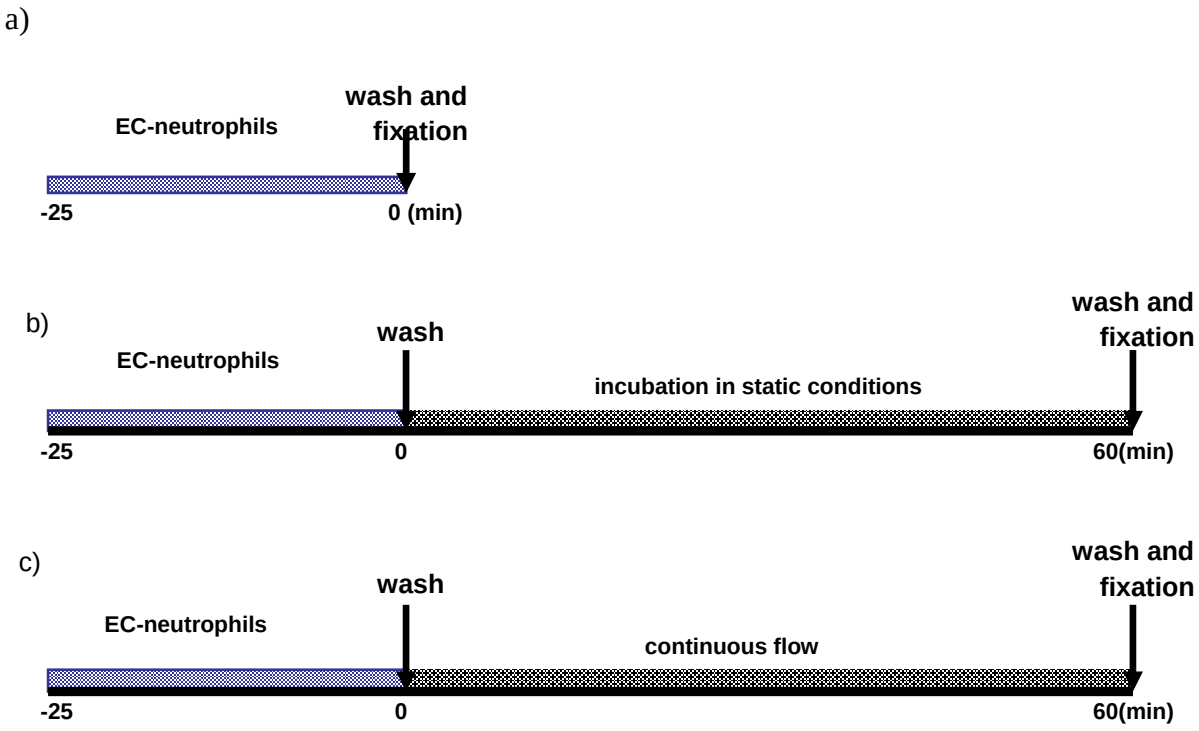


Figure 5.1 Diagram representing the different experimental conditions of the experiment

Approximately $2x10^5$ neutrophils in M199/BSA were added to each microslide, subsequently placed inside an incubator at 37^oC for 25 minutes. (a) one microslide was fixed with 2% PFA, as control, (b) a second was placed in the incubator for 1 hour at 37^oC and (c) a third was attached to a glass dish in a continuous flow system (0.05 Pa) at 37^oC for the same time period. At the end of that time, the microslides were washed and fixed with 2% PFA and the number of transmigrated neutrophils was determined.

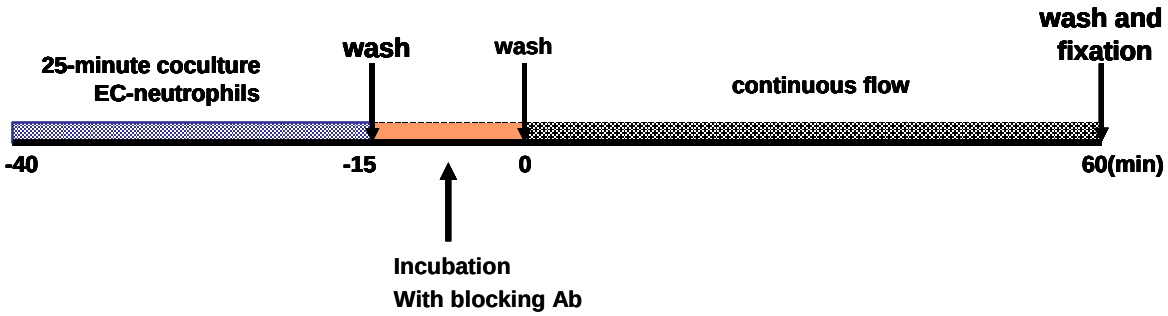


Figure 5.2 Diagram representing the experimental procedure using blocking antibodies
Approximately $2x10^5$ neutrophils in M199/BSA were added to each microslide, subsequently placed inside an incubator at 37^oC for 25 minutes. After washout the co-cultures were treated with monoclonal blocking antibodies against CD31, CD99, JAM-C or VE-cadherin (10μg/ml) for 15 minutes and then they were connected to a flow system.

5.2.3 Assessment of the effect of NO donor/inhibitor on neutrophil recruitment and reverse migration in static conditions

For neutrophil recruitment studies, HUVEC monolayers in a 12-well plate were stimulated with 100U/ml of TNF-α for 4 hours, except for one well which was used as unstimulated control. Neutrophils, after being isolated from whole blood, were resuspended in 1.5ml of M199/BSA at a final concentration of $1x10^6$ cells/ml. At the end of the 4-hour HUVEC stimulation with TNF-α, the monolayers in all wells were washed with M199/BSA to remove any residual cytokine.

A HUVEC monolayer in one well was incubated with L-NAME (100 μM) at 37^0C, for 30 minutes (neutrophils added to that well were also treated with L-NAME for the same time). Static assays were performed, adapted from Butler *et al*, 2005. Next, 1.5ml of the neutrophil suspension was added to each well. In one case, neutrophils added to one well were previously incubated with DEANO, at 37^oC, for 30 minutes.

Neutrophils in each well were allowed to settle for 5 minutes and then non-adherent neutrophils were removed by a gentle wash, with M199/BSA. Video recording of 10 random fields were taken between the 7^{th} and 9^{th} minute and between the 24^{th} and 26^{th} minute. The numbers of adherent and transmigrated neutrophils, at each time point, were determined by off-line analysis, and expressed as cells/mm^2.

For reverse migration studies, HUVEC monolayers in a 12-well plate were stimulated with 100U/ml of TNF-α for 4 hours, except for one used as untreated control. After that time, monolayers were washed with M199/BSA and 1.5ml of neutrophil suspension (1×10^6 cells

in M199/BSA) was added to each well for 30 minutes. After three washes, complete HUVEC medium was re-introduced to each well. In one well, medium was supplemented with DEANO, while another with L-NAME (both 100μM). Video recordings of 10 random fields were taken at that time point. The plate was placed inside an incubator, at 37^{o}C, 5% CO_2, for 2 hours, with changes in the medium every hour. The numbers of transmigrated neutrophils at each time point were counted by off-line analysis. The reduction in the number of transmigrated neutrophils, at 2 hours, compared to 0 hours, was calculated, and this difference was expressed as the percentage of once transmigrated neutrophils that had reverse migrated by the end of the experiment.

5.3 Results

5.3.1 The effect of constant shear stress on human neutrophil transmigration and reverse transmigration

Prolonged flow assays were performed on TNF-α and IL-1β stimulated HUVEC monolayers, as previously described, in order to directly compare transmigration after brief or continuous exposure to shear stress. Very few neutrophils adhered to unstimulated HUVEC monolayers. There was no marked difference in neutrophil adhesion after brief or continuous exposure to flow (Figure 5.3A). On EC stimulated with cytokines adhesion levels were above 1000 cells/mm^2/10^6 cells perfused and remained stable over time in both conditions. The number of adherent neutrophils was slightly higher in the short than in the continuous exposure to flow. However, this result could probably be attributed to the fact that rolling and non-firmly adherent neutrophils could no longer be washed away after the flow had been turned off. Therefore, the numbers of those cells (that otherwise would have been washed) were included in the total number of neutrophils adherent to HUVEC.

When comparing the patterns of transmigration between brief and continuous exposure to shear stress we saw significant differences (Figure 5.3B). Approximately 75% of adherent neutrophils had transmigrated by 25 minutes, in both conditions. However, while transmigration was stable over time in continuous flow, it gradually decreased to 50% in the absence of flow. The ‘disappearance’ of transmigrated neutrophils was a result of their reverse transmigration, as confirmed by direct observation under phase-contrast microscopy. The phase dark transmigrated neutrophils started reverse transmigrating back across the endothelial cell monolayer after 25 minutes and re-emerging on the top of HUVEC monolayer, as phase bright cells.

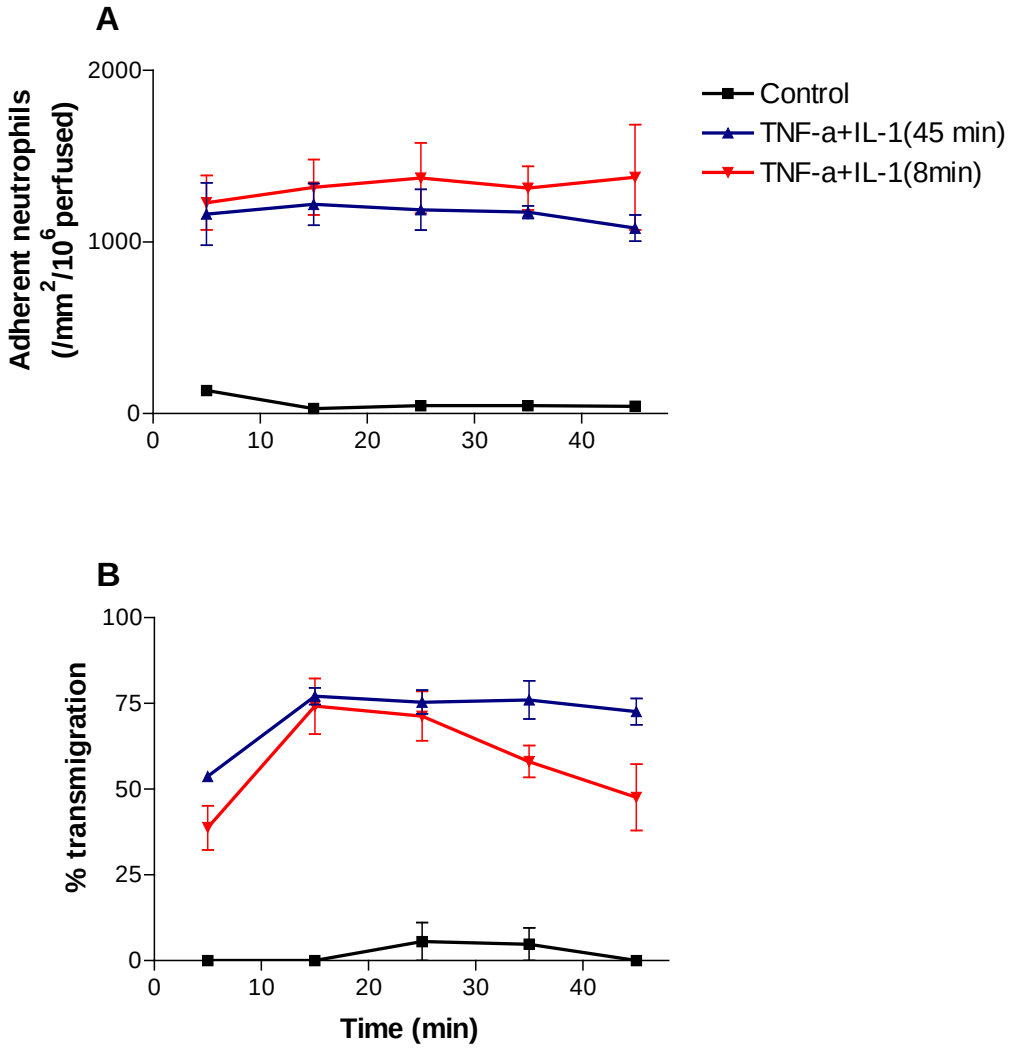


Figure 5.3 Effect of continuous shear stress on neutrophil adhesion and transmigration (A) ANOVA showed a significant effect of these three conditions (P<0.001) on adhesion levels. Bonferonni's Multiple comparison test showed a significant difference between 45-minute flow on adhesion and the 8-minute flow condition (*P*<0.05). (B) The effect of flow on the percentage of transmigration. ANOVA showed a significant effect on transmigration between 45-minute flow and 8-minute flow (p<0.001). Bonferonni's Multiple comparison test showed a significant difference between 45-minute flow on adhesion and the 8-minute flow condition (*P*<0.01). All data are the mean±SEM of three experiments.

The difference in the patterns of transmigration between brief and continuous exposure to flow led us to the question whether continuous flow was able to retain transmigrated neutrophils underneath the monolayer, not allowing them to reverse transmigrate. To address this we tracked migration and transmigration over 1, 2 and 4 hours of continuous flow (compared to static controls). The number of transmigrated neutrophils in the 25-minute static control was 1200-1500 cells/mm^2.

After 1 hour of continuous flow, we found no significant reduction in the number of transmigrated neutrophils, compared to 65% reduction, in the absence of flow (Figure 5.4). After 2 hours of continuous flow there was a significant drop of 25% in the number transmigrated neutrophils, however nearly twice this number were lost form beneath the monolayer, under static conditions. The same pattern was observed after 4 hours, but this time there was a more dramatic decrease in transmigration levels both in the presence or absence of flow (Figure 5.5). Ultimately, the presence of continuous flow could not inhibit the reverse migration of neutrophils, but it did significantly delay the process.

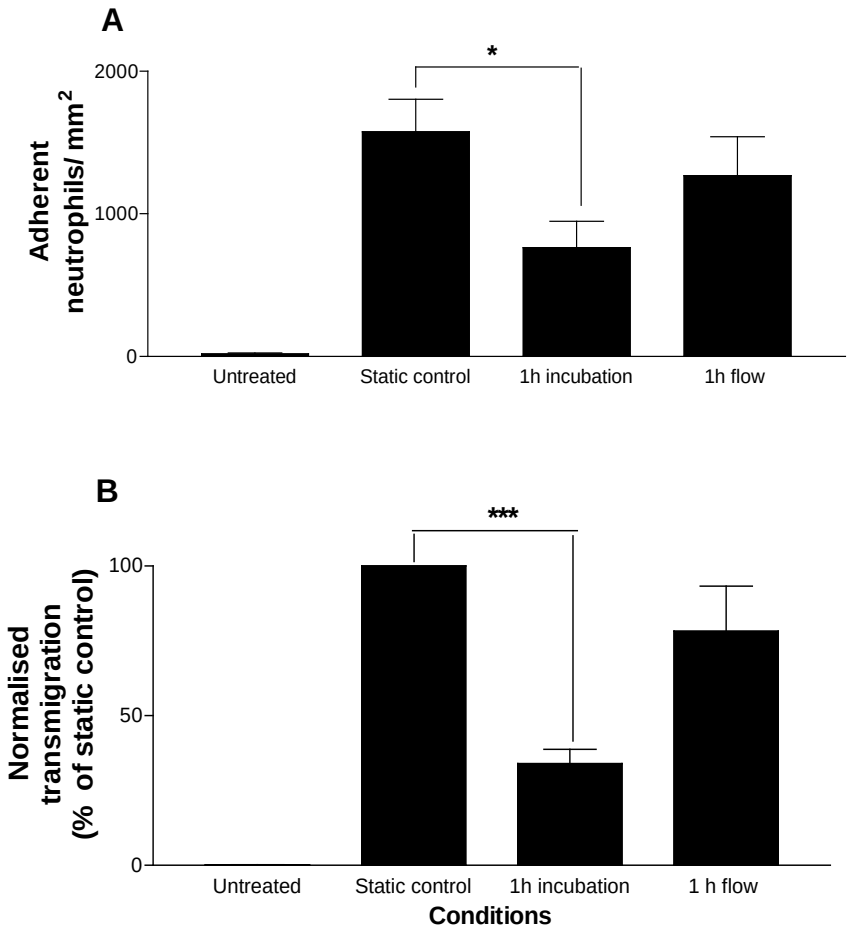


Figure 5.4 The effect of 1h incubation under flow or static conditions on adhesion and transmigration levels of neutrophils

(A) The levels of neutrophil adhesion significantly decreased after 1 hour incubation in static condition. In contrast, there was a slight decrease in adhesion levels under flow after 1h. There was a significant effect of experimental conditions on adhesion (P<0.001, by ANOVA).*, P<0.05, by Students't-test. (B) Similarly, the levels of transmigration significantly decreased after 1 h incubation in static condition. There was a significant effect of experimental conditions on adhesion (P<0.001, by ANOVA). There was significant difference between static control and 1 hour incubation condition (***, P<0.001, by Students't-test). All data are the mean$\pm$SEM of four different experiments.

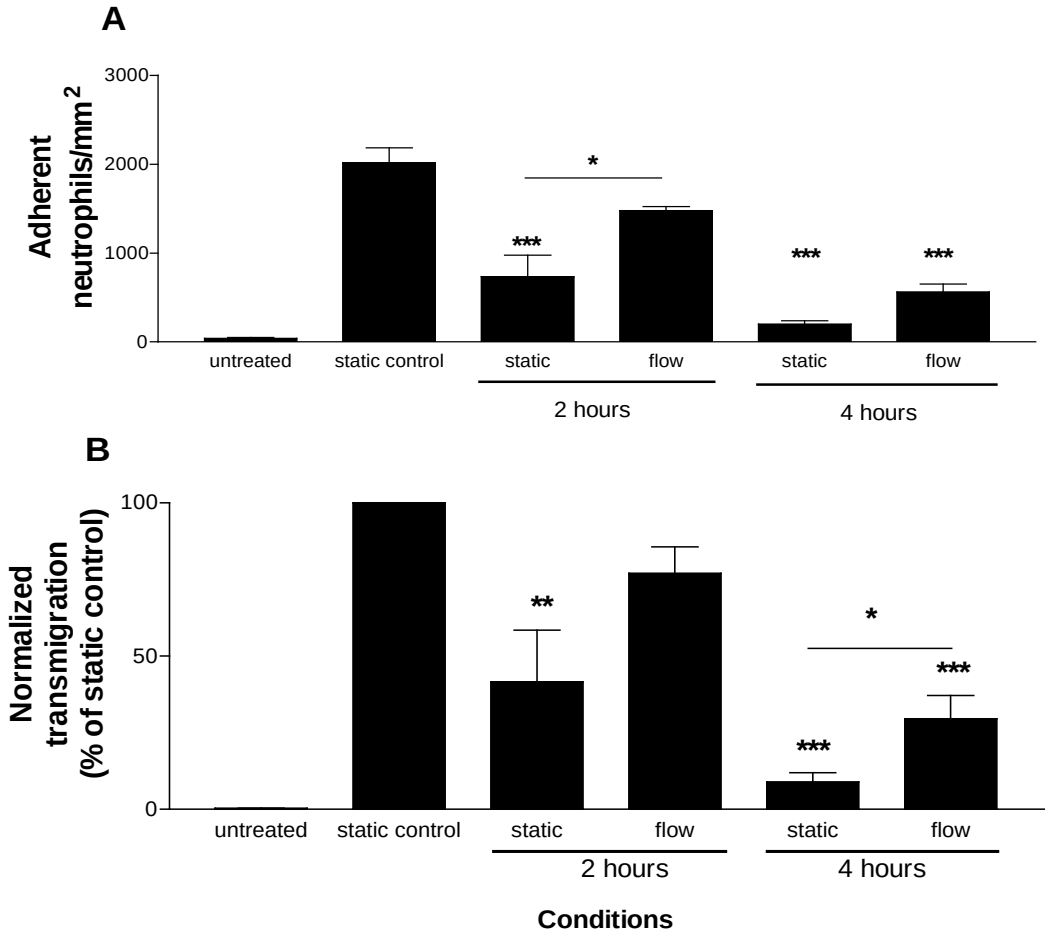


Figure 5.5 The effect of presence and absence of flow on neutrophil adhesion and transmigration

(A) The effect of presence and absence of flow on neutrophil adhesion. There was a significant effect of experimental conditions on adhesion (P<0.001, by ANOVA). *, P<0.05, ***, P<0.001, by Bonferroni's Multiple Comparison Test. (B) The effect of flow and static conditions in transmigration. Similarly, ANOVA showed that the effect of the presence of flow on transmigration is significant (P<0.001).*, P<0.05, **, P<0.01 and ***P< 0.001, by Bonferroni's Multiple Comparison Test. All data are the mean±SEM of four different experiments.

The previous findings showed that flow could retain the neutrophils underneath the endothelium for up to 1 hour, preventing them from reverse migration. The hypothesis we had to test here was whether blocking important molecules for transmigration could impair the inhibitory effect of continuous flow on reverse migration. A number of monoclonal antibodies that could block adhesion molecules, such as CD31, CD99, JAM-C and VE-cadherin, were used in order to study their role in reverse transmigration. Data was presented normalized transmigration compared to the 1 hour flow control.

Blocking of CD31 or JAM-C resulted only in a detectable decrease in transmigration, compared to their isotypes and 1 hour control. Blocking CD99 or VE-cadherin led to a greater reduction in transmigration, but it was not statistically significant. Overall, blocking of these molecules could not increase the numbers of reverse transmigrated neutrophils.

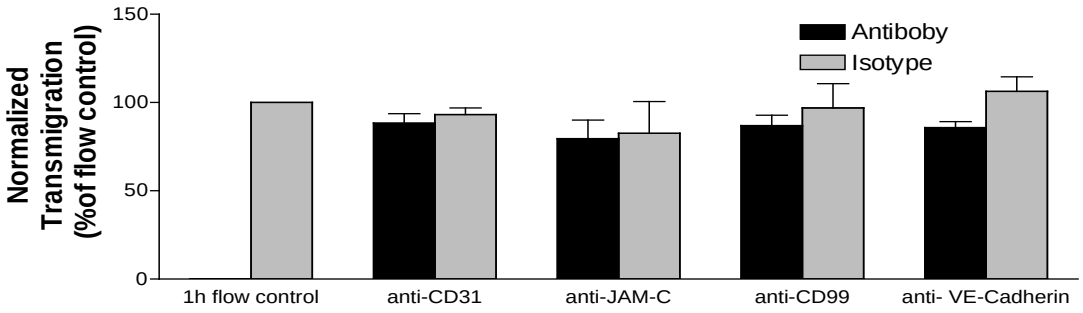


Figure 5.6 The effect of blocking antibodies on transmigration levels
Blockade of important adhesion molecules did not have an effect on reverse transmigration. All data are the mean±SEM of three different experiments.

5.3.2 The effect of NO donor/inhibitor treatment on human neutrophil recruitment and reverse transmigration

Static assays were performed in order to investigate the effect of NO on neutrophil recruitment. For this purpose, DEANO as NO donor and L-NAME as NO synthase (eNOS) inhibitor were used. Figure 5.7 shows adhesion levels and percentage of transmigrated neutrophils at 7 and 24 minutes. Neutrophils did not adhere to unstimulated HUVEC. In HUVEC stimulated with cytokines, treatment with L-NAME resulted in slightly lower adhesion levels at 7 minutes, compared to control, but ultimately it reached similar levels by 24 minutes. In contrast, neutrophil incubation with DEANO led to significantly lower adhesion at 7 minutes, compared to control. Despite the fact adhesion increased over time, it did not reach control adhesion levels (Figure 5.7A).

Transmigration patterns in various conditions were similar to the ones of adhesion. HUVEC treatment with L-NAME showed a slightly lower percentage of transmigrated neutrophils, at 7 and 24 minutes compared to control. Neutrophil incubation with DEANO showed that only 40.1±23% of adherent neutrophils had transmigrated by 7 minutes, compared to 75% of control. Transmigration did not further increase in all conditions (Figure 5.7B).

Static assays were also conducted in order to examine the effect of NO on reverse migration of neutrophils. After establishing a population of transmigrated neutrophils, the co-cultures were incubated with DEANO or L-NAME, for 2 hours. Transmigrated neutrophils were counted at 0 and 2 hours, and results were presented as the % of reduction in their numbers at 2 hours. This reduction represents the reverse migration of neutrophils. Treatment

with L-NAME did not change the percentage of reverse migrated neutrophils. Interestingly, incubation with DEANO resulted in lower levels of reverse transmigration, compared to control (Figure 5.8).

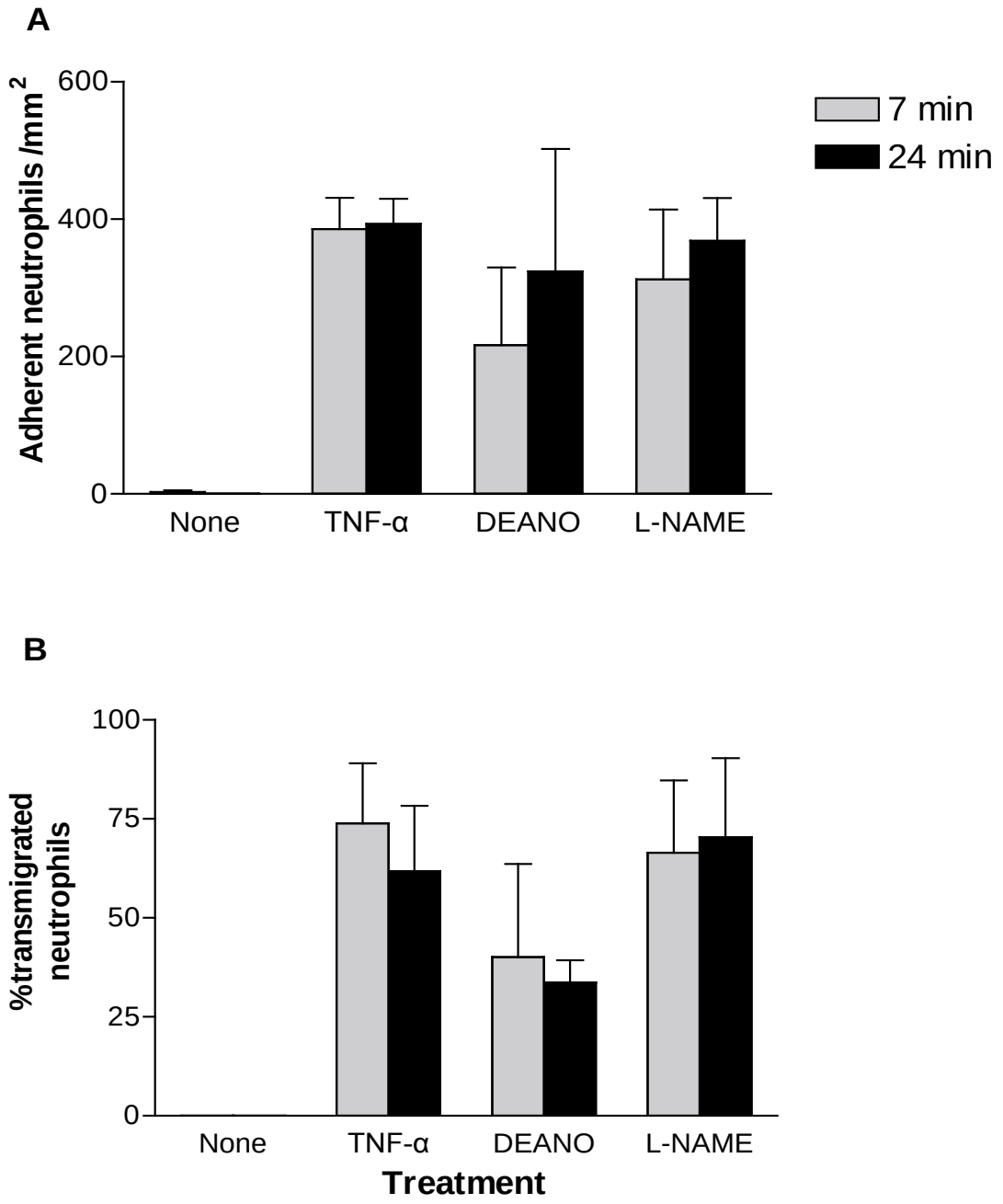


Figure 5.7 Effect of DEANO and L-NAME treatment on neutrophil adhesion and transmigration at 7 and 24 minutes of static culture
All data are the mean+SEM of three different experiments, except for L-NAME, where n=2.

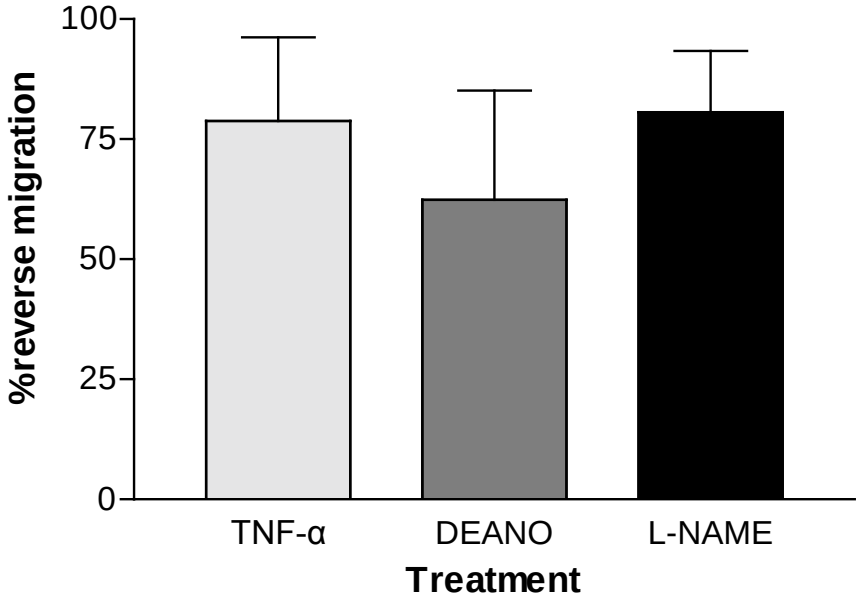


Figure 5.8 Effect of DEANO and L-NAME on neutrophil reverse migration
Treatment of co-cultures with DEANO decreased reverse migration. All data are the mean+SEM of two different experiments.

5.4 Discussion

There is increasing evidence that EC phenotype and the behaviour on leukocytes can be conditioned by the physicochemical environment, including wall shear stress (Nash *et al*., 2004). For example, a number of studies have investigated the role of wall shear stress on the responses of endothelial cells to cytokine stimulation and subsequent leukocyte adhesion and transmigration (Sheikh *et al*., 2004; Sheikh *et al*., 2003). In this chapter, we examined whether wall shear stress could modulate the migration, but more importantly the reverse migration of neutrophils, a process that might be dependent upon shear sensing by endothelial cells, neutrophils or both cell types. Firstly, we showed that flow generated wall shear stress is not required for neutrophil transmigration in the HUVEC system when driven by TNF-α stimulation. These findings are in accordance with previous studies in static cultures using neutrophils (Butler *et al*., 2005). Moreover, in our assay, transmigration could take place minutes after the flow had been turned off in prolonged flow-based adhesion assays. Previous studies have shown that the presence of shear stress did not lead to any increase in adhesion or transmigration of neutrophils, but resulted in faster kinetics of transmigration compared to static conditions (Kitayama *et al*., 2000). Others claim that T cell transmigration requires shear flow (Cinamon *et al*., 2001). Studies from our own group using peripheral blood lymphocytes demonstrate no such dependence upon flow generated shear forces (McGettrick *et al*., 2009). Interestingly, shear stress has been found to direct the migration of activated neutrophils on the surface of platelet monolayers and on the surface of endothelial cells. Neutrophils used $\alpha_v\beta_3$ integrin and CD31 as mechanotransductors and moved preferentially in the direction of flow (Rainger *et al*., 1999). However, these signals did not translate into more effective transmigration on an EC monolayer.

Many studies have shown effects of acute or prolonged changes in wall shear stress on endothelial cell phenotype. Of particular relevance here, our own group has shown that conditioning endothelial cells at increasing wall shear stress reduces their ability to respond to TNF-α and recruit neutrophils (Sheikh *et al*., 2004; Sheikh *et al*., 2003). However, nothing is known about whether wall shear stress is able to modify the long-term fate of the recruited neutrophils. For this reason, we investigated the fate of transmigrated neutrophils under different regimens of static or constant flow. In the presence of continuous flow, transmigration remained stable over a short period of time (1h). This was in contrast to neutrophil behaviour under static conditions where as quickly as 15 minutes after transmigration the process of reverse migration was evident. This is in agreement with a previous study from our group which remains the only published description of neutrophil reverse migration in a human system (Buckley *et al*., 2006). When we extended the period of flow out to 4 hours we found that the process of reverse migration was retarded but not inhibited. One possible explanation for this finding is the fact that under static conditions IL-8 (or other neutrophil chemotactic agents) generated by ECs accumulates in the medium and are able to attract neutrophils from beneath the endothelial cell monolayer (Huber *et al*., 1991). This hypothesis is readily tested in our assays by adding exogenous agents to the medium (Luu *et al*., 2000). These experiments have not been done, but would represent an obvious line of experimental progression.

An alternative hypothesis is that continuous shear stress is able to tighten EC junctions. However, when assays were performed using antibodies that inhibit the function of junctional molecules, a process that should permit accelerated reverse migration in the context of the prevailing hypothesis, we found no consistent effects. We chose targets that have previously

been reported to regulate EC permeability (CD31, JAM-C ,CD99 and VE-cadherin) (Muller, 2003), so to date it would appear that there is no evidence that changes in the integrity of EC junctions plays a major role in retaining neutrophils in the sub-endothelial cell environment. This is perhaps surprising considering that regulation of EC junctional integrity via modulation of VE-cadherin plays a role during neutrophil transmigration by forming transient gap in cell junctions (Alcaide *et al*., 2009).

As one endothelial cell response to shear stress is increased formation of nitric oxide (Li *et al*., 2004), and as nitric oxide has been shown to regulate neutrophil recruitment (Hickey *et al*., 2001; Kubes *et al*., 1991; Lefer *et al*., 1999), we tested the hypothesis that flow generated NO could regulate reverse migration. In the presence of a NO donor (DEANO) we found reduced levels of neutrophil adhesion, transmigration and reverse transmigration. The reduction in neutrophil recruitment by NO is in accordance with previous studies that have shown that NO may effect neutrophils ability to adhere to microvascular endothelium. Interestingly, in our experiments an inhibitor of eNOS did not have an effect on neutrophil transmigration. Again this in accordance with the studies of Kubes *et al.*, who showed that neutrophil recruitment in eNOS and iNOS knockout mice was predominantly regulated by the latter with little contribution from the former（Hickey *et al*., 2001).

In conclusion, shear stress could retard the process of reverse migration. Testing our hypothesis we found that indeed NO partially impairs neutrophil transmigration and reverse migration. Therefore, we could attribute shear stress effect on neutrophil transmigration and reverse migration to NO production. Our findings indicate that NO contributes to the mechanism, which shear stress acts upon endothelium, but this should be further investigated.

CHAPTER 6

INVESTIGATION OF THE DISTINCT PROTEIN PROFILE OF REVERSE MIGRATED NEUTROPHILS

6.1. Introduction

As examined in Chapter 4, human reverse migrated neutrophils (RPMNs) exhibit an altered phenotype, compared to naïve circulating neutrophils. According to Buckley *et al*., (2006) these cells are morphologically more rigid than the naïve, can be functionally primed and exhibit a distinct surface phenotype compared to naïve circulating cells, naïve activated neutrophils and tissue resident neutrophils. We were interested in examining the changes in the RPMNs phenotype at a proteomic level.

Two-dimensional (2D) gel electrophoresis is a powerful tool for studying protein expression. In 2D electrophoresis, proteins are separated according to their charge in the first dimension, by isoelectric focusing (IEF) and their size in the second dimension by SDS-PAGE. Traditional 2D gel electrophoresis is both time and effort-consuming (Alban and Olu, 2003). Moreover, variation between the gels makes it difficult to attribute any changes in protein expression to induced biological change and not the system variation. To address this problem, 2D fluorescence difference gel (DIGE) analysis technology has been developed (Marouga *et al*., 2005).

2D difference gel electrophoresis (DIGE) enables the separation of more than one sample in a single 2D polyacrylamide gel, enabling to run multiple samples on the same 2D gel. In this way, the sensitivity and accuracy of the method is improved. The protein samples are labelled with up to three CyDye DIGE fluorescent dyes prior to electrophoresis. The CyDye DIGE fluor minimal dyes (Cy2, Cy3, and Cy5) are charge and size matched and label only 3–5% of the total protein present in each sample. The properties of the dyes ensure the high linearity and sensitivity for accurate protein quantitation. Another major advantage of

this methodology is the presence of an internal standard which significantly reduces the system variation. The internal standard is prepared by pooling equal amounts of protein from each biological sample in the experiment and it is usually labelled with Cy2. In this way, each protein from all samples is represented in the internal standard (Marouga *et al*., 2005). The internal standard and all the other labelled samples are mixed and run on the same 2D gel. The different protein extracts labelled with different CyDye DIGE fluors can be visualized separately by different excitation profiles. Digital images of each sample in each dye are generated and analysed with DeCyder software (GE Healthcare, Uppsala, Sweden). The presence of a common internal standard as a reference sample in every gel enables the inter-gel analysis. Each sample within a gel can be normalized to the internal standard present on that gel. This allows the abundance of each protein spot in a biological sample to be measured as a ratio to its corresponding spot present in the internal standard, ensuring the accurate quantitation and spot statistics between gels. Moreover, biological variation can be distinguished from experimental variation (Marouga *et al.*, 2005).

The DeCyder differential analysis software (GE Healthcare, Uppsala, Sweden) is fully automated image analysis software for spot detection and accurate measurement of protein differences with statistical confidence. Images, after being loaded to the programme, are analysed with Differential in-gel analysis (DIA). The DeCyder DIA module processes the images from a single gel and performs background subtraction, detection, quantitation and in-gel normalization. The spots are then detected, using a co-detection algorithm. Spot volumes in each image are quantified in the DIA module and these are expressed as a ratio, between the spot volumes on the sample image and the corresponding spot volumes from the internal standard image. With this ratio, inter-gel protein abundance comparisons can be done. When

spot detection and quantitation of a single gel has been performed, analysis continues in BVA (biological variation analysis) module, where multiple images from different gels are matched (gel-to-gel matching) on pooled internal standard images only, making it possible to generate statistical data and thus comparing the different protein abundance levels between multiple groups. The presence of the same pooled internal standard on every gel enables accurate normalization of the individual experimental samples and therefore it reduces gel-to-gel and software analysis variation (From GE Healthcare).

Using DeCyder analysis software, the spots of interest are identified. The next step is the making of preparative gels where the correspondent gel plugs can be picked from to be further analysed for mass spectrometry. After protein identification is the verification of previous findings with Western blotting, as it represents an alternative method to measure protein abundance differences.

In this chapter, the protein expression profile of RPMNs was compared with those of four other control groups which represent a different level of neutrophil interaction with the EC. These were: 1) fresh neutrophils, 2) adherent neutrophils, 3) transmigrated neutrophils (TPMNs) and 4) neutrophils in culture for the same period with the experiment (4h control). Our hypothesis was that RPMNs are a distinct group with a series of proteins specifically regulated.

6.2. Methods

Experimental design

The protein lysates were prepared from 4 different experiments. The protein concentration of the samples was determined and the samples were labeled with CyDyes. Then, first and second electrophoresis were performed and the gels were scanned. The gel images were analysed with DeCyder software and the proteins of interest were determined. Preparative gels were made and the excised gel plugs of the proteins of interest were analysed by mass spectrometry for protein identification. The whole workflow is shown in Figure 6.1. After the preparation of protein lysates, the methodology used is described in the general methodology chapter (section 2.8-10).

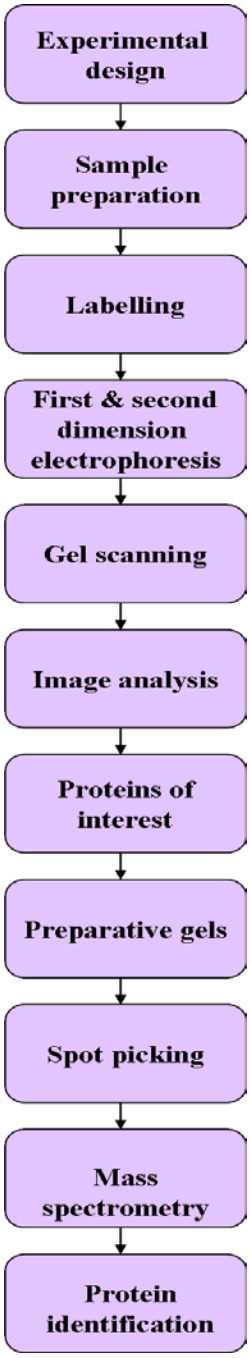


Figure 6.1 Analytical workflow in 2D-DIGE methodology (adapted from GE Healthcare)

Neutrophil isolation

Whole blood was isolated from healthy donors and transferred into 50ml tubes, each containing 10% of sodium citrate as anticoagulant. 10ml of PBS was added per 25ml of whole blood and was gently mixed. Then 15ml of Ficoll-paque was introduced with a T-needle to the bottom of the tube. The blood was centrifuged at 800g for 20 minutes. After plasma and the monocyte fraction being removed, 40ml of an isotonic ice-cold NH_4Cl solution was added to lyse the red cells. The mixture was left on ice for 15 minutes, followed by a 7-minute centrifugation at 400g at 4^o C. After two washes, neutrophils were incubated in a recovery buffer containing 5% human serum albumin in RPMI for 30 minutes at 37^oC.

Experimental groups

We aimed to include in our study as many control groups as possible. However, for practical reasons we had to conduct two series of 2-D DIGE experiments. In the first, protein lysates from RPMNs were compared to three other control groups (fresh, adherent, 4-hour control). In the second, transmigrated neutrophils (TPMNs) were compared to fresh.

Below the experimental groups are described, as shown in Figure 6.2:

1) Fresh neutrophils: $10x10^6$ neutrophils, resuspended in recovery medium, are centrifuged at 400g and washed once in PBS.

2) Adherent: $20x10^6$ neutrophils in M199/BSA were added into a 75 cm^2 flask with HUVEC, previously stimulated with 100U/ml TNF-α for 4 hours. The co-culture was kept for 1 hour at 37^oC. Next, the flasks were shaken and washed with M199/BSA and all the phase bright non-transmigrated neutrophils were collected. These cells were washed once in PBS.

3) RPMNs: This group represents transmigrated neutrophils that remained in co-culture with HUVEC (in complete medium) for 4 hours. A proportion of these cells had

reverse migrated by the end of this period. RPMNs were collected after 4 hours and centrifuged at 400g for 5 minutes.

4) 4-hour control: Neutrophils incubated in pre-conditioned medium for 4 hours in contact with extracellular matrix. In detail, HUVEC in a 75cm^2 flask were stimulated with TNF-α for 4 hours. After a wash, complete medium was introduced to the flask. After 2 hours, the pre-conditioned medium was collected. At the same time, the HUVEC monolayer was lysed using pre-heated to 37^{o}C PBS containing 0.5% Triton X-100 (v/v) and 20mM NH_4OH. The cell lysis was observed under a phase contrast microscope and after 5 washes with 4ml of PBS the extracellular matrix was exposed. Fresh neutrophils were resuspended in the pre-conditioned medium and transferred to that flash, where they remained for 4 hours in culture.

5) TPMNs: HUVEC were cultured for 4 days on Transwell filters and subsequently stimulated with 100U/ml TNF-α for 4 hours. Neutrophils in M199/BSA were added to the filters, allowed to settle, adhere to, and finally transmigrate through HUVEC monolayers for 4 hours. After that time, neutrophils that have transmigrated though the filters were collected from the bottom of the well.

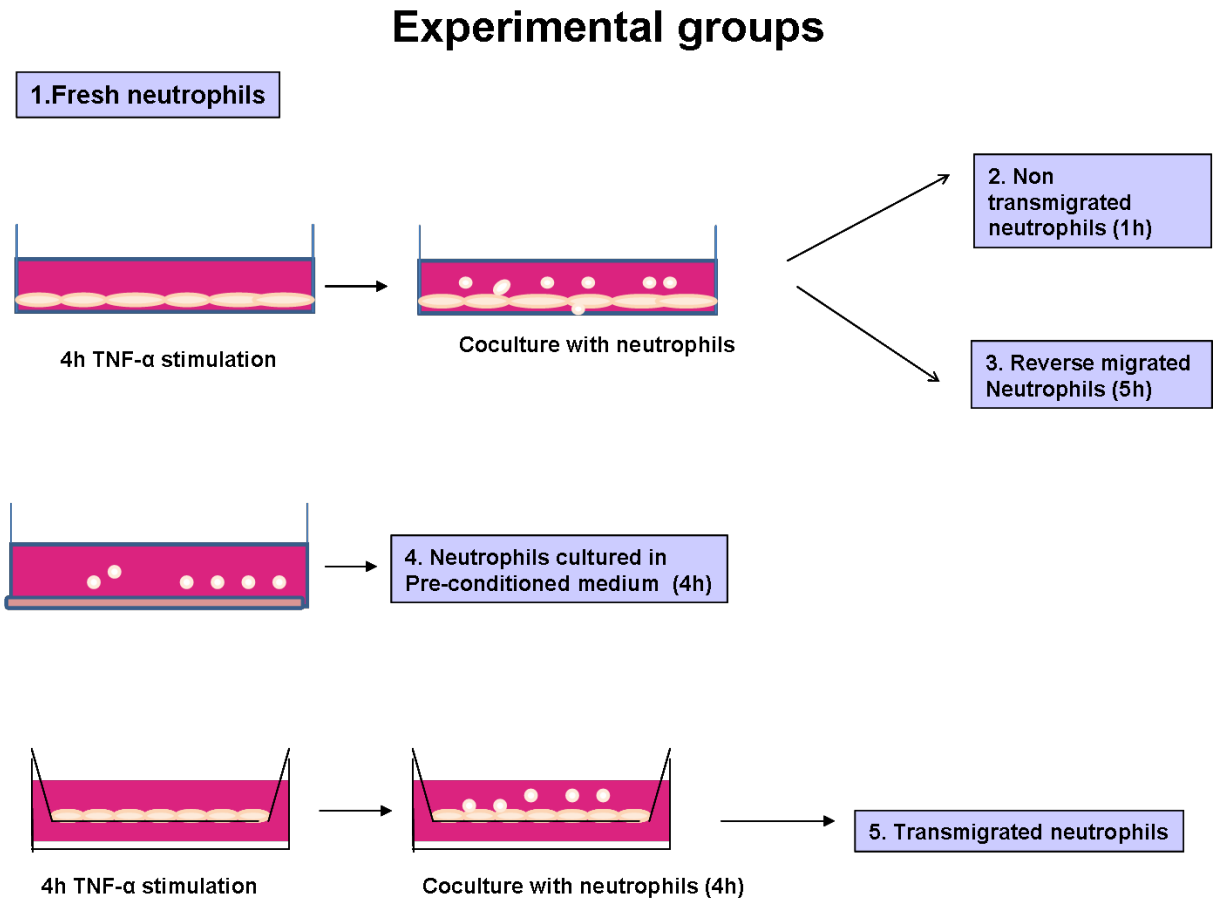


Figure 6.2 The experimental groups in this proteomic study
RPMNs were compared with four other control groups. These were: 1) fresh neutrophils, 2) adherent neutrophils (non-transmigrated neutrophils), 3) neutrophils in culture for the same period with the experiment (4h control) and 4) transmigrated neutrophils (TPMNs).

6.3 Results

6.3.1 Identification of differentially regulated protein spots by biological variance analysis

Protein samples from the four groups were labelled with CyDyes and separated by 2D-DIGE in four individual experiments. Gel images were acquired and analysed with DeCyder 7.0. Differential in-gel analysis (DIA) identified 1000-1500 protein spots, using a volume filter exclusion of 10000.

Performing statistical analysis in the BVA we identified the protein spots that were differentially expressed between the groups. In RPMNs, 13 protein spots were differentially expressed, compared to fresh neutrophils. Similarly, 10 spots were differentially expressed in RPMNs, compared to adherent and 4-hour control neutrophils. The differentially expressed proteins between these four groups were overlapped. Only two spots were differentially expressed only in a single group. Spot no.234 was found to be significantly differentially expressed between adherent neutrophils and RPMNs and spot no.838 between fresh and adherent neutrophils.

Table 6.1 shows 12 proteins spots that have been differentially expressed in reverse migrated neutrophils compared to fresh. The spot graphs also demonstrate the biological variation between the 4 different conditions (Figures 6.3, 4 and 5). Among the 13 differentially regulated proteins in RPMNs, 5 are down-regulated and 8 are up-regulated, compared to fresh neutrophils. The down-regulation of these proteins appears to be specific for reverse migration. In contrast, protein spot no 298 appears to be specifically up-regulated upon reverse migration, compared to the other conditions.

Table 6.1 Biological variation analysis (BVA) of differential regulated protein spots in reverse migrated neutrophils compared to fresh

Spot	Average	1-ANOVA
1833	-3.42	0.0017
433	-2.16	0.00021
737	-1.61	0.0057
1235	-1.36	0.0071
1214	-1.31	0.0076
961	1.23	0.00016
1533	1.32	0.0051
960	1.61	0.0033
298	1.82	0.0049
1701	1.87	0.0023
628	6.46	0.0048
623	6.7	0.0091
1742	7.43	0.00066

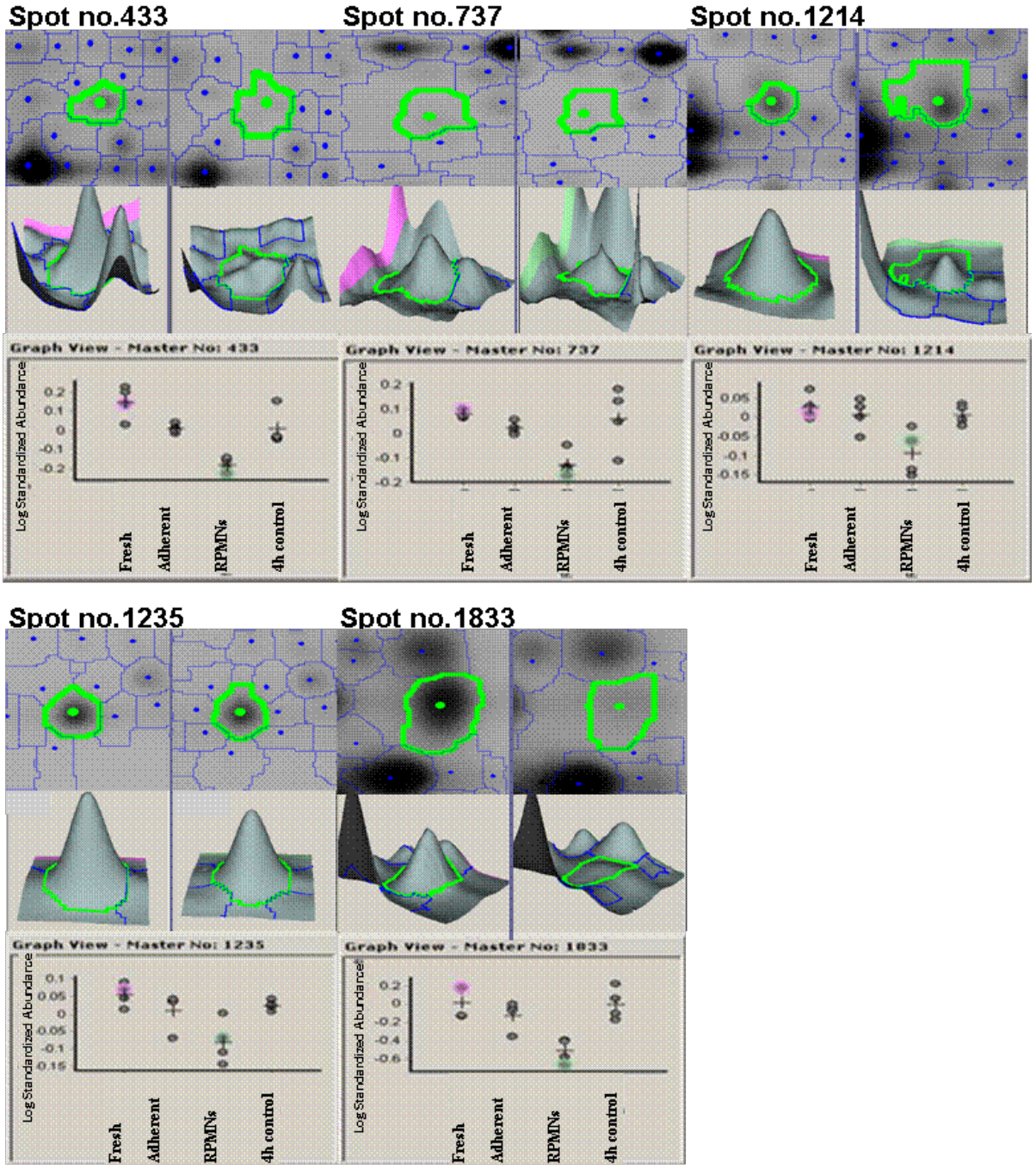


Figure 6.3 Down-regulated protein spots in RPMNs
DIGE spot maps were analysed with DeCyder 7.0. Significantly regulated spot graphs from BVA analysis are shown, supplemented by images of the spots on 2D gel and their 3D construction. Results in the spot graphs are presented as log standardized abundance. Spots no 433, 737, 1214, 1235 and 1833 are down-regulated in RPMNs compared with fresh neutrophils.

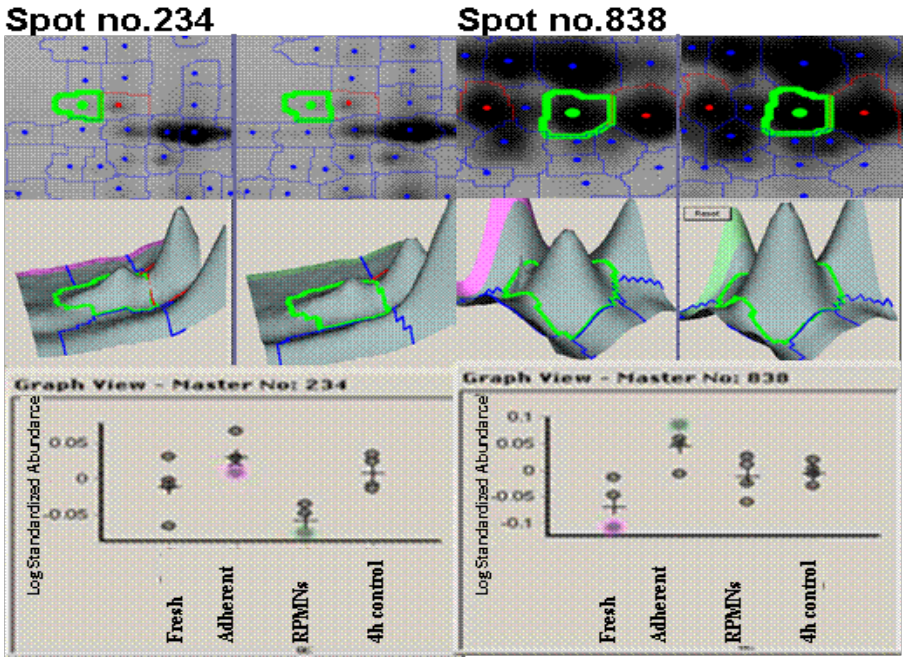


Figure 6.4 Differentially expressed protein spots between the four groups
DIGE spot maps were analysed with DeCyder 7.0. Significantly regulated spot graphs from BVA analysis are shown, supplemented by images of the spots on 2D gel and their 3D construction. Results in the spot graphs are presented as log standardized abundance. Spot no.234 was found to be significantly differentially expressed between adherent and RPMNs and spot no.838 between fresh and adherent neutrophils.

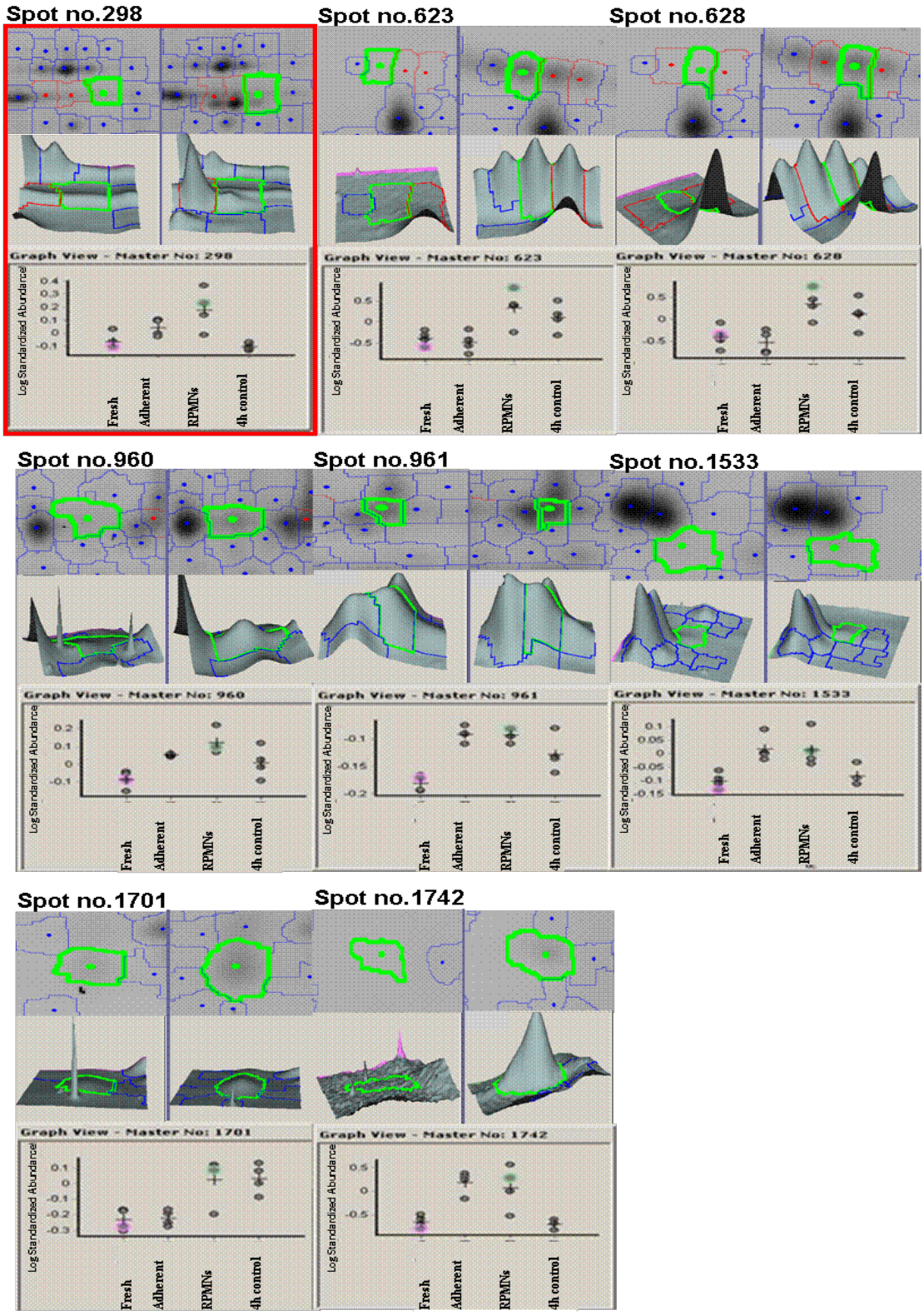


Figure 6.5 Up-regulated protein spots in RPMNs
All the above spot graphs show that these protein spots were up-regulated in RPMNs neutrophils. However, it appears that only protein spot no 298 (red frame) is specifically up-regulated in RPMNs neutrophils.

6.3.2 Identification of protein spots by mass spectrometry (I)

Trypsinization and analysis by mass spectrometry resulted in the identification of less than half of protein of interest (Table 6.2). From the down-regulated protein group, the ALDH16A1 isoform of aldehyde dehydrogenase (spot no.433), transaldolase (spot no.1214) and non-secretory ribonuclease (spot no.1833) were identified. Non-secretary ribonuclease is also known as eosinophil-derived neurotoxin and is an abundant protein in eosinophils. Also, transaldolase and serine-threonine-protein phosphatase PP1 were detected on the same spot (spot no.1235). Alpha enolase (spot no.960) and CAP1 (spot no.826 and 838) were the only up-regulated proteins in RPMNs that have been identified.

Table 6.2 Protein spot identification (I)

Spot no.	Spot ID	Protein name	Average ratio	1-ANOVA	Swiss prot	Mascot score	Peptides matched	Coverage%	Theoretical	
									MW	pI
1	234	n.i.								
2	433	TF Serotransferrin	-2.16	0.00021	P02787	69.8	8	27	77	7
		ALDH16A1 Isoform of aldehyde dehydrogenase			Q8IZ83-1	85.1	9	22.7		
3	737	n.i.								
4	1214	Transaldolase	-1.31	0.0076	P37837	181	7	54.6	37.5	6.4
5	1235	Transaldolase	-1.36	0.0071	P37837	137	8	50.5	37.5	6.4
		Serine/threonine-protein phosphatase PP1-beta catalytic subunit			P62140	39.7	1	4.9		
6	1833	Non-secretory ribonuclease	-3.42	0.0017	P10153	42.7	1	9.9	18.3	10.6
7	838	Adenylyl cyclase-associated protein 1 (CAP1)	1.14	0.0076	Q01518	91.6	9	36.7	51.8	9.1
8	960	Alpha enolase	1.61	0.0033	P06733	43.1	1	4.7	47.1	7.7

6.3.3 Extended data analysis (EDA) of all the experimental groups combined

For practical reasons, a series of 2D-DIGE experiment profiles between fresh and TPMNs was performed later on. Its analysis was linked to the one of the previous series of experiment via the Extended Data Analysis software. The combination of the two series of experiments introduced changes in the statistical data, as eight fresh samples were compared to four samples from each group (Table 6.3). Overall, the trends observed did not change (Figures 6.6 and 7). Also, a new protein spot that was significantly down-regulated was identified. This was spot no 1989 that was not included in the previous analysis, because it was located at the very bottom of the gel.

Table 6.3 Extended Data Analysis (EDA) of differential regulated protein spots in RPMNs compared to fresh

Spot ID	Average Ratio	1-ANOVA
1833	-3.42	0.0017
433	-2.16	0.00021
737	-1.61	0.0057
1235	-1.36	0.0071
1214	-1.31	0.0076
961	1.23	0.00016
1533	1.32	0.0051
960	1.61	0.0033
298	1.82	0.0049
1701	1.87	0.0023
628	6.46	0.0048
623	6.7	0.0091
1742	7.43	0.00066

Extended data analysis combined the 5 groups together (fresh, adherent, TPMNs, RPMNs and 4-hour control) and revealed differential regulated proteins in RPMNs neutrophils compared to fresh. The statistics are altered due to the fact that 8 fresh samples were compared to 4 samples from each group.

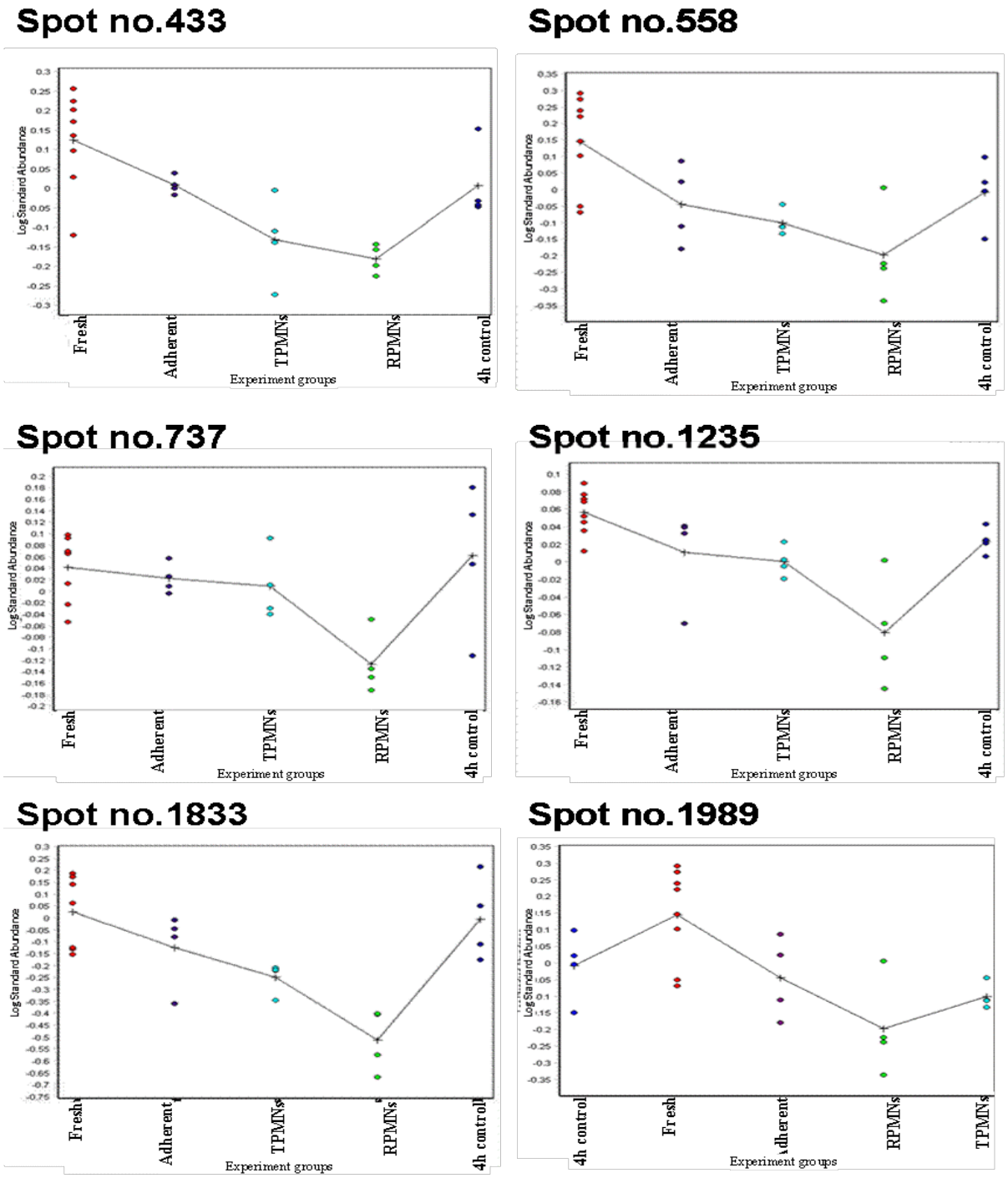


Figure 6.6 Differentially expressed protein spots after all groups were combined in Extended Data Analysis (EDA) (i)

Significantly down-regulated protein spots from EDA analysis are shown. Each group from left to right is represented by a colour. Red: fresh, purple: adherent, cyan: TPMNs, green: RPMNs, blue: 4h control. Results are presented as log standardized abundance.

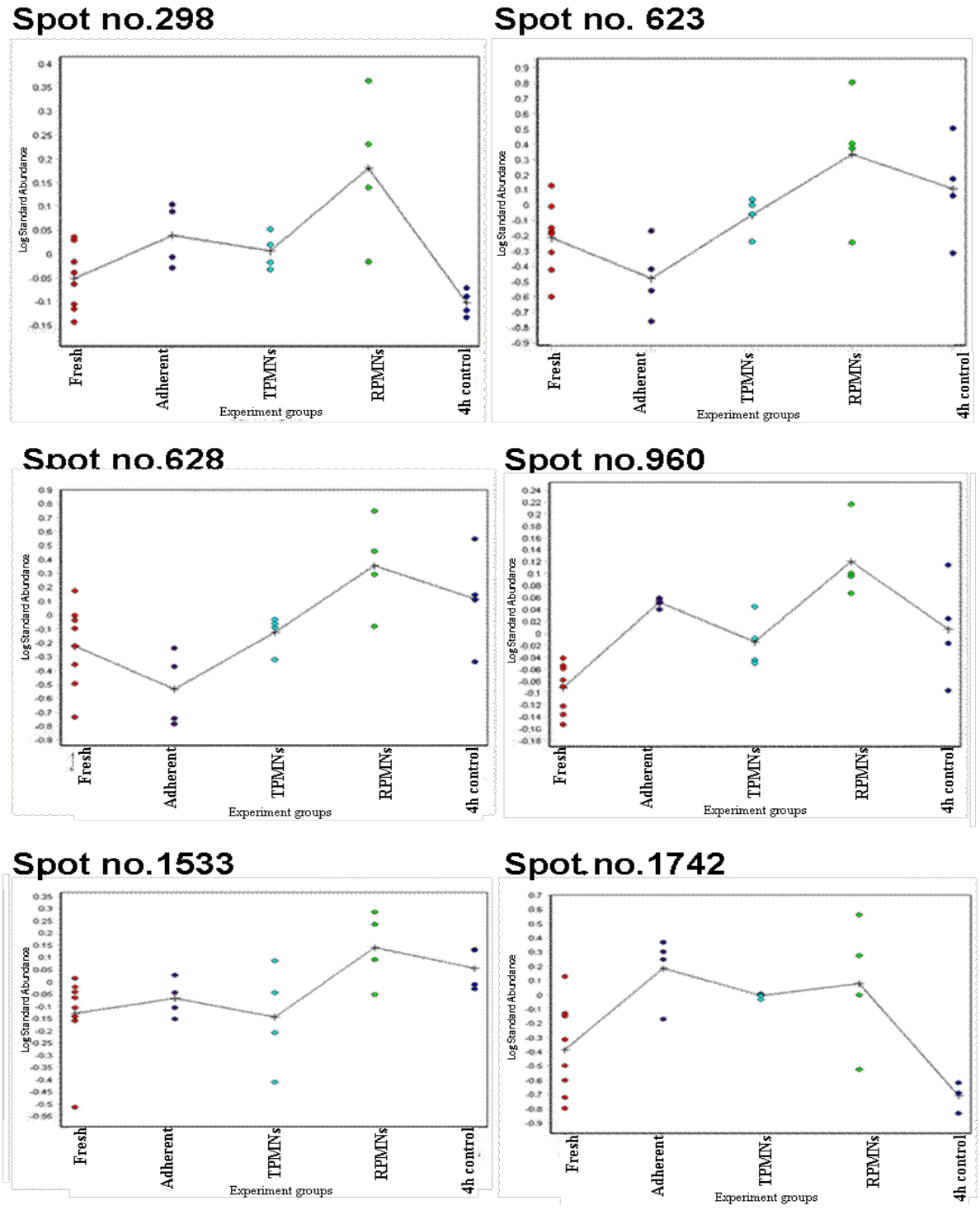


Figure 6.7 Differentially expressed protein spots after all groups were combined in Extended Data Analysis (EDA) (ii)

Significantly up-regulated protein spots from EDA analysis are shown. Each group from left to right is represented by a colour. Red: fresh, purple: adherent, cyan: TPMNs, green: RPMNs, blue: 4h control. Results are presented as log standardized abundance.

6.3.4 Identification of protein spots by mass spectrometry (II)

In order to increase the number of identified proteins, we repeated the process and analysed the gel plugs with mass spectrometry in the CRUK Institute for Cancer studies at the University of Birmingham. This time, 7 down-regulated and 10 up-regulated protein spots were identified. Tables 6.4 and 6.5 show the results from mass spectrometry. Alpha-enolase, transaldolase, CAP1 and non-secretory ribonuclease were identified again at the same spots, as previously. In contrast, ALDH16A1 and serine/threonine-protein phosphatase PP1-beta catalytic subunit were not detected again. The mass spectrometry methodology was more sensitive and allowed the identification of more than one protein in a single spot. However, on one occasion we could not identify with confidence the neighbouring spots no 623, 628 and 633 which were highly up-regulated in RPMNs. By mass spectrometry one peptide from each spot was identified as solute carrier family 12 member 7.

Table 6.4 Identification of downregulated protein spots in reverse migrated neutrophils

Spot no.	Spot ID	Protein name	Average ratio	1-ANOVA	Swiss prot	Mascot score	Peptides matched	Coverage%	Theoretical	
									MW	PI
1	234	Heat shock 70 kDa protein 4	-1.11	0.0058	P34932	258.8	6	8.57	94.3	5
2	433	Serotransferrin precursor	-2.16	0.00021	P02787	439.5	9	16.33	77	7
3	737	Serine/threonine-protein kinase PAK 2	-1.61	0.0057	Q13177	55.8	2	6.3	58	5.6
4	1214	Alcohol dehydrogenase [NADP+]	-1.31	0.0076	P14555	414.9	8	31.08	36.5	6.4
5	1235	Transaldolase	-1.36	0.0071	P37837	336.7	7	19.58	37.5	6.4
6	1833	Non-secretory ribonuclease	-3.42	0.0017	P10153	134	2	19.25	18.3	10.6
		Cofilin-1			P23528	109.6	2	12.65	18.5	9.1
7	1989	Ig gamma-1 chain C region	-4.17	0.026	P01857	259	6	25.76	36.1	9.4
		Eosinophil lysophospholipase			Q05315	180.6	3	20.42	16.5	7.7
		Ig gamma-2 chain C region			P01859	79.8	2	7.98	35.9	8.8

Table 6.5 Identification of upregulated protein spots in reverse migrated neutrophils

Spot no.	Spot ID	Protein name	Average ratio	1-ANOVA	Swiss prot	Mascot score	Peptides matched	Coverage%	Theoretical	
									MW	PI
1	298	Myosin-9	1.82	0.0049	P35579	304.4	6	3.88	226.4	5.4
2	623	Solute carrier family 12 member 7	6.7	0.0091	Q9Y666	41.6	1	0.65	119	6.3
3	628	Solute carrier family 12 member 7	6.46	0.0048	Q9Y666	43.3	1	0.65	119	6.3
4	633	Solute carrier family 12 member 7	5.29	0.026	Q9Y666	41.2	1	0.65	119	6.3
5	838	Adenylyl cyclase-associated protein 1 (CAP1)	1.14	0.0076	Q01518	509.2	9	18.95	51.8	9.1
6	960	Alpha-enolase	1.61	0.0033	P06733	176.2	4	10.6	47.1	7.7
		Rab GDP dissociation inhibitor beta			P50395	139.6	3	6.97	50.6	6.1
		Tryptophanyl-tRNA synthetase, cytoplasmic			P23381	116.1	2	5.1	53.1	5.8
7	961	Alpha-enolase	1.23	0.00016	P06733	473	8	21.43	47.1	7.7
		Rab GDP dissociation inhibitor beta			P50395	278.5	7	18.2	50.6	6.1
		Coronin-1A			P31146	131.8	3	7.81	51	6.3
		Actin-related protein 2			Q8TDY3	84.2	2	6.09	44.7	6.3
8	1533	14-3-3 protein beta/alpha	1.32	0.0051	P31946	250.4	6	22.36	28.1	4.6
9	1701	Protein S100-A9	1.87	0.0023	P06702	232.5	4	43.86	13.2	5.7
		Protein S100-A8			P05109	134.9	2	23.66	10.8	6.6
10	1742	Vimentin	7.43	0.00066	P08670	567.5	10	16.52	53.6	4.9

6.3.5 Principal component analysis and hierarchical clustering

All the experimental groups were combined in EDA. Principal component analysis (PCA) and hierarchical clustering of the spot maps of the differentially regulated proteins were performed (Figure 6.8). RPMNs showed a distinct protein expression profile compared to other groups. The previous observation was confirmed in hierarchical clustering (Figure 6.9).

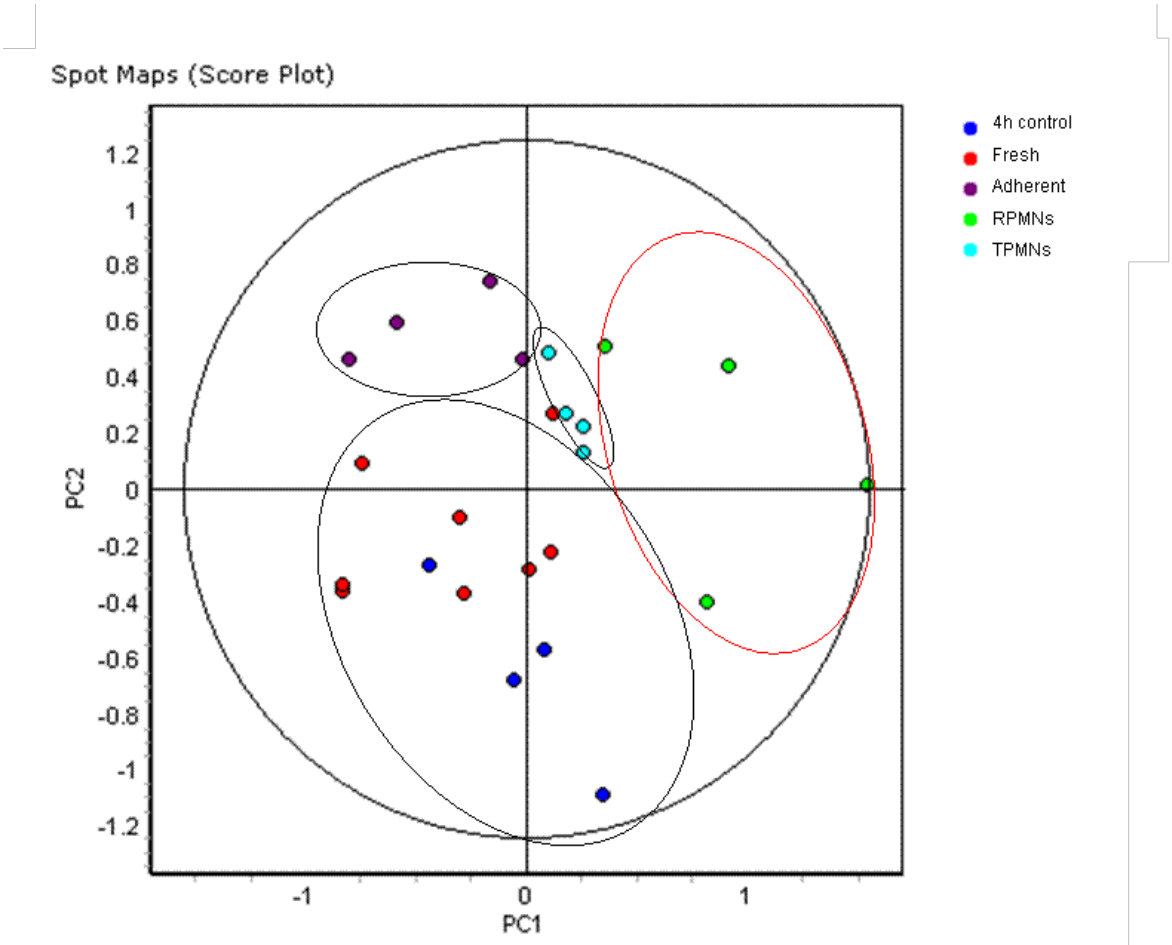


Figure 6.8 Principal Component analysis (PCA)
Significant differential regulated proteins were analysed with extended data analysis (EDA) and PCA on all spot maps was performed. Blue, red, purple, green and cyan represent 4h control, fresh, adherent, RPMNs and TPMNs respectively. RPMNs group (red circle) is distinct, compared to the other groups.

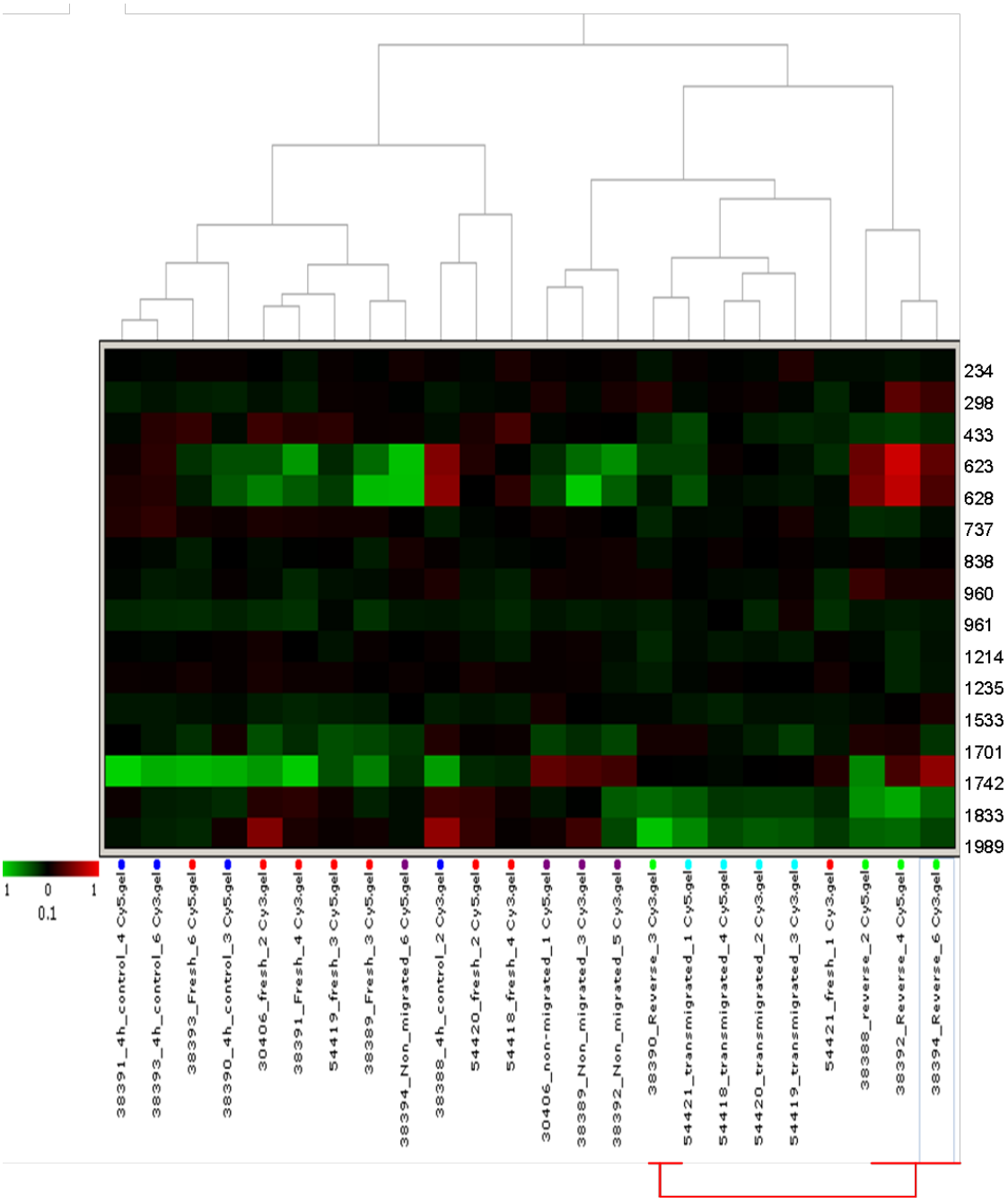


Figure 6.9 Hierarchical clustering of RPMNs and other control groups
Sixteen differentially regulated proteins were used for hierarchical clustering in the EDA. Hierarchical clustering (up-regulation in red, down-regulation in green) revealed two groups of spot maps. Again, RPMNs (red line) were distinct from the control groups.

6.3.6 Western Blot analysis of ENO1, CAP1, PP1 and RNAse2 expression in fresh and RPMNs

Western blots of ENO1, CAP1, PP1 and RNAse expression were performed as previously described. ENO1 expression was slightly higher in RPMNs, compared with the fresh. CAP1 was markedly increased in RPMNs, while PP1 expression was down-regulated (Figure 6.10). Our results confirmed the findings from BVA in 2D-DIGE.

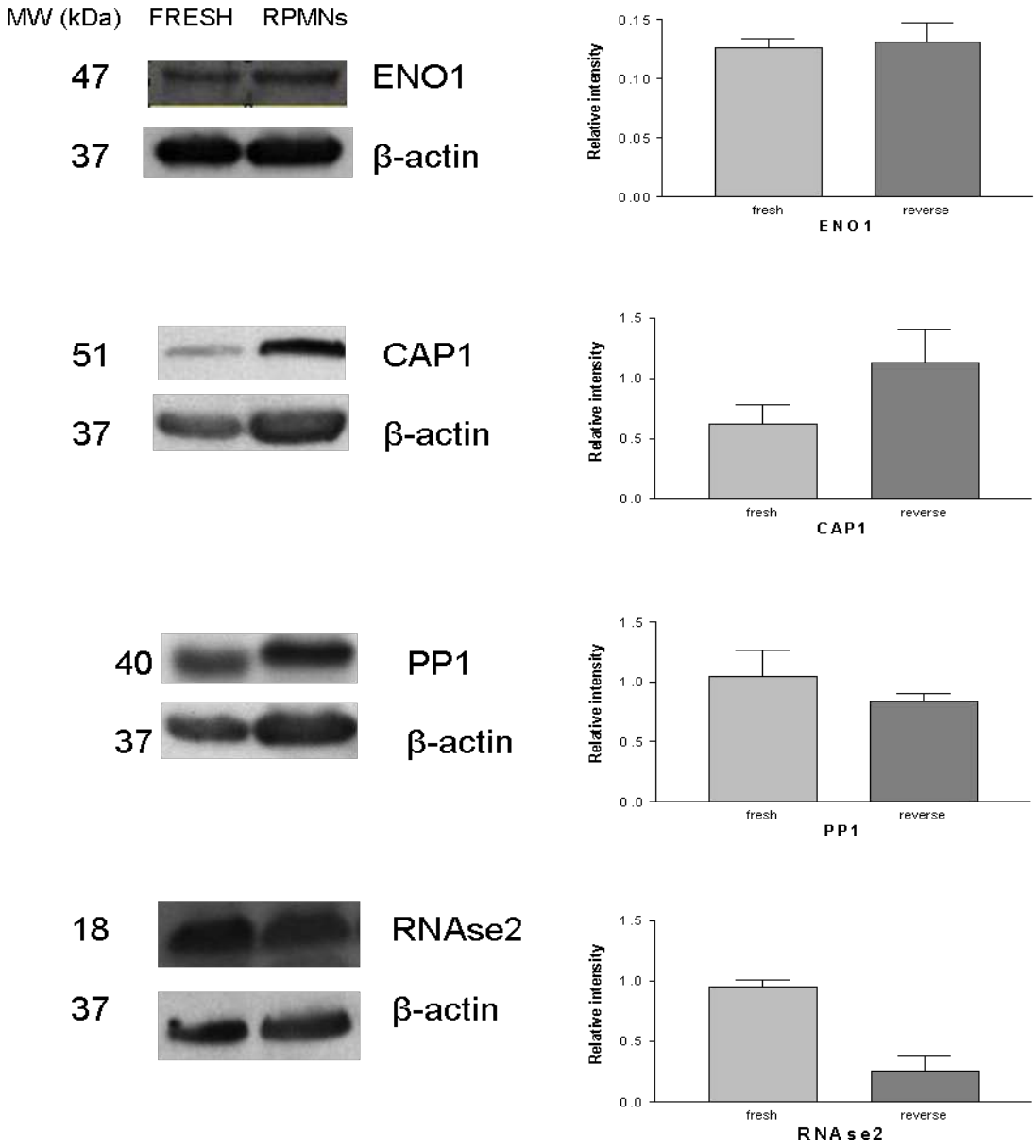


Figure 6.10 Western Blot analysis of ENO1, CAP1, PP1 and RNAse2 expression in fresh and RPMNs

Fresh and RPMNs ($5x10^6$/ml)were lysed in sample buffer and protein samples were analysed by Western blotting with anti-ENO1, anti-CAP1, anti-PP1, anti- RNAse2 or anti-β-actin as control. Results are shown as relative expression. The experiment is representative of three experiments, except for RNAse2, where n=2.

6.4 Discussion

In this chapter, we aimed to study the changes that take place inside human RPMNs at a proteomic level. The question we had to address was whether RPMNs showed a distinct protein expression profile compared with other control groups using 2D-DIGE methodology and mass spectrometry. We have identified 14 proteins that are regulated in reverse migration. All the down-regulated proteins showed a distinct expression profile, specific for the RPMNs. In contrast, only one up-regulated protein was found to exhibit a different profile from the other groups.

The proteins identified could be classified in three categories: a) metabolic enzymes, b) regulatory proteins and c) cytoskeletal proteins. Transaldolase and alpha enolase belong to the first group. The heat shock 70 kDa protein 4, serine/threonine-protein kinase PAK 2 and Rab GDP dissociation inhibitor beta, 14-3-3, S100-A9 and S100-A8 are regulatory proteins. Vimentin, myosin-9 and adenylyl cyclase associated protein 1 (CAP1) are those associated with the cytoskeleton.

Interestingly, changes in the expression pattern of metabolic enzymes take place in RPMNs. Alpha enolase (spots 960-961) is an enzyme of glycolysis, which catalyzes the dehydration of 2-phosphoglycerate to phosphoenolopyruvate. This enzyme consists of two subunits of 433 aminoacids. Each subunit has a molecular weight of 47k Da. It is a highly conserved enzyme that is abundantly expressed in cytoplasm (Pancholi, 2002). There is also surface expression of alpha enolase in stimulated immune cells (neutrophils, B and T-lymphocytes, monocytes), epithelial, neuronal and endothelial cells (Terrier *et al*., 2007). Cell surface alpha enolase acts as a strong receptor and activator of plasminogen (Miles *et al*.,

1991). Alpha enolase has been detected in the nucleus, as a Myc-binding protein (MBP-1), playing a role in the regulation of cell growth and differentiation (Pancholi, 2002). It is also found to be directly or indirectly associated in the site-specific tubule organization (Rattner *et al*., 1991). Interestingly, alpha enolase may be associated to a large number of autoimmune and inflammatory diseases (Rattner *et al*., 1991; Roozendaal *et al*., 1998; Terrier *et al*., 2007). Antibodies against alpha enolase have been found in the sera from patients with systemic rheumatic diseases (Pancholi, 2002).

Transaldolase (spot 1235) is a 37.5 KDa enzyme of the pentose phosphate pathway (PPP). PPP provides the cell with ribose-5 phosphate for nuclear acid synthesis and NADPH for lipid biosynthesis. PPP comprises an oxidative and a non-oxidative phase. Transaldolase is a rate-limiting enzyme of PPP's non-oxidative phase (Heinrich *et al*., 1976). It transfers a 3-carbon ketol, from sedoheptulose 7-phospate to glyceraldhehyde 3-phosphate (GA3P) to form fructose 6-phosphate and erythrose 4-phosphate (Banki *et al*., 1994b). Transaldolase and transketolase link PPP with glycolysis (Huang *et al*., 2005). GA3P can enter glycolysis for ATP production or enter glyconeogenesis to enter PPP for NADPH production.

In addition PPP maintains glutathione at a reduced state (GSH) via NADPH production and, thus, cellular integrity is protected from reactive oxygen intermediates (ROIs) (Banki *et al*., 1998). ROIs formation is associated with apoptosis. It has been suggested that transaldolase modulates glutathione levels and sensitivity to apoptosis (Banki *et al*., 1996). Reduction in transaldolase expression was found to be associated with increase in glucose 6-phosphate dehydrogenase activities and GSH levels and inhibition of apoptosis (Banki *et al*., 1996). In contrast, transaldolase up-regulation leads to an increased susceptibility to

apoptosis. Another study has demonstrated that transaldolase can regulate the production of ROI and subsequently, caspase activation and cell death, during HIV infection (Banki *et al*., 1998). There is evidence that transaldolase is associated with multiple sclerosis. Autoantibodies against transaldolase have been found in the serum and CFS of multiple sclerosis patients (Banki *et al*., 1994a; Esposito *et al*., 1999). The change in the levels of abundance of the above metabolic enzymes may imply a change in the metabolism of RPMNs, especially glucose, as these cells prolong their survival.

The majority or regulatory proteins were down-regulated in RPMNs. Serine/threonine-protein kinase PAK-2 (spot 737) belongs to protein kinase superfamily. It phosphorylates ribosomal protein S6, histone H4 and myelin basic protein and is associated with apoptosis (Rudel and Bokoch, 1997). Full-length PAK-2 stimulates cell survival by inhibiting via phosphorylation the pro-apoptotic BAD, whereas proteolytically activated PAK-2p34 is associated with cell death response (Jakobi *et al*., 2003), probably via JNK pathway (Vilas *et al*., 2006). PAK-2 is also involved in the regulation of various cytoskeletal functions, such as cell motility (Bokoch, 2003). Protein phosphatase 1 (PP1) is a serine/threonine phosphatase that regulates a large number of functions (Cohen, 2002). Another regulatory protein down regulated in RPMNs was the heat shock 70 kDa protein 4 (spot 234). Increased levels of protein from this family occur after environmental stress, infection, normal physiological processes, and gene transfer (Kiang and Tsokos, 1998). The Rab GDP dissociation inhibitor beta (spots 960-961) is a protein that inhibits the dissociation of GTP and the binding of GDP in Rab proteins, regulating the GTP/GDP exchange reaction in them（Sedlacek *et al*., 1998). The Rab GDP dissociation inhibitor beta may also play a role in protein transport (Bächner *et al*., 1995) . The down-regulation of the regulatory proteins probably may be attributed to the

fact that some cellular events that these proteins take part in are impaired after reverse migration.

The expression levels of a number of regulatory proteins in RPMNs were increased. The 14-3-3 proteins (spot 1533) are a family of conserved regulatory proteins, present in all eukaryotic cells. These proteins bind to a plethora of regulatory proteins, enabling 14-3-3 proteins to play an important role in mitogenic signal transduction, apoptotic cell death and cell cycle control (Fu *et al*., 2000). Recently, it has been shown that 14-3-3 proteins antagonize the pro-apoptotic activity of BAD. 14-3-3 protein binding to BAD ,via phosphorylation, prevents the latter from forming complexes with Bcl-X_L/Bcl-2, thus inhibiting apoptosis (Zha *et al*., 1996). Subsequently, the up-regulation of 14-3-3 proteins in RPMNs may be associated with inhibition of apoptosis.

Proteins S100A8 and S100A9 (spot 1701) were also up-regulated. These proteins belong to the group of S100 calcium-binding proteins and are mainly expressed by cells of the myeloid lineage, where they comprise up to the 30% of cytosolic proteins (Hessian *et al*., 1993). S100A8 and S100A9 proteins exhibit antimicrobial properties and play a role in neutrophil activation and adhesion (Kerkhoff *et al.*, 1998; Newton *et al.*, 1998; Steinbakk *et al*., 1990). They form an heterodimer, calprotectin, which exist in abundance in neutrophils (Foell *et al*., 2007). Calprotectin is also present in NETs (Urban *et al.*, 2009). Calprotectin levels are increased in the extracellular fluid of patients with inflammatory diseases, such as RA and vasculitis. This complex has been recently used as a marker of inflammation (Ehrchen *et al.*, 2009).

Interestingly, the majority of proteins associated with the cytoskeleton were up-regulated in RPMNs. The protein with the most significant increase in expression was vimentin. Vimentin is a major intermediate filament protein. Vimentin regulates secretion and plays an important role in the lysosomal vesicle transport (Quintanar *et al*., 2000; Styers *et al*., 2004). It has been recently demonstrated that vimentin is degraded in apoptotic neutrophils (Lavastre *et al.*, 2002a; Lavastre *et al.*, 2002b). Interestingly, when apoptosis was suppressed by GM-CSF, vimentin expression was increased (Moisan *et al*., 2003). The marked increase of vimentin levels in RPMNs suggests that these cells had not undergone apoptosis.

Myosin-9 (spot 298) is involved in cytokinesis and cell shape (Sellers, 2000). It is expressed in other leukocytes (Toothaker *et al*., 1991). Mutations of myosin 9 gene causes platelet disorders characterized by a triad of giant platelets, thrombocytopenia, and characteristic Döhle body-like leukocyte inclusions (Kunishima *et al*., 2001). Another cytoskeletal protein identified, CAP1 (spot 838) is a membrane protein associated with actin. CAP1 binds to adenylyl cyclase with the N-terminal domain and regulates filament dynamics by inhibiting actin polymerazation through the C-terminal domain (Moriyama and Yahara, 2002). The up-regulation in cytoskeletal proteins may suggest that RPMNs have a high requirement for these proteins dynamically regulate their cytoskeleton which after activation has an established polarity. These findings are consistent at the molecular level with our visual observations under phase contrast microscopy. i.e., RPMNs on the apical surface of HUVEC are not spherical, but polarised with protrusions and actively migrating across the EC monolayer.

This broad range of proteins that are differentially regulated in RPMNs is the very first evidence that changes in glucose metabolism, cell shape/motility and regulatory pathways occur within hours after the cells have reverse migrated. These changes might be associated with prolonged survival and immune function. Alternatively, they may have some as yet to be defined role in the clearance of these cells from the circulation as part of the resolution phase of inflammation.

CHAPTER 7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

This thesis explored neutrophil transmigration and reverse migration through the endothelial monolayer in human and murine *in vitro* models of inflammation. Neutrophils are believed to be terminally differentiated effector cells of the innate immune response, with a limited life span in the circulation. After recruitment to tissue and execution of their inflammatory functions, they are thought to die by apoptosis followed by clearance by tissue macrophages. During the last decade a number of studies have revealed that neutrophils play a very important role in the innate immune response and their functions are not restricted to killing invading microorganisms. Thus neutrophils: a) interact with other cell types (monocytes, dendritic cells, T cells and B cells) in a bidirectional way, b) recruit and activate APCs, c) play a key role in repairing inflamed tissue by controlling microbial contamination and attracting monocytes and/or macrophages and d) may also play a part in tumor destruction (reviewed by Nathan, 2006).

In this thesis, we used human and murine *in vitro* models of inflammation to investigate the reverse transmigration of neutrophils. This term describes a phenomenon where transmigrated neutrophils have the ability to reverse transmigrate back across the same endothelial monolayer by which they were recruited. Reverse migration of neutrophils has been reported *in vitro* (Buckley *et al*., 2006) and *in vivo* (Mathias *et al*., 2006). The process of reverse transmigration challenges well established paradigms of neutrophil behaviour and inevitably leads us to the following questions: a) do reverse migrated neutrophils show prolonged survival and b) what is their fate after re-entering the circulation. A previous study has confirmed the prolonged survival of human reverse migrated neutrophils and their presence in the blood of healthy people and patients with chronic inflammatory diseases

indicates that *in vivo* these cells also have prolonged residence in the systemic circulation. RPMNs *in vivo* were found to share the same phenotype with the *in vitro* generated reverse migrated neutrophils (Buckley *et al*., 2006). However, in this study the fate of these cells *in vivo* was not demonstrated. In this thesis, after generating mRPMNs in murine *in vitro* models of inflammation, we assessed the phenotype and the prolonged survival of mRPMNs and finally their fate *in vivo* using adoptive transfer methodology.

A schematic diagram illustrating the major findings in this thesis is shown in Figure 7.1. Murine *in vitro* models of inflammation were developed in order to investigate the recruitment and reverse migration of murine neutrophils to cytokine stimulated murine EC monolayers. A differential response of mECs to TNF-α stimulation compared to IL-1β was observed, implying that mECs are far less sensitive to TNF-α than IL-1β, which is not the case for human EC (Luu *et al*., 1999).

Next, mRPMNs were generated in static cultures and examined for CXCR2 and ICAM-1 surface expression, viability and apoptosis levels. Around 90% of mRPMNs were alive, as determined by Trypan Blue exclusion. Murine RPMNs showed a prolonged survival, as shown with human RPMNs (Buckley *et al*., 2006). This is also in accordance with previous studies showing prolonged survival of neutrophils after being in contact with the endothelium (Buckley *et al*., 2006; McGettrick *et al*., 2006). These findings suggest that under inflammatory conditions neutrophils are rescued from apoptosis in order to prosecute their immune functions, although how reverse migration contributes to the process of inflammation is still to be determined.

Murine reverse migrated neutrophils showed an altered phenotype compared to control populations. CXCR2 was not found to be down-regulated, but ICAM-1 expression increased significantly. ICAM-1 up-regulation in these cells has been suggested to facilitate their engagement by the macrophages in the liver and spleen and therefore their removal from circulation (Buckley *et al*., 2006). However, when adoptive transfer experiments were performed in order to examine the fate of RPMNs *in vivo,* our results did not give a clear indication as to the tissue localisation of RPMNs. We attributed this to the limitations of the particular methodology used and investigation of the fate of mRPMNs may be possible using more sensitive imaging platforms to track their localisation *in vivo* after adoptive transfer.

A chapter of this thesis was also dedicated to the role of shear stress and nitric oxide on the recruitment and reverse migration of human neutrophils. There is increasing evidence that EC phenotype can be conditioned by the physicochemical environment, including wall shear stress (Nash *et al*., 2004). A number of studies have investigated the role of wall shear stress on leukocyte adhesion and transmigration (Sheikh *et al*., 2004; Sheikh *et al*., 2003). We examined whether wall shear stress could modulate the reverse migration of neutrophils. In the absence of continuous flow, efficient reverse migration proceeded over a timeframe of several hours. Continuous flow delayed but did not inhibit reverse migration. In our hands, NO seemed to impair neutrophil recruitment and transmigration, which is in agreement with previous observations in animal models of inflammation (Kubes *et al*., 1991). Importantly, the availability of NO also appeared to delay reverse migration. It is thus tempting to speculate that the well documented regulation of NO synthesis by shear stress (Li *et al*., 2004), might be a possible mechanism by which neutrophils are retained in tissue and in the absence of these cues, neutrophils spontaneously undergo reverse migration.

Finally, using 2D-DIGE methodology, we examined whether reverse migrated neutrophils had a distinct protein expression profile. The proteins identified as characterising reverse migrated cells could be classified in three categories: a) metabolic enzymes, b) regulatory proteins and c) cytoskeletal proteins. Transaldolase and alpha enolase are metabolic enzymes. The heat shock 70 kDa protein 4, Serine/threonine-protein kinase PAK 2 and Rab GDP dissociation inhibitor beta are regulatory proteins. Myosin-9, adenylyl cyclase associated protein 1 (CAP1), vimentin, protein S100-A9 and S100-A8 are those associated with the cytoskeleton. All cytoskeletal proteins were up-regulated in reverse migrated neutrophils. These changes in expression profiles depict changes in metabolism, motility and regulatory pathways in these cells and could be associated with prolonged survival and immune function, although the relevance of their regulation during the process of reverse migration remains to be described in future studies.

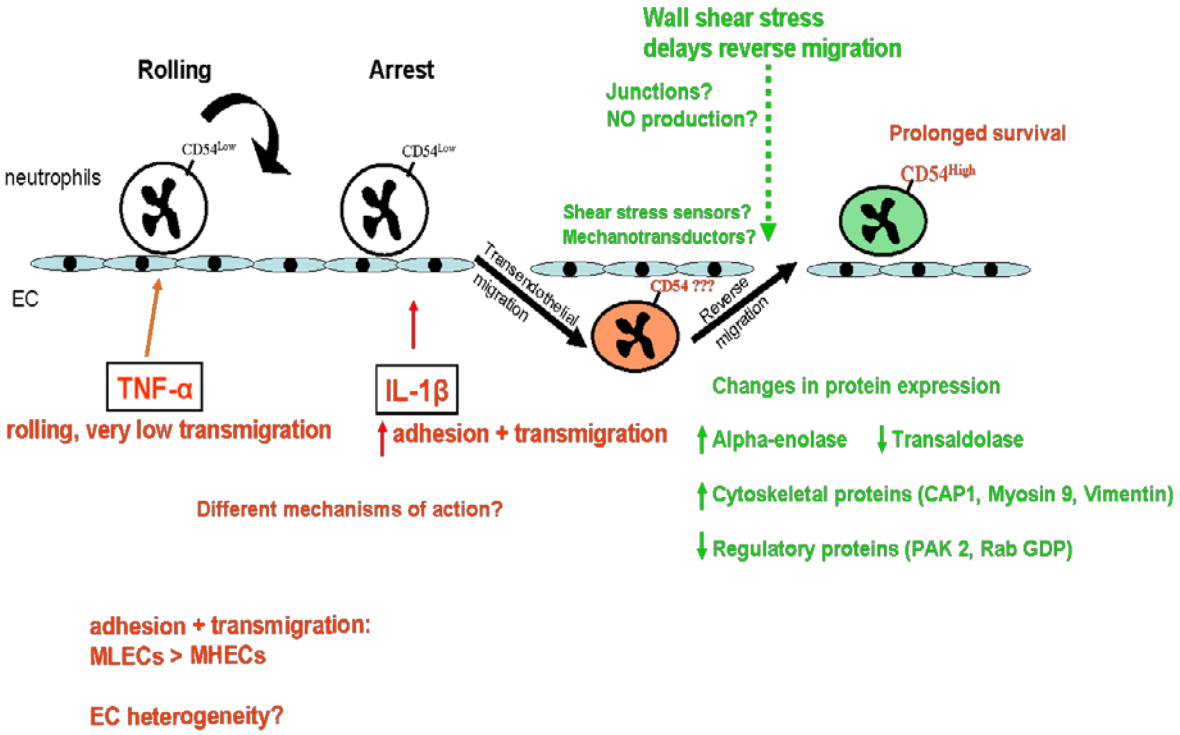


Figure 7.1 Schematic overview of the major findings of this thesis
The major findings from experiments in murine (in red) and human (in green) *in vitro* models of inflammation are shown. MLECs were more sensitive to cytokine treatment than MHECs. Stimulation of mECs with IL-1β could promote neutrophil adhesion and transmigration, whereas treatment with TNF-α could support rolling only. Murine RPMNs showed prolonged life and were ICAM-1^{high} ($CD54^{high}$). In human *in vitro* models of inflammation, continuous flow retarded the process of reverse migration and there was an indication that NO could mediate in this process. Finally, it was demonstrated that human RPMNs have a distinct protein expression profile.

The bidirectional transmigration of neutrophils implies that these are able to return back to the circulation *in vivo* after they had been recruited to inflammatory sites. The physiological relevance of this process is unknown, but it has been suggested as an alternative route of resolution by aiding removal of the inflammatory infiltrate once it is no longer required. In addition, increased incidence of neutrophil reverse migration might be associated with chronic inflammatory diseases such as atherosclerosis and rheumatoid arthritis (Buckley *et al*., 2006). Interestingly, the process of reverse migration has also been reported in monocytes *in vivo* (Bradfield *et al*., 2007) and in lymphocytes *in vitro* (McGettrick *et al*., 2009), although importantly, the relevance of the process to lymphocyte and monocyte biology is also unknown.

Future work

The work described in this thesis could be further developed in a number of ways. The future directions of this work are listed below.

- Investigate the surface expression of important adhesion molecules (such as P- and E-selectin, VCAM-1, CD31) after stimulation with TNF-α or IL-1β in MLECs and MHECs. Use blocking antibodies against the adhesion molecules to further investigate the different mechanisms of endothelial activation by these cytokines.

- Examine the role of important adhesion molecules (such as CD31) in the process of neutrophil adhesion and transmigration on mECs using cells from knock-out mice in our models of inflammation.

- Further investigate the changes in expression of other molecules on the surface of mRPMNs. Perform functional studies to examine the mRPMNs ability for adhesion

and transmigration on cytokine stimulated mECs, and for ROS production. Also, assess their rigidity.

- Investigate the fate of mRPMNs with a more sensitive *in vivo* imaging technique such as *in vivo* imaging system methodology (IVIS).

- Further explore the effect of wall shear stress on reverse migration. Examine the possible role of NO in this process, by assessing eNOS induction. Also, assess the release of IL-8 or other chemotactic agents in the medium in the presence or absence of wall shear stress.

- Verify the data obtained from 2D-DIGE electrophoresis with Western Blot.

- Investigate whether the specific regulated proteins in human RPMNs can serve as biological markers of reverse migration. For example, examine their expression by flow cytometry, in order to obtain information on a specific RPMN phenotype. Based on that phenotype, human RPMNs can be identified and isolated from the whole blood of healthy volunteers or patients with chronic diseases. Thus, these biological markers could act as a powerful tool to investigate any correlation between the numbers of RPMNs and chronic inflammation.

- Further examine the significance of the changes in the proteomic profile of human RPMNs, by extending our proteomic study to metabolomics, using analytical methodology, such as nuclear magnetic resonance (NMR).

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Online material:

http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/B9A1E643A0DEB1D1C1257628001D1C81/$file/11001340AD.pdf

APPENDIX

PUBLICATIONS ARISING FROM THIS THESIS

Papers

Woodfin A., Voisin M.B., Bauer M., Colom B., Caille D., Diapouli F.M., Nash G.B, Chavakis T., Albelda S.M., Rainger G.E , Meda P., Imhof B.A. & Nourshargh S. Junctional adhesion molecule-C (JAM-C) is a key regulator of polarized neutrophil transendothelial cell migration *in vivo. Nature Immunology* (In press*)*

Poster presentation

2010 Graduate School Poster conference, University of Birmingham, UK.
Diapouli F.M., Luu N.T., Rainger G.E , Nash G.B. The effect of shear flow on reverse migration of neutrophils.